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L. 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 alveolar epithelium. We tested whether *L. pneumophila*-infection of lung epithelial cells (A549) resulted in proinflammatory activation.

L. pneumophila-infection induced liberation of IL-2, IL-4, IL-6, IL-8, IL-17, MCP-1, TNFα, IL-1β, IFNγ, G-CSF, but not of IL-5, IL-7, IL-10, IL-12 (p70), IL-13 or GM-CSF. We focused on IL-8 and found induction by *L. pneumophila* strains 130b, Philadelphia 1, Corby, and to a lower extent, JR32. Knock out 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 MAP kinase was activated and kinase inhibition reduced secretion of of induced cytokines with exception of IL-2 and G-CSF. In contrast, inhibition of MEK1/ERK pathway only reduced expression of few cytokines. *L. pneumophila* also induced binding of NF-κB subunit RelA/p65 and the RNA polymerase II to the *il8* promoter and a specific inhibitor of the IκBα- complex dose-dependently lowered IL-8 expression.

Taken together, *L. pneumophila* activated p38 MAP kinase- and NF-κB/RelA pathway-dependent expression of a complex pattern of cytokines by human alveolar epithelial cells, presumably contributing to immune response in Legionellosis.

Keywords: alveolar epithelium, cytokines, bacteria, signal transduction pathways

INTRODUCTION

Legionella pneumophila is an important causative agent of severe community-acquired pneumonia and the second most commonly detected pathogen in pneumonia that are admitted to intensive care units in industrialized countries [1]. About 15 % of Legionellosis appears in community outbreaks. Although more than 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 occurred after inhalation of contaminated water aerosol droplets. *L. pneumophila*-containing phagosomes initially do not fuse with lysosomes and the bacteria induce remodeling of their membrane-bound compartment into an endoplasmatic reticulum-like organelle [3]. This remodeling depended on the Dot/Icm type IVB secretion apparatus of *L. pneumophila* [4]. Besides delivery of proteins by the type IVB secretion system, *L. pneumophila* contain a battery of additional virulence factors including a type II secretion apparatus [3]. Sequencing of *L. pneumophila* serogroup 1 genomes revealed about 3,000 genes among which are many genes with possible function in manipulation of host cell signaling [5].

According to the *Legionella*-host interaction, recent studies demonstrated, that *L. pneumophila* lipopolysaccharide was recognized by TLR2 and flagellin by TLR5 [6, 7]. A stop-codon in the human TLR5 gene lead to an increased susceptibility for Legionellosis [7]. Interestingly, although some studies suggested a minor role of TLR4 in Legionellosis [6], Hawn et al showed recently that TLR4 polymophisms are associated with resistance to Legionnaires´ disease [8].

Essential results according *L. pneumophila* pathogenesis were obtained by analyzing infection of protozoans or immune cells like 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 signaling pathways in lung

epithelial cells, including p38 MAP kinase and NF-κB dependent gene transcription, by bacterial infection contributed significantly to cytokine release [10, 11]. Although *Legionella* efficiently infected and stimulated lung epithelial cells [7, 12], mechanisms of *L. pneumophila*-induced activation of and cytokine release in lung epithelial cells are widely unknown. Therefore, we analyzed the pro-inflammatory activation of lung epithelial cells by *Legionella*-infection in detail. In this study we show that *L. pneumophila* induced the release of several important cytokines in human alveolar epithelial A549 cells, e.g. IL-2, IL-4, IL-6, IL-8, IL-17, MCP-1, TNFα, IL-1β, IFNγ, and G-CSF, as well as activated p38 MAP kinase-, ERK-, and NF-κB pathways. Blocking of p38 MAP kinase reduced secretion of all cytokines in *Legionella*-infected cells with exception of IL-2 and G-CSF, while blocking of ERK pathway diminished only release of IFNγ, IL-1β, IL-6 and TNFα. By addressing expression of IL-8 as a model cytokine in more detail, we verified the important role of p38 MAP kinase and NF-κB/RelA-dependent gene transcription for activation of *L. pneumophila*-infected epithelial cells. Moreover, activation of IL-8 expression was reduced by a flagellin deletion mutant implicating a role of TLR5 or possible intracellular receptors in *Legionella* sensing by A549 cells. However, IL-8 expression in A549 cells was not affected by a *dotA*-knock out mutant, suggesting that the type IVB Dot/Icm secretion system and intracellular replication were not needed for IL-8 expression in A549 cells.

MATERIALS AND METHODS

Materials

DMEM, FCS, trypsin-EDTA-solution, CA-650, and antibiotics were obtained from Life Technologies (Karlsruhe, Germany). Protease inhibitors, Triton X-100, and Tween-20 were purchased from Sigma Chem. Co. (Munich, Germany), TNFα from R&D Systems (Wiesbaden, Germany), SB202190, SB203580 and SB202474 from Calbiochem-Merck (Darmstadt, Germany), and IKK-NBD from Biomol (Plymouth Meeting, PA). All other chemicals used were of analytical grade and obtained from commercial sources.

Cell lines

Alveolar epithelial cell line A549 was purchased from ATCC (Rockville, USA) and cultured in HAM'S F 12 with L-glutamine, 10% FCS without antibiotics. The NF-κB-dependent reporter cell line, A549 6Btkluc, was a kind gift of Dr. Robert Newton (Department of Biological Sciences, University of Warwick, Coventry, UK). They contain a stably integrated plasmid with three tandem repeats of the sequence 5'-AGC TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GA-3', which has two copies of the decameric NF-κB binding site upstream of a minimal thymidine kinase promoter (-105 to +51) driving a luciferase gene.

Infection with bacterial strains and isogenic mutants

L. pneumophila sg1 strains 130b (ATCC BAA-74, kindly provided by Nicholas P. Cianciotto, Northwestern University Medical School, Chicago, USA [13]), Philadelphia 1 (ATCC 33152, kindly provided by Birgid Neumeister, Tübingen University, Germany [14]), JR32 wild type [15] and JR32 *dotA* mutant (LELA 3118, both kindly provided by Howard Shuman, Columbia University, New York, USA [16]) Corby wild type and a Corby *flaA* mutant, defective in

flagellin (both kindly provided by Klaus Heuner, Würzburg University) were routinely grown on buffered charcoal-yeast extract (BCYE) agar for 2 or 3 days at 37°C [17] and subsequently inoculated in plain RPMI to an OD_{660} of 0.2 to 0.4. A549 cells (10^5/ml) were infected with 10^5-10^8 cfu/ml *L. pneumophila*, i.e. a multiplicity of infection of 1:1 - 1:1000, for the indicated durations in 1 ml of epithelial cell growth medium. Extracellular bacteria were not routinely killed with antibiotics. *L. pneumophila* strains did not significantly grow in epithelial cell growth medium as controlled by serial dilutions were plated on BCYE agar.

To verify intracellular infection, A549 cells were incubated for 2 h with the added bacteria with or without kinase inhibitors, then gentamycin (100 μg/ml) was added for 2 more hours and cells were washed three times with plain medium to remove unbound bacteria and were treated with 10% (wt/vol) saponin (Sigma Chemicals, Munich, Germany) to lyse the host cells. Serial dilutions were plated on BCYE agar.

IL-8 ELISA

Confluent A549 cells were infected for 15 h as indicated in a humidified atmosphere. 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 gentamycin (100 μg/ml) and then incubated for the rest of the time in this medium before IL-8 was analyzed in the supernatant.

Bioplex Protein Array System

Confluent A549 cells were infected for 15 h as indicated in a humidified atmosphere. After incubation supernatants were collected and cytokine release was analyzed with the Bioplex Protein Array system (BioRad, Hercules, CA) using beads specific for IL-2, IL-4, IL-5, IL-6, IL-

7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, MCP-1, TNFα, IL-1β, IFNγ, GM-CSF and G-CSF, according to the manufacturers instructions [19, 20].

Western Blot

For determination of p38 MAP and ERK kinase phosphorylation, A549 cells were infected as indicated, washed twice, and harvested. Cells were lysed in buffer containing Triton X-100, subjected to SDS-PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection of phosphorylated MAP kinases was carried out with phospho-specific p38-MAP kinase or ERK antibodies (Cell Signaling, Frankfurt, Germany) [11]. Degradation of IκBα was analyzed using a rabbit polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) as described previously [11]. In all experiments, unphosphorylated ERK2 or p38 MAP kinase (Santa Cruz Biotechnologies, Santa Cruz, CA) was detected simultaneously to confirm equal protein load. Proteins were visualized by incubation with secondary IRDye 800- or Cy5.5-labeled antibodies, respectively (Odyssey infrared imaging system, LI-COR Inc.) [11, 21].

RT-PCR

For analysis of IL-8 and GAPDH gene expression in A549 cells, total RNA was isolated by means of the RNEasy Mini kit (Quiagen, Hilden, Germany) and reversely transcribed using AMV reverse transcriptase (Promega, Heidelberg, Germany). Generated cDNA was amplified by PCR using specific intron-spanning specific primers for IL-8 and GAPDH [11]. All primers were purchased from TIB MOLBIOL, Berlin, Germany. After 35 amplification cycles, PCR products were analyzed on 1.5 % agarose gels, stained with ethidium bromide and subsequently

visualized. To confirm use of equal amounts of RNA in each experiment, all samples were checked for GAPDH mRNA expression.

Reporter gene assay

A549 cells stably transfected with a NF-κB-dependent luciferase reporter [22] were cultured in 12-well plates with DMEM. Cells were incubated with *Legionella* for 15 h, lysed, and luciferase activity was measured by using a luciferase-reporter-gene assay (Promega, Mannheim, Germany).

Chromatin Immunoprecipitation (ChIP)

A549 cells in 75 cm culture flacks were infected with *L. pneumophila* 130b as indicated and then subjected to ChIP assay as previously described using anti-p65 (Santa Cruz), or anti-RNA polymerase II (Santa Cruz) antibodies [11, 20]. The *il8* promoter was amplified by PCR using HotstarTaq polymerase (Qiagen) and specific primers as followed: 5'–AAG AAA ACT TTC GTC ATA CTC CG–3'; antisense 5'–TGG CTT TTT ATA TCA TCA CCC TAC–3'. PCR amplifications of the total input DNA in each sample is shown as a control [18, 19].

Statistical methods

Data are shown as means ± SEM of at least three independent experiments. A one-way ANOVA was used for data of Fig. 1, 2 (A/D/E), 3 (C), 4 (A/B), and 5. Main effects were then compared by a Newman-Keuls´ post-test. $p < 0.01$ was considered to be significant and indicated by * or #, respectively.

RESULTS

L. pneumophila-induced release of cytokines was mainly dependent on p38 MAP kinase

To characterize inflammatory activation of human alveolar epithelial cells by *L. pneumophila*, we infected 10^5 A549 cells with *L. pneumophila* strain 130b with an infection dose of 10^7 cfu/ml, i.e. a multiplicity of infection of 1:100. Cytokine release was analyzed using a Bioplex-assay. After 15 h of incubation, we observed significant induction of IL-2, IL-4, IL-6, IL-8, IL-17, MCP-1, TNFα, IL-1β, IFNγ, G-CSF, but not of IL-5, IL-7, IL-10, IL-12 (p70), IL-13 or GM-CSF (Fig. 1). Pre-incubation of A549 cells with specific p38 MAP kinase inhibitor SB202190 reduced levels of IL-4, IL-6, IL-8, IL-17, MCP-1, TNFα, IL-1β, IFNγ, but not of IL-2 and G-CSF. Inhibition of MEK1 by U0126 only reduced release of IFNγ, IL-1β, IL-6 and TNFα (Fig. 1). Incubation with inhibitors alone showed no cytotoxicity and did not alter cytokine expression or infection of epithelial cells (data not shown).

L. pneumophila strains induced flagellin-dependent but *dotA*-independent IL-8 expression of alveolar epithelial cells

We analyzed alveolar epithelial cell activation in more detail by addressing the expression of the important chemotactic cytokine IL-8. A549 cells were infected with *L. pneumophila* strains 130b, Philadelphia 1, JR32, and Corby with different concentrations for 15 h (Fig. 2A/D/E). *L. pneumophila* 130b, Philadelphia 1 and Corby similarly induced dose-dependent IL-8 release (Fig. 2A/D), whereas JR32-provoked cytokine secretion was lower (Fig. 2E). *L. pneumophila* 130b also induced dose- (Fig. 2B), and time-dependent (Fig. 2C) expression of IL-8 mRNA. Gene expression started as early as 60 min after infection. A *flaA* knock out mutant, defective in flagellin production, showed strongly reduced IL-8 release by *L. pneumophila* Corby-infected A549 cells during 15 h (Fig. 2 D). In contrast, a *dotA* knock out mutant, defective in a gene

essential for the establishment of a functional type IVB secretion apparatus of *L. pneumophila* [3], did not alter IL-8 release by *L. pneumophila* JR32-infected A549 cells during 15 h (Fig. 2 E) or time-course of IL-8 mRNA induction up to 4 h (Fig. 2F).

L. pneumophila-induced IL-8 release was dependent on p38 MAP kinase activation in alveolar epithelial cells

Next, we analyzed *L. pneumophila*-induced activation of mitogen-activated kinase pathways: A549 cells were infected with *L. pneumophila* 130b and phosphorylation of p38 MAP kinase (Fig. 3A) and ERK2 (Fig. 3B) was assessed by Western blot. *L. pneumophila*-infection induced phosphorylation of both kinases within 60-120 min. Blocking of p38 MAP kinase with SB202190 reduced IL-8 mRNA accumulation (Fig. 3D). Moreover, p38 MAP kinase inhibitors SB202190 and SB203580 dose-dependently reduced *L. pneumophila*-induced IL-8 release (Fig. 3C). Control compound SB202474 had no effect on cytokine release (Fig. 3C). Neither inhibitors nor control compound did reduce cell number or induce morphological signs of cytotoxicity.

L. pneumophila-induced IL-8 release depended on NF-κB activation in alveolar epithelial cells

Activation of the IL-8 promoter is considered to require activation of the transcription factor NF-κB. We blocked IκBα-kinase complex with the specific peptide inhibitor IKK-NBD, and observed dose-dependent reduction of IL-8 secretion by *L. pneumophila* 130b-infected A549 cells (Fig. 4A). In A549 cells, transfected with a NF-κB-dependent reporter gene construct, we found dose-dependent induction of reporter gene expression (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). By Western Blot analysis we observed degradation of cytosolic NF-

κB inhibitor IκBα starting at 60 min after stimulation of A549 cells with *L. pneumophila* 130b (Fig. 4C) and recruitment of NF-κB/p65 and RNA polymerase II (Pol II) to the *il8* promoter by chromatin immunoprecipitation (Fig 4D). IKK-NBD did not reduce cell number or induce morphological signs of cytotoxicity. These data indicate, that *L. pneumophila* induce IL-8 expression by activation of the canonical NF-κB pathway.

L. pneumophila-induced IL-8 release was reduced when extracellular bacteria were killed by the addition gentamycin

To address the importance of *L. pneumophila* remaining extracellular for cellular activation, we changed medium after an infection period of 1, 2 or 4 h with or without addition of gentamycin and analyzed IL-8 release after a total infection time of 15 h (Fig. 5). Removal of extracellular bacteria early in infection resulted in a significant lower IL-8 release in comparison with a late removal after 4 hours of infection. Moreover, killing of extracellular bacteria with gentamycin further reduced IL-8 release.

DISCUSSION

In the present study we 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 p38 MAP kinase- and canonical NF-κB/RelA-pathway, but to a lesser extend on MEK1-ERK1/2 pathway (Fig. 6).

Lung epithelial cells have important functions in innate immunity, e.g. they recognize pathogens, including bacteria, by 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 with the *Legionella*-permissive *naip5* locus-defect A/J mouse strain [24].

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

L. pneumophila-infected A549 cells released chemoattractants IL-8 (for PMNs) and MCP-1 (for monocytes), Th1 cytokines IL-2, TNFα and IFNγ, and 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 our findings, Chang et al. described IL-6, IL-8 as well as TNFα expression in *Legionella*-infected A549 cells [23].

In humans, *Legionella* infection increased e.g. serum levels of IFNγ, IL-6, IL-12, and IL-10 [25, 26]. In experimental studies using murine models or isolated macrophages, mainly cytokines

attracting and activating PMNs and moncytes/macrophages have been analyzed. In experimental *Legionella*-pneumonia in A/J mice, Tateda et al. found induction of the chemotactic cytokines KC, MIP-2 and LIX, recruiting PMNs into the lung [27]. Accordingly, attachment of *L. pneumophila* to cultured mouse peritoneal macrophages increased the steady-state levels of cellular mRNAs for 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 man [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 Th1 cytokines IFNγ and TNFα: IFNγ promoted *Legionella* clearance in macrophages [31] and transgenic overexpression of IFNγ in A/J mice reduced bacterial burden [32]. Since IFNγ was also found to be crucial for immune defense against *Listeria monocytogenes* in mice [33] and humans [34], an important role of IFNγ in host defense against intracellular bacteria, including *Legionella*, has to be considered. Similarly, TNFα 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 MAP kinase, this pathway might be crucial for 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, IL-10 and IL-13, as well as the lymphoid and myeloid growth factors IL-7 and GM-CSF. Interestingly, high IL-12 (p70) levels went along with decreased mortality in A/J mice *L. pneumophila* infection [27], expression was also found in human *L. pneumophila* pneumonia [25], and could be produced by *Legionella*-exposed dendritic cells [36], but alveolar epithelium seems not to be a source for this cytokine. In contrast, IL-10 reverted the *Legionella*-protective effects of IFNγ [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, TNFα, IL-1β, IL-6, IL-8, G-CSF were considered as uniform inflammatory reaction factors, e.g. induced by TLR2, while IFNγ, IL-2, IL-4, IL-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 host immune response in Legionellosis.

To gain more insight into alveolar epithelial cell activation by *L. pneumophila*, we analyzed expression of the important chemotatic cytokine IL-8 in more detail. *L. pneumophila* serogroup 1 strains 130b, Philadelphia1, JR32, as well as Corby induced IL-8 secretion of infected A549 cells. Philadelphia1-derived strain JR32 induced IL-8 expression to a lesser extent in lung epithelial cells, underlining existing differences between these strains. In accordance with recent findings from Hawn et al., Ren et al. and Molofsky et al., respectively [7, 39, 40], experiments using a Corby *flaA* knock out mutant strain indicated that recognition of flagellin by TLR5 or other possibly intracellular receptors seem to be essential for the early induction of IL-8 release in alveolar epithelial cells in vitro. Moreover, removal or killing (Gentamycin) of extracellular bacteria reduced IL-8 release by A549 cells significantly.

In addition, data obtained with a JR32 *dotA* knock out mutant indicated that type IVB-secreted effectors seem to be not essential for the early induction of IL-8 release in alveolar epithelial cells in vitro. Furthermore, it implies that bacterial replication is not necessary for induction of IL-8 release. However, Chang et al. have found that knock out 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 the e.g. strain Paris and Lens [5] and showed different expression pattern of pathogenetic factors. The physiological importance of such differences furthermore highlighted by e.g. the observation that Philadelphia 1-derived strain JR32 induced IL-8 expression to a lesser extent in lung epithelial cells. Thus, it cannot rule out that in infections with different *L. pneumophila* strains the importance of a particular

virulence factor may vary. Moreover, different genes within the *icm/dot* loci where manipulated by mutagenesis. In accordance with Chang et al., the time-course of IL-8 mRNA induction by JR32 strain or *dotA*-knock out mutant did not differ up to 4 h. Overall, it seems reasonable that recognition of extracellular *Legionella* by toll like receptors initially contributed to alveolar epithelial activation [6, 7].

Activation of p38 MAP kinase was shown to contribute to bacteria-related expression of IL-8 in infected lung epithelial cells [11] and Welsh et al. found that p38 MAP kinase and JNK were activated early during the uptake of *L. pneumophila* by macrophages [41]. In lung epithelial cells, p38 MAP kinase activation 60 min after infection was critical for 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 have to 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 MAP kinase activity, stimulation of IL-8 expression was dependent on activation of the transcription factor NF-κB in *L. pneumophila*-infected epithelium, as it was shown for *S. pneumoniae* in lung epithelial cells [11]. Further experiments addressing the role of the different pathogenic factors of *Legionella*, e.g. like LPS, flagella or hydrolyzing enzymes, for the activation of these important pro-inflammatory pathways are needed to gain more insight in the molecular mechanisms involved.

In summary, here we show that L. pneumophila-infection induced strong chemokine and cytokine release in human alveolar epithelial A549 cells. Expression of these molecules depended predominantly on activation of the p38 MAP kinase pathway and NF-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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FIGURE LEGENDS

Figure 1: *L. pneumophila* induce release of a cytokine pattern mainly dependent on p38 MAP kinase activity. A549 cells (10^5 cells/ml) were preincubated (30 min) with 5 μM of p38 MAP kinase inhibitor SB202190 or MEK1 inhibitor U0126 and infected with *L. pneumophila* 130b (10^7 cfu/ml). Cytokine release in the supernatant was measured by Bioplex assay. *, $p<0.01$ vs. control; #, $p<0.01$ vs. infected cells without pre-incubation with inhibitors at least in three independent experiments.

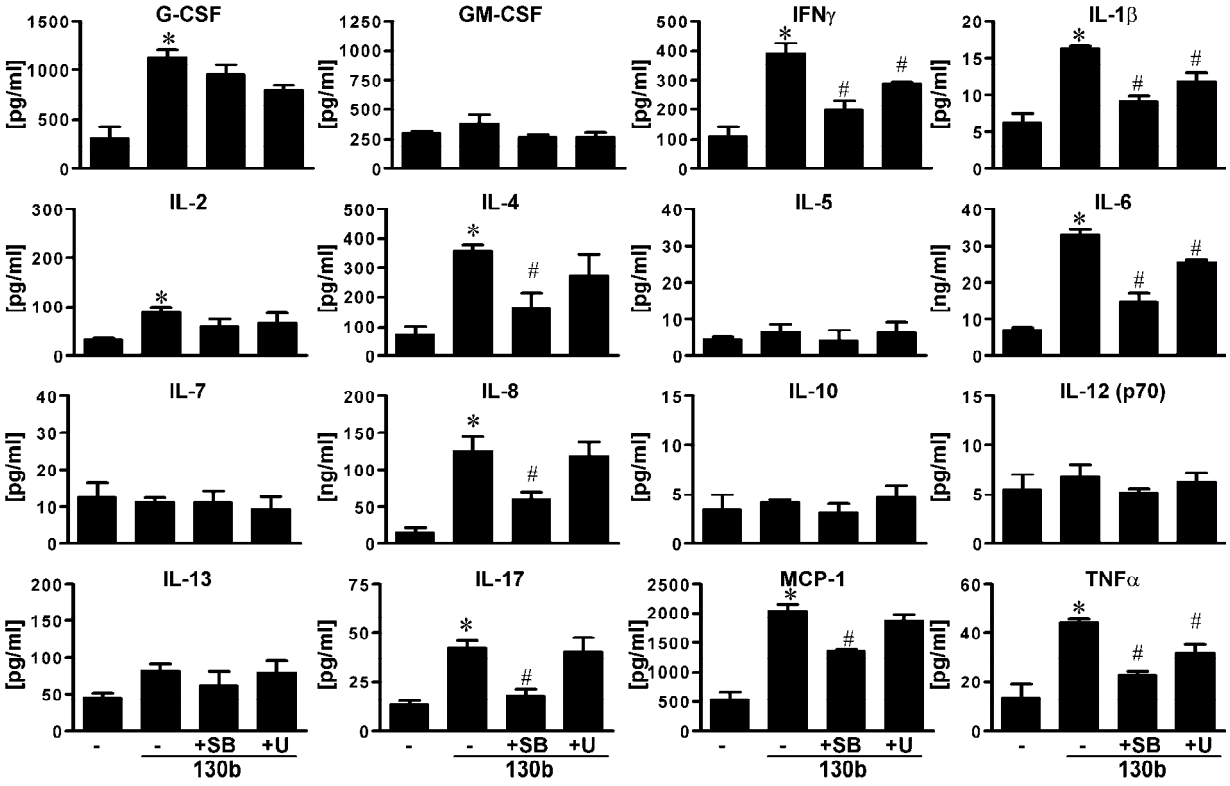

Figure 2: *L. pneumophila* strains induce flagellin-dependent, *dotA*-independent IL-8 expression by alveolar epithelial cells. A549 cells (10^5 cells/ml) were infected with *L. pneumophila* 130b (A, B, C), Philadelphia 1 (A), Corby wildtype and Corby Δ*flaA* knock out mutant (D), or JR32 wildtype and JR32 Δ*dotA* knock out mutant (E, F) with the indicated concentrations. After 15 h, IL-8 release in the supernatant was measured by ELISA (A, D, E). *, $p<0.01$ vs. uninfected

control; #, p<0.01 between different strains or wild type and mutant; n.s., no significant difference. IL-8 and GAPDH mRNA expression were determined by RT-PCR 4 h after infection with the indicated concentrations of *L. pneumophila* 130b (B) or at the indicated time points after infection with 10^7 cfu/ml *L. pneumophila* 130b (C), JR32 or JR32 *dotA* knock out mutant (F) (representative gels out of three are shown).

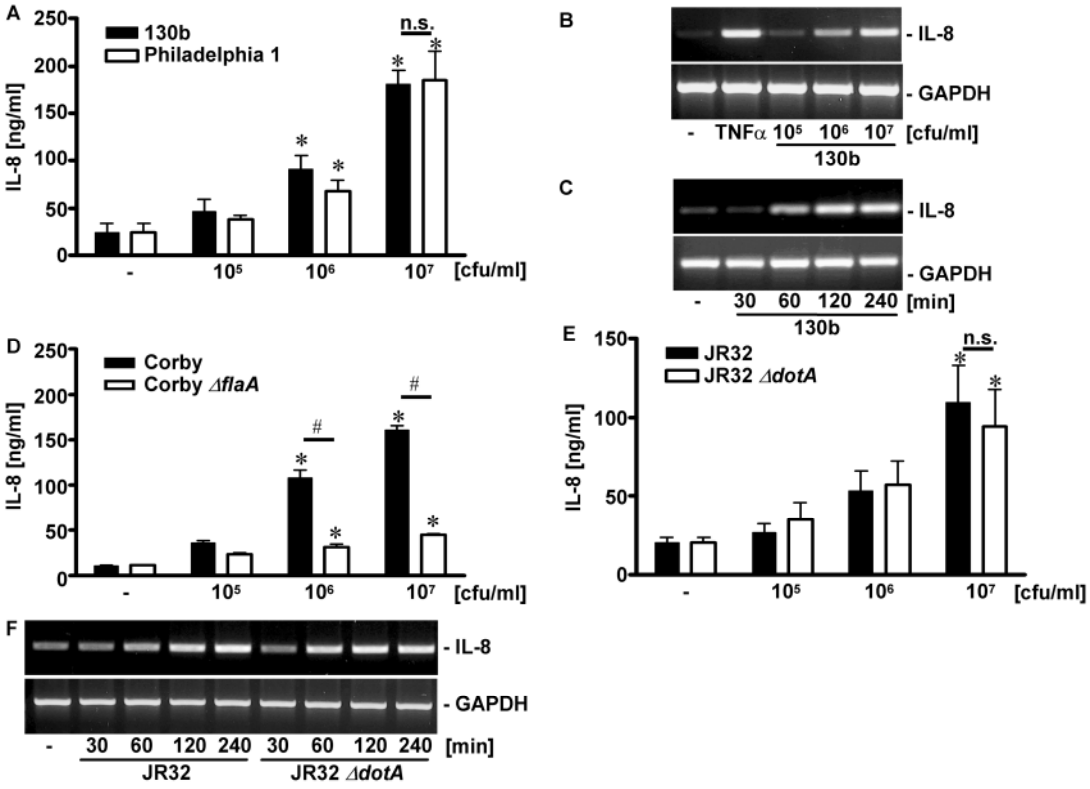

Figure 3: *L. pneumophila*-induced IL-8 release depended on p38 MAP kinase activation in alveolar epithelial cells. A549 cells (10^5 cells/ml) were infected with *L. pneumophila* 130b (10^7 cfu/ml). Phosphorylation of p38 MAP kinase (A) or ERK2-kinase (B) were determined at the indicated time points by Western blot analysis, (representative blots out of three are shown). A549 cells were pre-incubated (30 min) with 10 µM of SB202190 (C/D), SB203580 (C), and SB202474 (C) and infected with *L. pneumophila* 130b. IL-8 release in the supernatant was

measured by ELISA after 15 h (C) and IL-8 mRNA was detected after 4 h by RT-PCR (D). *, p<0.01 vs. control; #, p<0.01 vs. infected cells without pre-incubation with inhibitors at least in three independent experiments.

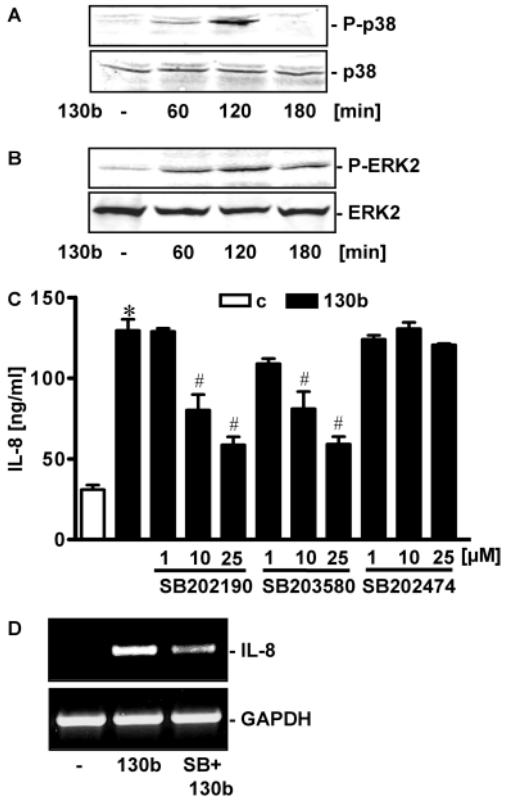

Figure 4: *L. pneumophila*-induced IL-8 release depended on NF-κB activation in alveolar epithelial cells. A549 cells (10^5 cells/ml) were pre-incubated with the indicated concentrations of IKK-NBD (A) and infected with *L. pneumophila* 130b. IL-8 release in the supernatant was measured by ELISA. *, p<0.01 vs. control; #, p<0.01 vs. infected cells without pre-incubation with inhibitors at least in three independent experiments. (B) A549 cells stably transfected with a NF-κB-dependent reportergene construct were infected with *L. pneumophila* 130b and luciferase activity was determined after 6 h. *, p<0.01 vs. uninfected cells. (C) Degradation of IκBα was determined at the indicated time points after infection with 10^7 cfu/ml *L. pneumophila* 130b by

Western blot analysis, (representative blots out 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 10^7 cfu/ml *L. pneumophila* 130b by chromatin immunoprecipoitation, (representative blots/gels out of three are shown).

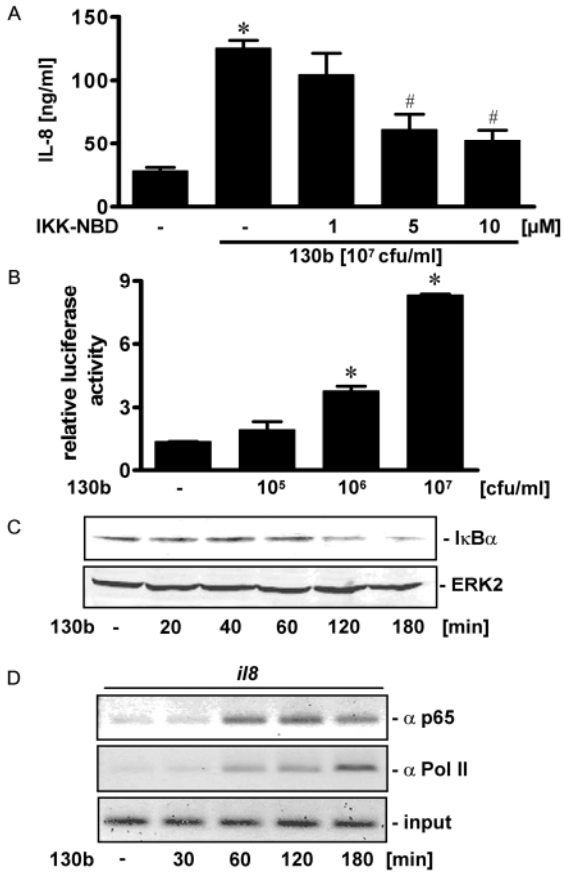

Figure 5: *L. pneumophila*-induced IL-8 release was reduced by gentamycin. A549 cells (10^5 cells/ml) were infected with *L. pneumophila* 130b. After the indicated time period, medium was changed, either with or without addition of gentamycin. IL-8 release in the supernatant was measured by ELISA after a total incubation time of 15 h. *, $p<0.01$ vs. control; #, $p<0.01$ vs. infected cells with addition of gentamycin at least in three independent experiments.

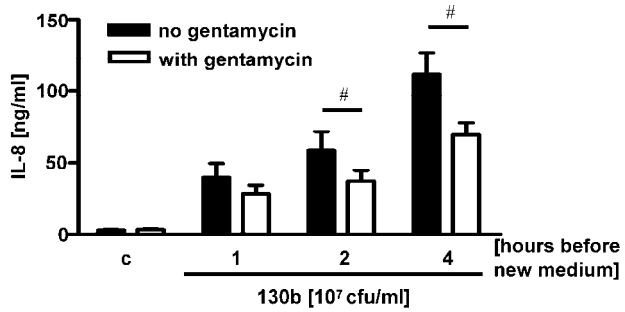

Figure 6: *L. pneumophila* induce release of a cytokine pattern differentially depending on p38 MAP or ERK1/2 kinase activity. *L. pneumophila* infection of human alveolar A549 cell line led to activation of p38 MAP and EKR1/2 kinase and cytokine release. Secretion of IL-2 and G-CSF was not effected by kinase inhibition, IL-4, IL-8, IL-17, and MCP-1 were blocked by p38 MAP kinase inhibition and IL-1β, IL-6, TNFα, and IFNγ also depended in ERK1/2 activity.