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Legionella pneumophila-induced IκBζ-dependent expression of interleukin-6 in lung epithelium

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ABSTRACT: Severe community- and hospital-acquired pneumonia is caused by *Legionella pneumophila*. Lung airway and alveolar epithelial cells comprise an important sentinel system in airborne infections. Although interleukin (IL)-6 is known as a central regulator of the immune response in pneumonia, its regulation in the lung is widely unknown.

Herein, we demonstrate that different *L. pneumophila* strains induce delayed expression of IL-6 in comparison with IL-8 by human lung epithelial cells. IL-6 expression depended, at early time points, on flagellin recognition by Toll-like receptor (TLR)5, activity of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK)1 and p38 mitogen-activated protein (MAP) kinase, and, at later time points, on the type-IV secretion system. In the same manner, but more rapidly, the recently described transcription factor IκBζ was induced by *Legionella* infection and, binding to the nuclear factor (NF)-κB subunit p50 - recruited to the *il6* promoter together with CCAAT-enhancer-binding protein β and phosphorylated activator protein-1 subunit cJun. Similarly, histone modifications and NF-κB subunit p65/RelA appeared at the *iκbζ* and subsequently at the *il6* gene promoter, thereby initiating gene expression. Gene silencing of IκBζ reduced *Legionella*-related IL-6 expression by 41%.

Overall, these data indicate a sequence of flagellin/TLR5- and type IV-dependent IκBζ expression, recruitment of IκBζ/p50 to the *il6* promoter, chromatin remodelling and subsequent IL-6 transcription in *L. pneumophila*-infected lung epithelial cells.

KEYWORDS: Cytokines, gene regulation, *Legionella*, pneumonia, signal transduction

An important causative agent of severe community-acquired pneumonia and the second most commonly detected pathogen in pneumonia in those admitted to intensive care units in industrialised countries is *Legionella pneumophila* [1, 2]. Although >40 *Legionella* species are known, the majority of human infections are caused by *L. pneumophila* serogroup 1 [3]. *L. pneumophila* is a Gram-negative, facultative intracellular pathogen of amoebae in natural and man-made aquatic environments. Infection of humans occurs after inhalation of contaminated water aerosol droplets. Recognition of *Legionella* by transmembraneous toll-like receptor (TLR)2 and TLR5 and cytosolic Naip5 (and possibly other unknown receptors) seems to activate the eukaryotic immune response [4–7].

With respect to *L. pneumophila* pathogenesis, essential results were obtained by analysing infection of protozoans or immune cells like macrophages [8, 9]. However, although *Legionella* replicates efficiently within lung epithelial cells and recent studies pointing to the lung epithelium as an important sentinel and effector of innate immunity [7, 10–16], little is known of the consequences of pulmonary epithelial cell infection with *Legionella*.

Interleukin (IL)-6 concentrations in blood and bronchoalveolar lavage fluid of patients suffering from pneumonia are positively associated with disease severity [17–19] and IL-6 promoter polymorphisms are associated with extrapulmonary dissemination in pneumococcal pneumonia [20]. Studies using IL-6-deficient mice further indicated a prominent role of the cytokine IL-6 in pneumonia [21, 22]. IL-6 influenced important innate immune mechanisms by, for example, reducing neutrophil apoptosis and increasing

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their cytotoxic capabilities [23, 24]. Its expression is regulated by several transcription factors, including nuclear factor (NF)-κB, activator protein (AP)-1, and CCAAT-enhancer-binding protein (C/EBP) [25, 26]. More recently, a critical role of inducible nuclear protein IκBζ for IL-6 expression was demonstrated [27].

Considering the important role of IL-6 in pneumonia, we here analysed mechanisms of *Legionella*-related IL-6 expression in human lung epithelial cells. *L. pneumophila* induced expression of IL-6 in a flagellin-TLR5- and type-IV secretion system-dependent manner. NF-κB-related expression of IκBζ was critical for IL-6 expression in *Legionella*-infected cells. *Legionella*-induced histone modifications and subsequent recruitment of p50 and p65/RelA to the *il6* and *iκbζ* gene promoter to initiate gene transcription. At the *il6* promotor, IκBζ and p50 interact in *Legionella*-infected cells and gene silencing of IκBζ reduced *Legionella*-related IL-6 expression. Overall, *L. pneumophila* flagellin activated the expression of IκBζ and subsequent transcription of IL-6 in human lung epithelial cells.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS) and trypsin-EDTA solution were obtained from Life Technologies (Karlsruhe, Germany). Pyrrolidine dithiocarbamate (PDTC), protease inhibitors, NP-40, Triton X-100 and Tween-20 were purchased from Sigma Chemical (Munich, Germany); MG-132 was from Calbiochem-Merck (Darmstadt, Germany) and recombinant flagellin from Alexis (Lörrach, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

Cell lines

Type-II alveolar cell line A549 was obtained from ATCC (Rockville, MD, USA) and cultured in Ham's F-12 medium with L-glutamine (PAA Laboratories, Pasching, Austria) and 10% FCS without antibiotics [28]. Primary human small airway epithelial cells (SAEC) were obtained from Clonetics/Cambrex (SAEC System; Cambrex, Baltimore, MD, USA) and cultured in SAEC BulletKit® (Clonetics/Cambrex) according to the supplier's instruction [15].

Infection with bacterial strains

L. pneumophila sg1 130b wildtype (ATCC BAA-74, kindly provided by N.P. Cianciotto, Northwestern University Medical School, Chicago, IL, USA) [29], JR32 wildtype [30] and JR32 Δ*dotA* mutant (LELA 3118, both kindly provided by H. Shuman, Columbia University, New York, NY, USA) [31], Corby wildtype and Corby Δ*flaA* mutant (both kindly provided by K. Heuner, Berlin, Germany) were routinely grown on buffered charcoal-yeast extract (BCYE) agar for 2 days at 37°C [32] and subsequently resuspended in epithelial cell medium at 37°C. Bacterial density was checked by determining the optical density at 660 nm (OD_{660}) with a Beckman spectrophotometer DU520 (Beckman Coulter, Unterschleissheim, Germany). A549 or SAEC (10^6) were infected with 10^5, 10^6 and 10^7 colony-forming units (CFU) bacteria, *i.e.* a multiplicity of infection of 0.1:1–10:1, per milliliter and incubated in infection medium (Ham's F-12 with L-glutamine without antibiotics or SAEC medium) for a given time at 37°C and 5% CO_2. 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. For heat-inactivating *L. pneumophila*, bacteria were incubated at 95°C for 30 min [14].

IL-6 ELISA

Confluent A549 cells or SAECs were infected as indicated in a humidified atmosphere. After incubation, supernatants were collected and processed for IL-6 quantification by immunoassay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN USA).

Chromatin immunoprecipitation (ChIP)

A549 cells were infected with *L. pneumophila* as indicated and then subjected to ChIP assay as previously described [14, 28, 33, 34] using anti-mRNA polymerase II (Pol II) (N-20), anti-p65 (C-20), anti-p50, anti-C/EBPβ (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), anti-P-cJun (Cell Signalling Technology, Danvers, MA, USA), anti-P^{Ser10}/Ac^{Lys14}-H3, Pan-Ac-H4 (Upstate, Lake Placid, NY, USA) and anti-IκBζ (Acris Antibodies GmbH, Hiddenhausen, Germany). The *il6* and *iκbζ* enhancer region was amplified by PCR using HotStarTaq polymerase (Qiagen, Hilden, Germany) and specific primers as followed: *il6* sense 5′-ACAAATTAACTGGAACGCT-3′, antisense 5′-ATTGGGGG TTGAGACTCTAA-3′ and *iκbζ* sense 5′-AGGGGAATGTCC GGGACT-3′, antisense 5′-TAATGTCTGACCTCGTGGCAA-3′. PCR amplification of total input DNA in each sample is shown as a control.

For Chromatin-IP-IP, DNA was eluted after first IP, refilled with ChIP-RIPA buffer and incubated with anti-IκBζ and anti-p50 antibodies, respectively for the second IP. As a control an IP with anti-sheep immunoglobulin (Ig)G (Li-COR) was performed.

Western blot

For determination of IκBζ and COX-2 induction, A549 or SAE cells were infected as indicated, washed twice, and nuclear (IκBζ, actin), membrane (TLR5) or whole cell extract (COX-2, ERK2) were harvested. Cells were lysed in buffer containing NP-40 or Triton X-100, subjected to SDS-PAGE, and blotted on nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection of target proteins was carried out with specific antibodies: IκBζ (Acris Antibodies GmbH, Hiddenhausen, Germany), COX-2 (Santa Cruz Biotechnologies, Santa Cruz, USA) and subsequently incubated with secondary antibodies (IRDye 800-labeled anti-mouse or Cy5.5-labeled anti-rabbit, respectively, and IRDye 800-labeled anti-goat) (Li-COR Inc., Bad Homburg, Germany). Simultaneous detection of actin or ERK2 (Santa Cruz Biotechnologies) by using an Odyssey infrared imaging system (Li-COR Inc.) confirmed equal protein load as described [28, 35].

RT-PCR

For analysis of IκBζ, IL-6, IL-8, granulocyte macrophage colony-stimulating factor (GM-CSF) and GAPDH gene expression in A549 and SAECs, total RNA was isolated with the RNeasy Mini kit (Qiagen) and reversely transcribed using moloney murine leukaemia virus reverse transcriptase (Invitrogen, Karlsruhe, Germany). Generated cDNA was amplified by PCR using specific primers for IκBζ (forward:

5′-TGAATGCACTTCACATGCTG-3′; reverse: 5′-TTCGTTCT CCAAGTTCCGAGT-3′), IL-6 (forward: 5′-TTCTCCACAAGC GCCTTC-3′; reverse: 5′-TGGACTGCAGGAACTCCTTA-3′), IL-8 (forward: 5′-CTAGGACAAGAGCCAGGAAGA-3′; reverse: 5′-AACCCTCTCTGCACCCAGTTTTC-3′), GM-CSF (forward: 5′-GTCTCCTGAACCTGAGTAGAGACA-3′; reverse: 5′-AAGG GGATGACAAGCAGAAAGTCC-3′), GAPDH (forward: 5′-C CACCCATGGCAAATTCCATGGCA-3′; reverse: 5′-TCTAGA CGGCAGGCAGGTCAGGTCCACC-3′). All primers were purchased from TIB MOLBIOL (Berlin, Germany). After 20–35 amplification cycles, PCR products were analysed on 1.5% agarose gels, stained with ethidium bromide, and subsequently visualised. To confirm use of equal amounts of RNA in each experiment, all samples were checked for GAPDH mRNA expression.

RNA interference

siRNA targeting IκBζ (sense: GGAAAAAGGUAAAUACAG CTT; antisense: GCUGUAUUUACCUUUUUCCTT) and TLR5 (sense: GGAGCAAUUUCCAACUUAUTT; antisense: AUAAGU UGGAAAUUGCUCCTT) were obtained from Ambion. Control non-silencing siRNA (sense: UUCUCCGAACGUGUCACGUTT; antisense: CGUGACACGUUCGGAGAATT) were purchased from MWG (Ebersberg, Germany). A549 cells were transfected by using Amaxa Nucleofector™ according to the manufacturer's protocol (Nucleofector™ Solution V, Nucleofector™ program G-16; Amaxa, Cologne, Germany) with 2 μg siRNA per 10^6 cells. For A549 cells stably expressing nonspecific or IκBζ-specific shRNA, cells were nucleofected with the IκBζ SureSilencing™ shRNA vector (pGeneClip-Neo) or an control vector (both Superarray, Frederick, MD, USA), and selected with 400 $\mu g \cdot mL^{-1}$ G418 for neomycin resistance over 4 weeks.

Statistical methods

Data are presented as mean ± SE of at least three independent experiments. Effects were statistically evaluated employing paired t-tests. A p-value <0.05 was considered to be significant (if not indicated otherwise, test was performed *versus* control).

RESULTS

L. pneumophila*-induced expression of IL-6 and IκBζ in human lung epithelial cells*

Infection of human lung epithelial cell line A549 (fig. 1a and fig. S1A in the supplementary material) with *L. pneumophila* strain 130b, strain Corby (fig. 2a and b), and strain JR32 (fig. 3a and b) as well as of primary human lung SAEC with *L. pneumophila* strain 130b (fig. 1a and fig. S1B in the supplementary material) induced the expression of IL-6 as well as of IL-8 mRNA. IL-6 protein liberation was shown in *L. pneumophila* 130b- (fig. 1c), Corby- (fig. 2c and d), and JR32-infected (fig. 3c) A549 and 130b-exposed SAEC (fig. 1d and e). Heat-inactivation of *L. pneumophila* 130b reduced, but did not block, liberation of IL-6 in infected A549 cells (fig. 1c).

As the mRNA expression of IL-6 was observed later than that for IL-8, a possible role for IκBζ protein expression in the regulation of IL-6 expression was considered, and IκBζ expression was analysed. Early induction of IκBζ mRNA (fig. 1a) and protein (fig. 1b) was observed before the start of IL-6 mRNA expression in *L. pneumophila* 130b-infected A549 and SAEC (fig. 1a) as well as in Corby- (fig. 2a and b) and JR32-infected (fig. 3a and b) A549 cells. Expression of IκBζ, IL-6 and IL-8 mRNA persisted for 24 h (fig. 1a). Thus, different strains of *L. pneumophila* induced both, IκBζ and IL-6 expression in human lung epithelial cells in a specific temporal order.

L. pneumophila*-induced expression of IL-6 and IκBζ dependent on flagellin-related activation of TLR5 and on the* Legionella *type-IVB Dot/Icm secretion system*

Detection of *Legionella* flagellin by TLR5 seems to be important for activation of eukaryotic host cells [5, 14]. Indeed, *Legionella* deficient for flagellin (FlaA) showed to be less potent with respect to the induction of mRNA for IκBζ and IL-6 as well as of IL-8, another important pro-inflammatory cytokine in pneumonia [36], especially at early time points (fig. 2b). In addition, liberation of IL-6 protein was significantly lower in cells infected with flagellin-deficient *Legionella* compared with wildtype bacteria (fig. 2c and d). As published previously, FlaA-deficient *Legionella* replicated twice as well as wildtype bacteria over 24 h in lung epithelial cells but did not alter cell death as determined by lactate dehydrogenase release (data not shown and VINZING *et al.* [37]). Addition of recombinant flagellin to cell cultures demonstrated to be sufficient to induce expression of mRNA of IκBζ, IL-6 as well as of IL-8 in human lung epithelial cells (fig. 4a). Finally, exogenously added recombinant flagellin induced IL-6 protein secretion (fig. 4b). Suppression of endogenous TLR5 expression by specific siRNA (fig. 4c) significantly reduced flagellin-related IL-6 liberation in A549 cells (fig. 4d). Overall, these data indicate a critical role of TLR5-related detection of *Legionella* flagellin for IκBζ and IL-6 expression.

Type-IVB Dot/Icm secretion system-related activity is known to be important for cytosolic recognition of *Legionella* flagellin and subsequent cell activation [38]. Upon infection of A549 cells with a *L. pneumophila* JR32 mutant deficient for *dotA*, an integral part of the *Legionella* type-IVB system [8], we observed a similar induction of IκBζ, IL-6, and IL-8 mRNA at early time points compared with infection of cells with JR32 wildtype, but a clear reduction after 5 h (fig. 3a and b) as well as reduced liberation of IL-6 (fig. 3c). Overall, intracellular, type-IV-dependent recognition of *Legionella* flagellin seems to be important for the persistent induction of IκBζ and IL-6 in A549 lung cells.

NF-κB-related transcription of cytokines and IκBζ in L. pneumophila*-infected epithelial cells*

Since NF-κB seems to be central for the expression of IκBζ and important pro-inflammatory cytokines, including IL-6, two unrelated inhibitors of the NF-κB pathway, PDTC and MG-132 [39, 40], were tested with respect to *Legionella*-related cell activation. On mRNA level, both inhibitors reduced the expression of IκBζ, IL-6, GM-CSF, and IL-8 (fig. 5a and c). Moreover, pre-incubation of cells with the inhibitors significantly reduced *L. pneumophila* 130b-induced IL-6 (fig. 5b and d) and IL-8 (data not shown) protein expression as demonstrated by ELISA indicating the central role of NF-κB pathway for *Legionella*-related epithelial cell activation.

Impact of Legionella*-related signalling on the* iκbζ *and* il6 *gene promoter*

Since our data indicated an important role of IκBζ and NF-κB in IL-6 expression, we made use of ChIP to assess the

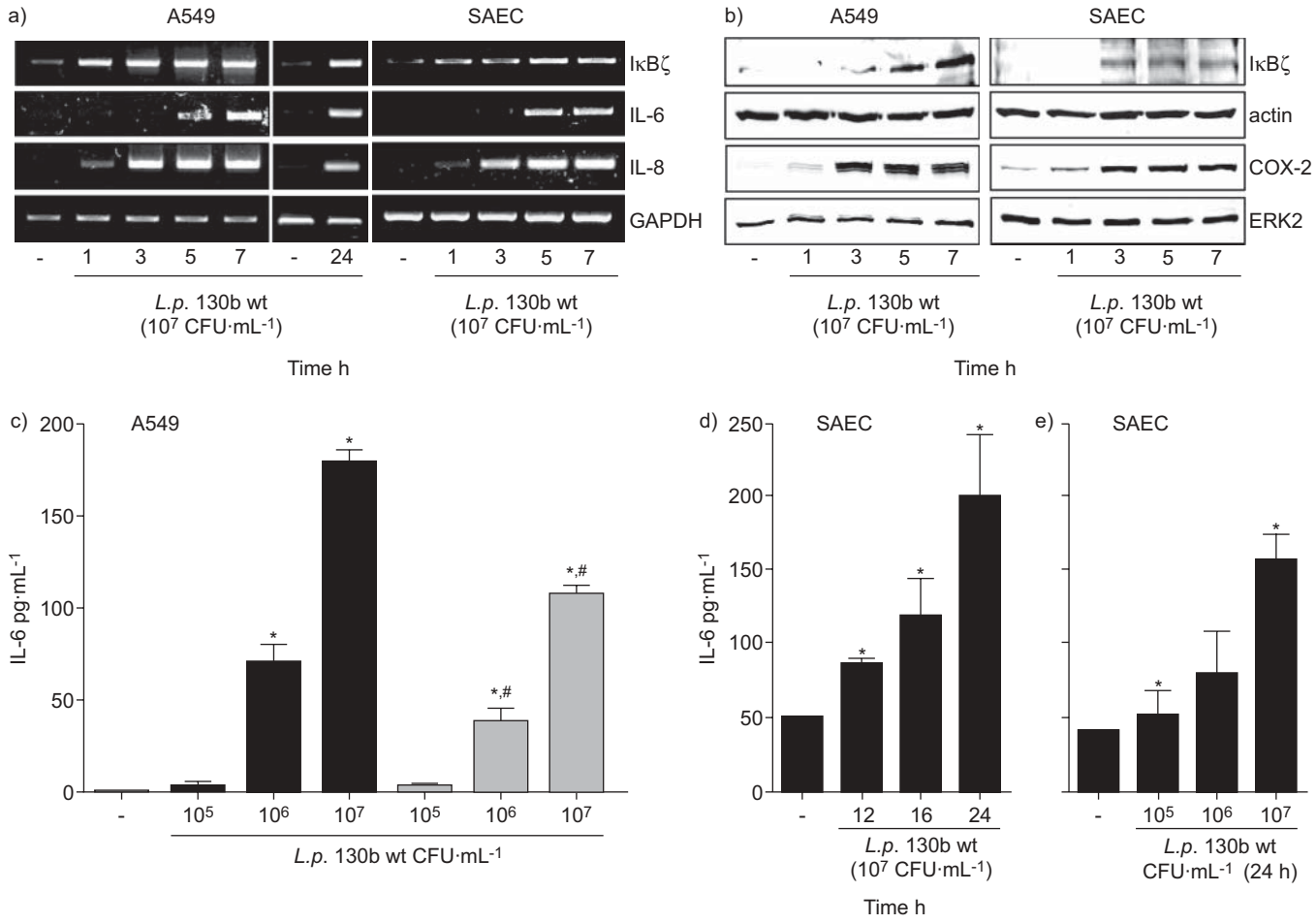

FIGURE 1. *Legionella pneumophila*-induced expression of interleukin (IL)-6 and IκBζ in human lung epithelial cells dependent on bacterial viability. Alveolar epithelial cells (A549) or human primary small airway epithelial cells (SAEC) were infected with *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1}) for a, b, d) the indicated times or c, e) with the indicated concentrations for 24 h. a) mRNA levels of IκBζ, IL-6, IL-8, and GAPDH were detected by RT-PCR, and b) protein expression of IκBζ, COX-2, actin and ERK2 were detected by western blot. Representative gels/blots of three independent experiments are shown. (c) Alveolar epithelial cells (A549) were infected with the indicated concentrations of viable (■) or heat-inactivated (■) *L. pneumophila* wildtype strain 130b for 24 h and IL-6 release was determined in the supernatant. Human primary SAEC were infected with *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1}) for d) the indicated times or e) with the indicated concentrations for 24 h, and IL-6 release was determined in the supernatant. *: $p<0.05$ unstimulated *versus* stimulated cells; $^{\#}$: $p<0.05$ viable *versus* heat-inactivated bacteria.

endogenous *iκbζ* and *il6* gene promoters in more detail in *L. pneumophila*-infected cells (fig. 6). At the *iκbζ* gene promotor, *Legionella* infection resulted in phospho-acetylation of histone H3 (P^{Ser10}/Ac^{Lys14}) and pan-acetylation of H4 indicating gene-transcription promoting remodeling of the gene (fig. 6a). Recruitment of NF-κB subunits p50 and p65 was observed and accompanied by binding of Pol II indicative for start of gene transcription (fig. 6a). At the *il6* gene promoter, we noted the same pattern of histone modification and recruitment of p50- and p65-NF-κB subunits (fig. 6b). Moreover, recruitment of IκBζ protein as well as of Pol II to the *il6* gene promoter was documented in *L. pneumophila*-infected epithelium.

Since YAMAZAKI *et al*. [41] evidenced strong interaction of IκBζ with p50, but weak binding to p65, we tested the interaction of both proteins (IκBζ and p50) at the *il6* gene promoter in *Legionella*-exposed cells. In ChIP-IP, experiments we found an association of endogenous p50 and IκBζ proteins indicating cooperative action of both molecules at the *il6* gene promoter (fig. 6c).

Finally, we wondered if a defect in *Legionella* flagellin expression would result in reduced stimulation of transcription factor and Pol II recruitment to both gene promoters. As it is shown in fig. 6d, flagellin-deficient *Legionella* induced weaker p65, p50 and Pol II recruitment to the *iκbζ* gene promoter. Along those lines, reduced p65, p50, and IκBζ binding and Pol II recruitment was also demonstrated at the *il6* gene promoter (fig. 6e). Quantification of the gels in figure 6d and e showed a statistically significant decrease for Pol II as well as for all transcription factors (data not shown).

Depletion of IκBζ reduced IL-6 expression in L. pneumophila*-infected epithelial cells*

To further analyse the role of IκBζ in *Legionella*-related IL-6 expression, we made use of IκBζ siRNA. IκBζ-specific, but not control siRNA, reduced IκBζ as well as IL-6 mRNA expression in *L. pneumophila* infected A549 cells (fig. 7a). Furthermore, we observed significant reduction of IL-6 protein expression (fig. 7b). The relatively low effect on the protein level may be

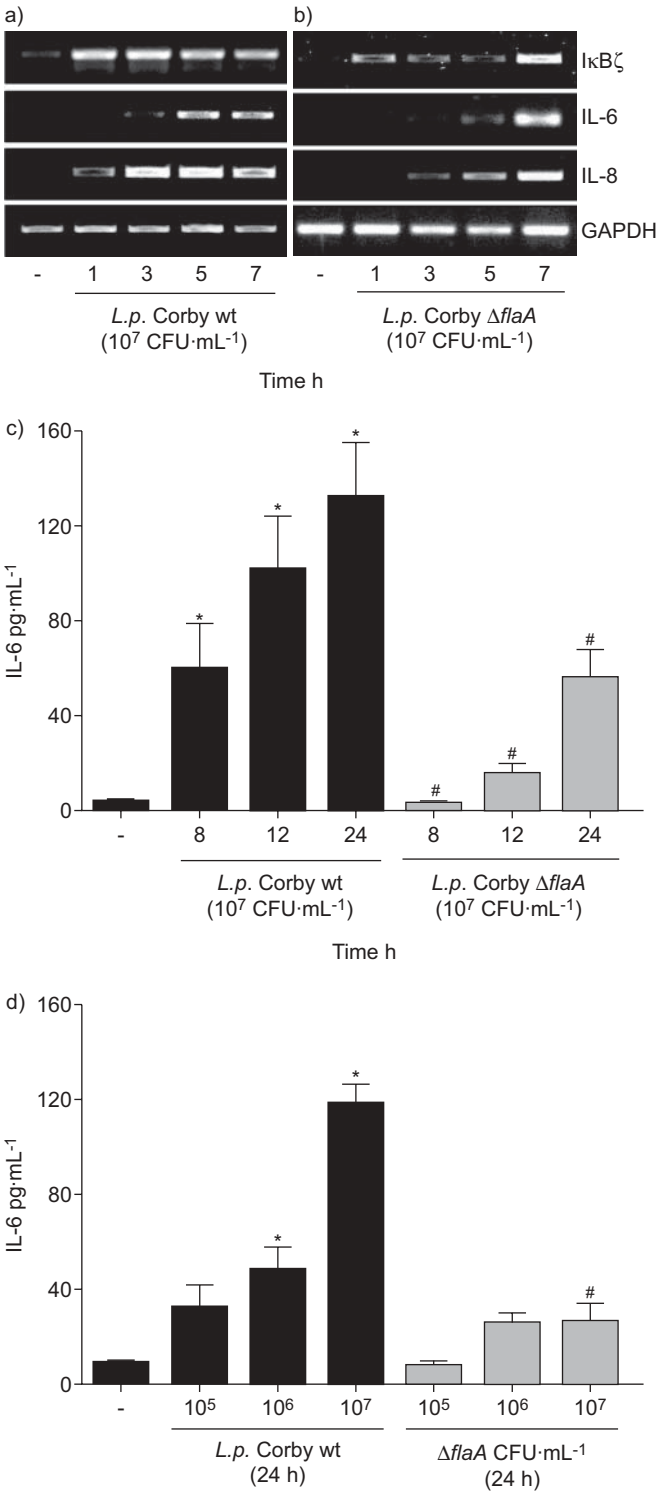

FIGURE 2. *Legionella pneumophila* induced expression of interleukin (IL)-6 and IκBζ dependent on flagellin. a) Alveolar epithelial cells (A549) were infected with *L. pneumophila* wildtype strain a) Corby or b) *ΔflaA*-knock out mutant (10^7 CFU·mL^{-1}) for the indicated times and mRNA expression was determined by RT-PCR. Representative gels of three independent experiments were shown. A549 cells were infected with *L. pneumophila* wild type strain Corby (■) or *ΔflaA*-knock out mutant (■)(10^7 CFU·mL^{-1}) for c) the indicated times or d) with the indicated concentrations for 24 h, and IL-6 release was determined in the supernatant. *: $p<0.05$ unstimulated *versus* stimulated cells; #: $p<0.05$ Corby *versus* Corby*ΔflaA*.

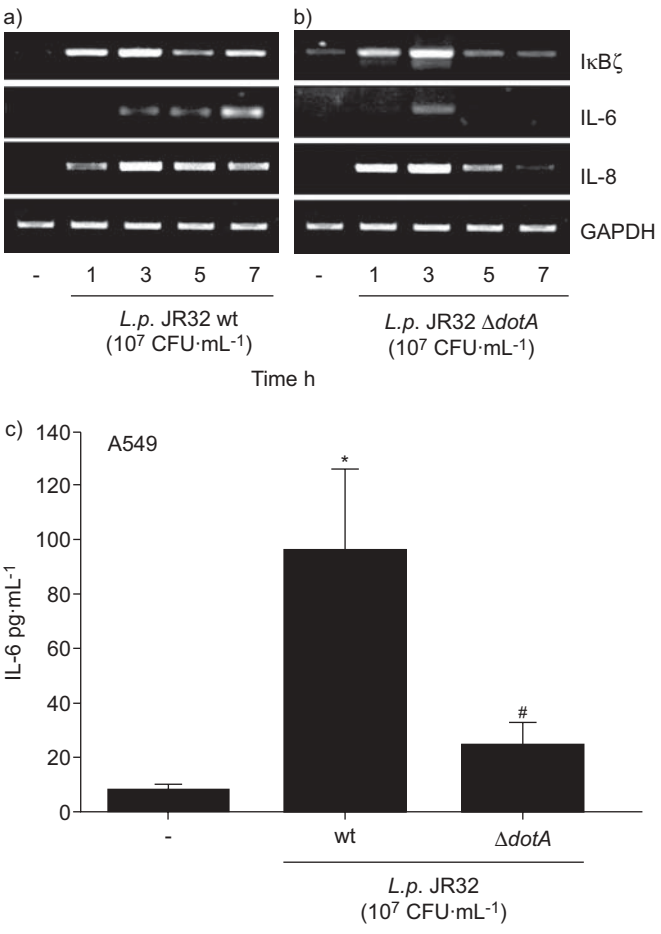

FIGURE 3. *Legionella pneumophila*-induced expression of interleukin (IL)-6 and IκBζ dependent on the *Legionella* type-IVB Dot/Icm secretion system. a) Alveolar epithelial cells (A549) were infected with *L. pneumophila* a) wildtype strain JR32 or b) *ΔdotA*-knock-out mutant (10^7 CFU·mL^{-1}) for the indicated times and mRNA expression was determined by RT-PCR. Representative gels of three independent experiments were shown. c) A549 cells were infected with *L. pneumophila* wild type strain JR32 or *ΔdotA*-knock out mutant (10^7 CFU·mL^{-1}) for 24 h and IL-6 release was determined in the supernatant. *: $p<0.05$ unstimulated *versus* stimulated cells; #: $p<0.05$ *L. pneumophila* JR32 *versus* JR32*ΔdotA*.

due to limited transfection efficiency and thus be overcome by siRNA-unaffected cells. To rule out effects of incomplete nucleofection, we created A549 cells stably expressing unspecific or IκBζ-specific shRNA. Cells were infected with *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1}) for 24 h and IL-6 release was detected by ELISA (fig. 7c). In IκBζ-depleted cells, *L. pneumophila*-induced IL-6 release was reduced by 41% in comparison to cells expressing unspecific shRNA.

Inhibition of MEK1- and p38 MAP kinase pathway blocked expression of IL-6 but not IκBζ

Besides IκBζ and the canonical NF-κB pathway, several kinase pathways were suspected to be important for bacteria-related activation of eukaryotic cells [10, 14, 42]. In particular, their role in *Legionella*-related IκBζ and IL-6 expression is unknown. Therefore, we made use of several chemical inhibitors for important kinases to test their role in IκBζ-, and cytokine-expression in *Legionella* infected cells (fig. 8a and fig. S2 in the

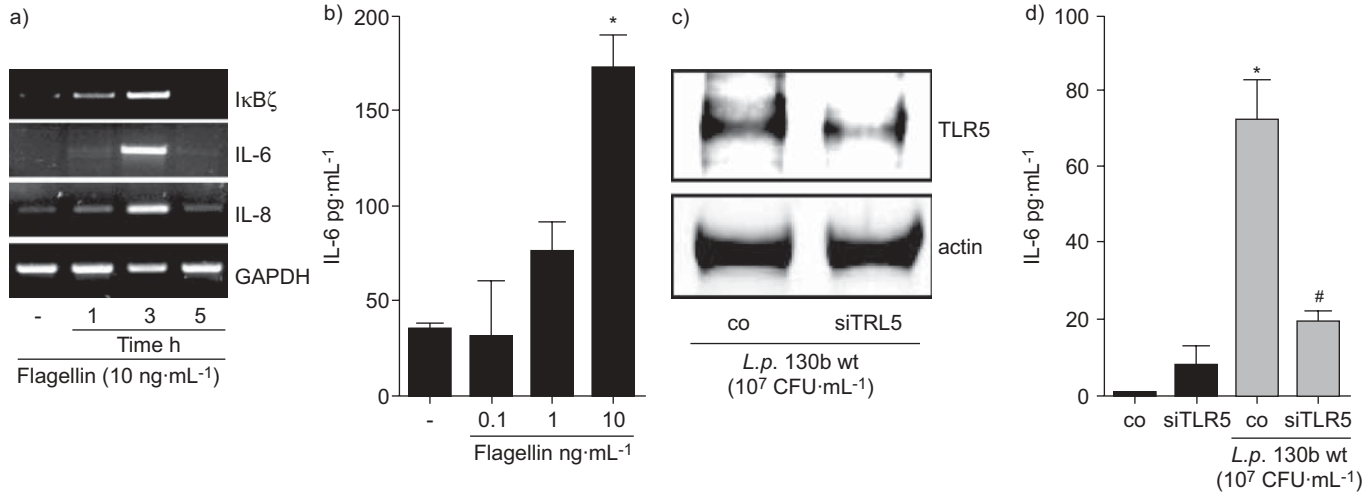

FIGURE 4. *Legionella pneumophila* induced expression of interleukin (IL)-6 and IκBζ activation of Toll-like receptor (TLR)5. A549 cells were stimulated with flagellin (10 ng·mL^{-1}) for a) the indicated times and mRNA expression was determined, or b) with the indicated concentrations for 24 h, and IL-6 release was determined in the supernatant. A549 cells were nucleofected with unspecific (co) or TLR5-specific (siTLR5) siRNA and after 48 h infected with *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1}) for 24 h. c) TLR5 expression was determined by western blot and d) IL-6 release by ELISA with (■) and without (■) *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1}). *: $p<0.05$ unstimulated *versus* stimulated cells; #: $p<0.05$ unspecific *versus* TLR5 siRNA. In c), representative blots of three independent experiments were shown.

supplementary material). Inhibition of MEK1 kinase (U0126, 10 μM) and p38 MAP kinase (SB202190, 10 μM) both blocked *L. pneumophila*-related IL-6, GM-CSF and IL-8 expression. Blocking of PI3 kinase (Ly294002, 10 μM) and JAK isoforms 1–3 (JAK inhibitor I, 10 μM) prevented expression of IL-6 and GM-CSF, but not of IL-8 in *Legionella*-infected cells. However, inhibitors of PKC (Gö6976, 10 μM) and JNK (SP600125, 10 μM) only reduced GM-CSF expression. Remarkably, neither inhibitor showed an effect on *L. pneumophila*-induced IκBζ expression in lung epithelial cells although effective kinase inhibition was indicated by effects of the inhibitors on the addressed cytokines.

Transcription factors C/EBPβ and AP-1 have been implicated in IL-6 expression and are downstream targets of MEK1 and p38 MAP kinase, respectively [43]. After infection with *L. pneumophila*, an early recruitment of both C/EBPβ and the phosphorylated form of the AP-1 subunit cJun occurred at the *il6* promoter (fig. 8b and c). Inhibition of MEK1 blocked

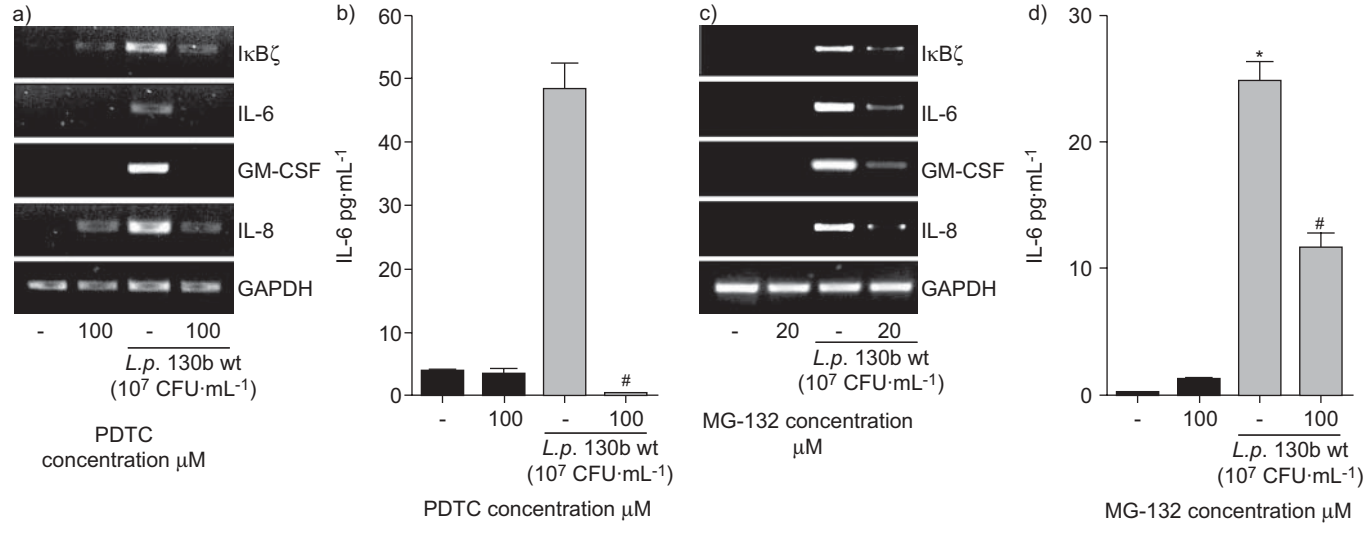

FIGURE 5. Nuclear factor (NF)-κB-related transcription of cytokines and IκBζ in *Legionella pneumophila*-infected epithelial cells. Alveolar epithelial cells (A549) were preincubated for 30 min with inhibitors a, b) PDTC (100 μM) or c, d) MG-132 (indicated concentration) and then infected with *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1})(■). After 7 h, mRNA expression was determined by RT-PCR (a and c). a, c) Representative gels of three independent experiments are shown. After 24 h, interleukin (IL)-6 release was determined in the supernatant (b and d). *: $p<0.05$ unstimulated *versus* stimulated cells; #: $p<0.05$ cells with *versus* without inhibitor.

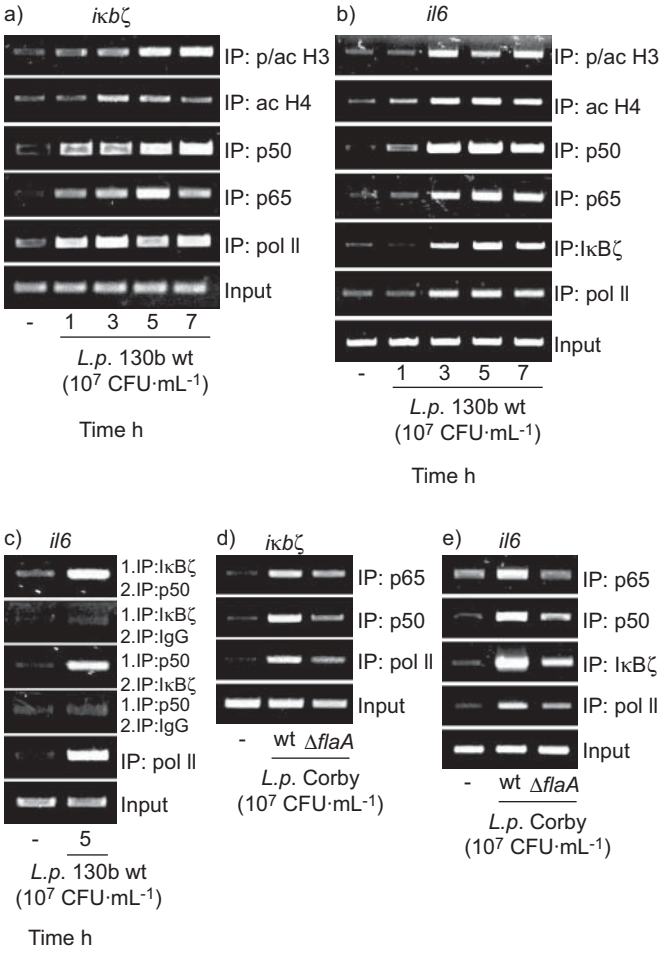

FIGURE 6. Impact of *Legionella*-related signalling on the *iκbζ* and *il6* gene promoter. A549 cells were infected with 10^7 CFU·mL^{-1} *Legionella pneumophila* strains 130b (a–c) or Corby wildtype (wt)/*ΔflaA*-knock out deletion mutant (*ΔflaA*) (d and e) for the indicated times (a and b), or for 5 h (c–e). Chromatin immunoprecipitation (ChIP) was performed against the indicated targets on the endogenous promoters of *il6* (b, c and e) or *iκbζ* (a and d). Representative gels of three independent experiments are shown.

L. pneumophila-induced C/EBPβ recruitment to the *il6* promoter (fig. 8b).

DISCUSSION

Here we demonstrate that *L. pneumophila* induced flagellin-dependent IκBζ expression and subsequent IL-6 expression in human lung epithelial cells. *Legionella* induced histone modifications and recruitment of p50 and p65/RelA to the *iκbζ* and subsequently to the *il6* gene promoter to initiate gene transcription. Both, IκBζ and p50 interact at the *il6* gene promotor in *Legionella*-infected cells and gene silencing of IκBζ reduced *Legionella*-related IL-6 expression.

Little is known about the molecular mechanisms of host–pathogen interaction in the lung. This limits the development of innovative intervention strategies in pneumonia despite the emergence of antibiotic-resistant bacteria complicating antibiotic therapy. Therefore, we aimed to elucidate mechanisms of IL-6 expression in *Legionella* infection.

Expression of the pleiotropic cytokine IL-6 during pneumonia is suspected to contribute to the local control of infection and inflammation [20–22] by regulating for example neutrophil recruitment and function [23, 24]. The development of interstitial pneumonia in transgenic mice overexpressing human IL-6 highlights the power of IL-6 to contribute to inflammatory processes in the lung [44]. Infection of lung epithelial cells by *L. pneumophila* strains 130b, JR32 and Corby (this study, [15, 45]) as well as of murine macrophages [46] resulted in strong release of IL-6.

Detection of *Legionella* flagellin by host cell pattern recognition receptors (PRR) seems to be a critical step in legionellosis. At early infection timepoints, flagellin is recognised by transmembraneous TLR5 [47] and a common dominant TLR5 stop codon polymorphism is associated with susceptibility to Legionnaires' disease in humans [5]. Recombinant flagellin induced IL-6 expression, whereas flagellin-deficient *L. pneumophila* showed reduced IL-6 induction and TLR5-depletion in eukaryotic cells reduced *Legionella*-related IL-6 expression indicating flagellin-TLR5-related induction of IL-6. In addition, cytosolic recognition of flagellin monomers by Naip5 in macrophages contributes to the restriction of *L. pneumophila* infection in mice [38]. Noteworthy, the difference of mRNA levels of IκBζ and of IL-6, induced by wildtype or flaA-deletion mutants disappear after 7 h. As the difference in IL-6 protein release persists for 24 h, an additional mechanism, *e.g.* in translation or release of IL-6 might be involved. In addition, a role of TLR2 can not be ruled out from these data [48].

Flagellin is suspected to reach the host cell cytoplasm by type-IV secretion system-induced perforation of phagosomes [38], and there activating, for example, NAIP5 or IPAF [37]. In agreement, we observed reduced IL-6 mRNA expression at later time points in cells infected with *Legionella* strain JR32 lacking DotA, an integral part of the type-IVB system in these bacteria [8]. Overall, extracellular and possibly intracellular recognition of *L. pneumophila* seems to be important for IL-6 expression in lung epithelium. Interestingly, *Legionella longbeachae* (the common cause of Legionellosis in Western Australia) which contains flagellin but no detectable pore-forming activity, does not activate Naip5-related activity, thus implicating possible important strain-specific effects in *Legionella*-related cell activation [49].

Expression of IL-6 is regulated by several transcription factors, including NF-κB, AP-1, and C/EBP [25, 26]. NF-κB-related gene expression is observed in *L. pneumophila*-infected lung cells [7, 11, 15]. In addition, transcription factors AP-1/cJun and C/EBPβ are recruited to the *il6* promoter after infection with *L. pneumophila*. However, much less is known about the role of the inducible nuclear protein IκBζ in lung infection, which seems to be critical for IL-6 expression [27]. We observed induction of IκBζ in *L. pneumophila* 130b-, Corby-, and JR32-infected A549 and primary human lung epithelial cells. Its expression persisted for 24 hours. In line with the study of YAMAMOTO *et al.* [27], experiments using recombinant flagellin, and flagellin-deficient *L. pneumophila* indicated flagellin-related induction of IκBζ in lung epithelium. Analysis of host cell signalling pathways demonstrated NF-κB dependency of IκBζ expression. However, although inhibition of MEK1, p38, JAK or PI3 kinase suppressed IL-6

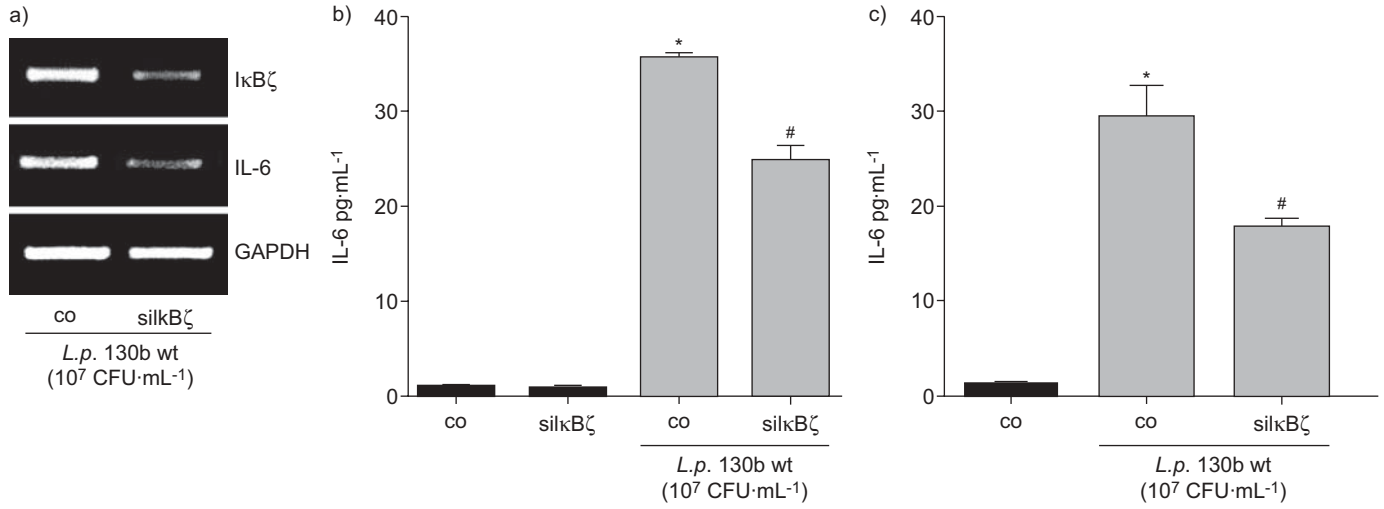

FIGURE 7. Depletion of IκBζ reduced interleukin (IL)-6 expression in *Legionella pneumophila*-infected epithelial cells. (a, b) A549 cells were nucleofected with unspecific (co) or IκBζ-specific (siIκBζ) siRNA and after 36 h infected with *L. pneumophila* wildtype strain 130b (10^7 CFU·mL^{-1}) for 24 h. IκBζ expression was determined by a) RT-PCR and b) IL-6 release by ELISA. c) A549 stably expressing unspecific (co) or IκBζ-specific shRNA (shIκBζ) were infected with *L. pneumophila* wt strain 130b (10^7 CFU·mL^{-1}) for 24 h and IL-6 release was detected by ELISA. *: $p<0.05$ unstimulated *versus* stimulated cells; #: $p<0.05$ unspecific *versus* IκBζ si/shRNA. In a), representative gels of three independent experiments were shown.

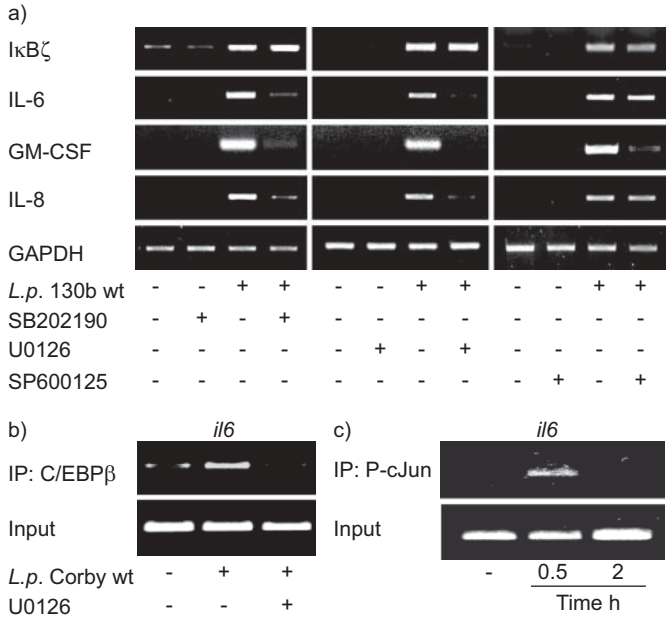

FIGURE 8. Expression of interleukin (IL)-6, but not IκBζ mRNA, depended on p38 MAP kinase and MEK1. a) Alveolar epithelial cells (A549) were preincubated for 120 min with inhibitors SB202190 (10 μM), U0126 (10 μM) or SP600125 (10 μM) and then infected with *L. pneumophila* (*L. p.*) wildtype strain 130b (10^7 CFU·mL^{-1}). After 7 h, mRNA expression was determined by RT-PCR. Representative gels of three independent experiments were shown. A549 cells were infected with 10^7 CFU·mL^{-1} *L. pneumophila* Corby for 30 min b) after preincubation for 120 min with inhibitor U0126 (10 μM) or c) for the indicated times without preincubation. ChIP was performed against the indicated targets on the endogenous promoter of *il6*. Representative gels of three independent experiments are shown.

expression, we observed no effect on IκBζ expression. In addition, *L. pneumophila*-induced recruitment of C/EBPβ to the *il6* promoter was blocked by MEK1 inhibitor U0126. These data suggest that kinase-related, IκBζ-independent signals also contributed to IL-6 expression in *L. pneumophila*-infected epithelium. In addition, PKC and JNK pathway neither contributed to IL-6 nor IκBζ expression, but reduced *Legionella*-induced GM-CSF expression. Both kinases have been shown to play important roles in the activation of epithelial cells and macrophages, respectively [11, 50].

Upon infection of cells with *L. pneumophila*, histone H3 gets phosphoacetylated and H4 acetylated at the *ikbζ* gene promoter, indicative of chromatin remodeling associated with transcriptional activity [14, 33, 34, 51]. This was accompanied by recruitment of p50 and p65 NF-κB subunits, which are known to be important for IκBζ expression [52]. In addition to these modifications and recruitments also documented at the *il6* gene promoter, we observed IκBζ binding at the *il6* gene promoter in *L. pneumophila*-infected epithelial cells.

Recently, KAYAMA *et al.* [52] demonstrated that LPS-induced histone H3 trimethylation on a subset of promoters depended on IκBζ in mouse macrophages. Together with our results, it seems possible that IκBζ is involved in TLR- or NLR-induced nucleosome remodeling regulating the expression of secondary genes. This might also explain the species- and cell type-dependent degree of IκBζ-dependence [27].

IκBζ seems to preferentially interact with NF-κB subunit p50 [41]. In fact, interaction of p50 with IκBζ was observed at the *il6* gene promoter in *Legionella*-exposed cells by Chromatin-IP-IP. Silencing of IκBζ expression by specific siRNA or shRNA resulted in decreased, but not completely abolished, IL-6 expression in *L. pneumophila*-infected cells, suggesting that IκBζ plays an important but not indispensable role in IL-6 expression in the system tested.

Finally, flagellin-deficient *L. pneumophila* induced weaker transcription factor recruitment and Pol II binding at both, the *iκbζ* and *il6* gene promoter, further highlighting the role of flagellin in activation of gene transcription. Overall, these data indicate a sequence of flagellin-dependent chromatin remodeling, p50, p65, and Pol II recruitment, IκBζ expression, and subsequent IL-6 expression in *L. pneumophila*-infected lung epithelial cells. In addition, other pathways like MEK-1-C/EBPβ and p38 MAP kinase-AP-1 as well as the canonical NF-κB pathway seem to be involved in IL-6 expression.

From the observation of the complex nature of *Legionella*-related IL-6 expression several questions arise: For example, which cytosolic receptors contribute to IκBζ and IL-6 expression? In addition, besides the contribution of NF-κB molecules, the signalling mechanisms leading to IκBζ expression are widely unknown and different kinase pathways could be excluded in this study. Besides *L. pneumophila*, other important pathogens which do not express flagella (*e.g.* pneumococci) cause pneumonia and induce IL-6 expression [42]. Furthermore, although *e.g.* the important role of IL-6 for pneumococci dissemination [21, 22] is known, underlying molecular mechanisms of pneumococci-related IL-6 (and potentially IκBζ) expression are unknown. Finally, *in vivo* studies with IκBζ deficient mice will help to dissect its role in pneumonia.

In conclusion, our data highlighted a complex pathway of IL-6 induction in human lung epithelium, composed of early flagellin-TLR5-dependent histone modifications, IκBζ expression, and subsequent IκBζ-p50-related IL-6 expression.

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STATEMENT OF INTEREST

None declared.

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REFERENCES

1 Vergis EN, Akbas EA, Yu VL. *Legionella* as a cause of severe pneumonia. *Sem Resp Crit Care Med* 2000; 21: 295–304.
2 Sabria M, Campins M. Legionnaires' disease: update on epidemiology and management options. *Am J Respir Med* 2003; 2: 235–243.
3 Swanson MS, Hammer BK. *Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. *Annu Rev Microbiol* 2000; 54: 567–613.
4 Girard R, Pedron T, Uematsu S, *et al.* Lipopolysaccharides from *Legionella* and *Rhizobium* stimulate mouse bone marrow granulocytes *via* Toll-like receptor 2. *J Cell Sci* 2003; 116: 293–302.
5 Hawn TR, Verbon A, Lettinga KD, *et al.* A common dominant TLR5 stop codon polymorphism abolishes flagellin signalling and is associated with susceptibility to legionnaires' disease. *J Exp Med* 2003; 198: 1563–1572.
6 Ren T, Zamboni DS, Roy CR, *et al.* Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog* 2006; 2: e18.
7 Opitz B, Vinzing M, van Laak V, *et al. Legionella pneumophila* induces IFNbeta in lung epithelial cells *via* IPS-1 and IRF3, which also control bacterial replication. *J Biol Chem* 2006; 281: 36173–36179.
8 Neild AL, Roy CR. Immunity to vacuolar pathogens: What can we learn from *Legionella*? *Cell Microbiol* 2004; 6: 1011–1018.
9 Molmeret M, Bitar DM, Han L, *et al.* Cell biology of the intracellular infection by *Legionella pneumophila*. *Microbes Infect* 2004; 6: 129–139.
10 Schmeck B, Zahlten J, Moog K, *et al. Streptococcus pneumoniae*-induced p38 MAPK-dependent phosphorylation of RelA at the interleukin-8 promotor. *J Biol Chem* 2004; 279: 53241–53247.
11 N'Guessan PD, Etouem MO, Schmeck B, *et al.* Legionella pneumophila-induced PKC α-, MAPK-, and NF-κB-dependent COX-2 expression in human lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: L267–L277.
12 Hippenstiel S, Opitz B, Schmeck B, *et al.* Lung epithelium as a sentinel and effector system in pneumonia – molecular mechanisms of pathogen recognition and signal transduction. *Respir Res* 2006; 7: 97.
13 Broide DH, Lawrence T, Doherty T, *et al.* Allergen-induced peribronchial fibrosis and mucus production mediated by IκB kinase β-dependent genes in airway epithelium. *Proc Natl Acad Sci USA* 2005; 102: 17723–17728.
14 Schmeck B, Lorenz J, N'Guessan PD, *et al.* Histone acetylation and flagellin are essential for *Legionella pneumophila*-induced cytokine expression. *J Immunol* 2008; 181: 940–947.
15 Schmeck B, N'Guessan PD, Ollomang M, *et al. Legionella pneumophila*-induced NF-κB- and MAPK-dependent cytokine release by lung epithelial cells. *Eur Respir J* 2007; 29: 25–33.
16 Strieter RM, Belperio JA, Keane MP. Host innate defenses in the lung: the role of cytokines. *Curr Opin Infect Dis* 2003; 16: 193–198.
17 Marik PE, Zaloga GP. Hypothermia and cytokines in septic shock. Norasept II Study Investigators. North American study of the safety and efficacy of murine monoclonal antibody to tumor necrosis factor for the treatment of septic shock. *Intensive Care Med* 2000; 26: 716–721.
18 Antunes G, Evans SA, Lordan JL, *et al.* Systemic cytokine levels in community-acquired pneumonia and their association with disease severity. *Eur Respir J* 2002; 20: 990–995.
19 Kolsuz M, Erginel S, Alatas O, *et al.* Acute phase reactants and cytokine levels in unilateral community-acquired pneumonia. *Respiration* 2003; 70: 615–622.
20 Schaaf B, Rupp J, Muller-Steinhardt M, *et al.* The interleukin-6 -174 promoter polymorphism is associated with extrapulmonary bacterial dissemination in *Streptococcus pneumoniae* infection. *Cytokine* 2005; 31: 324–328.
21 Bergeron Y, Ouellet N, Deslauriers AM, *et al.* Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice. *Infect Immun* 1998; 66: 912–922.
22 van der PT, Keogh CV, Guirao X, *et al.* Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia. *J Infect Dis* 1997; 176: 439–444.
23 Biffl WL, Moore EE, Moore FA, *et al.* Interleukin-6 delays neutrophil apoptosis *via* a mechanism involving platelet-activating factor. *J Trauma* 1996; 40: 575–578.
24 Mullen PG, Windsor AC, Walsh CJ, *et al.* Tumor necrosis factor-α and interleukin-6 selectively regulate neutrophil function *in vitro*. *J Surg Res* 1995; 58: 124–130.

25 Baccam M, Woo SY, Vinson C, *et al.* CD40-mediated transcriptional regulation of the IL-6 gene in B lymphocytes: involvement of NF-κB, AP-1, and C/EBP. *J Immunol* 2003; 170: 3099–3108.

26 Mann J, Oakley F, Johnson PW, *et al.* CD40 induces interleukin-6 gene transcription in dendritic cells: regulation by TRAF2, AP-1, NF-κB, AND CBF1. J Biol Chem 2002; 277: 17125–17138.

27 Yamamoto M, Yamazaki S, Uematsu S, *et al.* Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IκBζ. *Nature* 2004; 430: 218–222.

28 Schmeck B, Gross R, N'Guessan PD, *et al. Streptococcus pneumoniae*-induced caspase 6-dependent apoptosis in lung epithelium. *Infect Immun* 2004; 72: 4940–4947.

29 Edelstein PH, Nakahama C, Tobin JO, *et al.* Paleoepidemiologic investigation of Legionnaires disease at Wadsworth Veterans Administration Hospital by using three typing methods for comparison of legionellae from clinical and environmental sources. *J Clin Microbiol* 1986; 23: 1121–1126.

30 Wiater LA, Sadosky AB, Shuman HA. Mutagenesis of *Legionella pneumophila* using Tn903 dlllacZ: identification of a growth-phase-regulated pigmentation gene. *Mol Microbiol* 1994; 11: 641–653.

31 Sadosky AB, Wiater LA, Shuman HA. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect Immun* 1993; 61: 5361–5373.

32 Edelstein PH. Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. *J Clin Microbiol* 1981; 14: 298–303.

33 Schmeck B, Beermann W, van Laak V, *et al.* Intracellular bacteria differentially regulated endothelial cytokine release by MAPK-dependent histone modification. *J Immunol* 2005; 175: 2843–2850.

34 Schmeck B, Beermann W, N'Guessan PD, *et al.* Simvastatin reduces *Chlamydophila pneumoniae*-mediated histone modifications and gene expression in cultured human endothelial cells. *Circ Res* 2008; 102: 888–895.

35 Opitz B, Puschel A, Schmeck B, *et al.* Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae*. *J Biol Chem* 2004; 279: 36426–36432.

36 Strieter RM. Interleukin-8: a very important chemokine of the human airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 2002; 283: L688–L689.

37 Vinzing M, Eitel J, Lippmann J, *et al.* NAIP and Ipaf control *Legionella pneumophila* replication in human cells. *J Immunol* 2008; 180: 6808–6815.

38 Molofsky AB, Byrne BG, Whitfield NN, *et al.* Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J Exp Med* 2006; 203: 1093–1104.

39 Schreck R, Meier B, Mannel DN, *et al.* Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. *J Exp Med* 1992; 175: 1181–1194.

40 Rock KL, Gramm C, Rothstein L, *et al.* Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 1994; 78: 761–771.

41 Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-κB in the nuclei. *J Biol Chem* 2001; 276: 27657–27662.

42 Schmeck B, Moog K, Zahlten J, *et al. Streptococcus pneumoniae* induced c-Jun-N-terminal kinase- and AP-1-dependent IL-8 release by lung epithelial BEAS-2B cells. *Respir Res* 2006; 7: 98.

43 Xiao W, Hodge DR, Wang L, *et al.* Co-operative functions between nuclear factors NFκB and CCAT/enhancer-binding protein-β (C/EBP-β) regulate the IL-6 promoter in autocrine human prostate cancer cells. *Prostate* 2004; 61: 354–370.

44 Suematsu S, Matsuda T, Aozasa K, *et al.* IgG1 plasmacytosis in interleukin 6 transgenic mice. *Proc Natl Acad Sci USA* 1989; 86: 7547–7551.

45 Chang B, Amemura-Maekawa J, Kura F, *et al.* Expression of IL-6 and TNF-α in human alveolar epithelial cells is induced by invading, but not by adhering, *Legionella pneumophila*. *Microb Pathog* 2004; 37: 295–302.

46 McHugh SL, Yamamoto Y, Klein TW, *et al.* Murine macrophages differentially produce proinflammatory cytokines after infection with virulent *versus* avirulent Legionella pneumophila. *J Leukoc Biol* 2000; 67: 863–868.

47 Hayashi F, Smith KD, Ozinsky A, *et al.* The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* 2001; 410: 1099–1103.

48 Fuse ET, Tateda K, Kikuchi Y, *et al.* Role of Toll-like receptor 2 in recognition of *Legionella pneumophila* in a murine pneumonia model. *J Med Microbiol* 2007; 56: 305–312.

49 Asare R, Santic M, Gobin I, *et al.* Genetic susceptibility and caspase activation in mouse and human macrophages are distinct for *Legionella longbeachae* and *L. pneumophila*. *Infect Immun* 2007; 75: 1933–1945.

50 Shin S, Case CL, Archer KA, *et al.* Type IV secretion-dependent activation of host MAP kinases induces an increased proinflammatory cytokine response to *Legionella pneumophila*. *PLoS Pathog* 2008; 4: e1000220.

51 Muegge K. Preparing the target for the bullet. *Nat Immunol* 2002; 3: 16–17.

52 Kayama H, Ramirez-Carrozzi VR, Yamamoto M, *et al.* Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IκBζ. *J Biol Chem* 2008; 283: 12468–12477.