PKCα and PKCε differentially regulate *Legionella pneumophila*-induced GM-CSF

K. Vardarova*, S. Scharf*, F. Lang*, B. Schmeck*,#, B. Opitz*, J. Eitel*, A.C. Hocke*,
H. Slevogt*, A. Flieger¶, S. Hippenstiel*, N. Suttorp* and P.D. N'Guessan*

ABSTRACT: *Legionella pneumophila* is an important causative agent of severe pneumonia in humans. The human alveolar epithelium is an effective barrier for inhaled microorganisms and actively participates in the initiation of innate host defense. Although secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) is essential for the elimination of invading *Legionella* spp., mechanisms of *Legionella pneumophila*-induced release of this cytokine are widely unknown.

In this study, we have demonstrated a toll-like receptor (TLR)2- and TLR5-dependent release of GM-CSF in *L. pneumophila*-infected human alveolar epithelial cells. GM-CSF secretion was not dependent on the bacteria type II or type IV secretion system. Furthermore, an increase in protein kinase C (PKC) activity, particularly PKCα and PKCε, was noted. Blocking of PKCα and PKCε activity or expression, but not of PKCβ, PKCδ, PKCη, PKCθ, and PKCζ, significantly reduced the synthesis of GM-CSF in infected cells. While PKCα was critical for the initiation of a nuclear factor-κB-mediated GM-CSF expression, PKCε regulated GM-CSF production *via* activator protein 1.

Thus, differential regulation of GM-CSF, production by PKC isoforms, contributes to the host response in Legionnaires' disease.

AFFILIATIONS
*Dept of Internal Medicine/Infectious Diseases and Pulmonary Medicine,
#FORSYS Junior Research Group, Systems Biology of Lung Inflammation, Charité-Universitätsmedizin Berlin, Berlin, and
¶NG5 Pathogenesis of *Legionella* Infection, Robert Koch-Institut, Wernigerode, Germany.

CORRESPONDENCE
P.D. N'Guessan
Dept of Internal Medicine/Infectious Diseases and Pulmonary Medicine
Charité-Universitätsmedizin Berlin
Augustenburger Platz 1
13353 Berlin
Germany
E-mail: dje_philippe.nguessan@charite.de

egionnaires' disease is a severe pneumonia with high mortality caused by *Legionella pneumophila* [1, 2]. The bacterium enters the human body by aerosol droplets and successfully establishes itself in macrophages and the alveolar epithelium, which normally offer an efficient barrier against infections [3–7]. Among the various putative virulence factors of this pathogen that have been identified to date, the type II (lspDE) and IVB (Dot/Icm) secretion system enables the bacteria to export proteins into the host cell cytoplasm and, therefore, activates diverse cell signaling pathways [8]. Furthermore, flagellin, a major virulence factor of *L. pneumophila* has been shown to be essential for activating the innate defense in human alveolar epithelium as well as in macrophages [6, 8, 9]. Pro-inflammatory immune responses of the airway epithelium play an important role in the immune defense mechanisms of the respiratory tract by detecting microbes and pathogen-related factors by means of toll-like receptors (TLR) or cytosolic pathogen pattern recognition receptors followed by the subsequent release of pro-inflammatory mediators [10]. To clear *L. pneumophila* from the lung, a functionally intact

innate immune system, particularly macrophages and polymorphonuclear leukocytes (PMNs), must be present [3, 8]. Epithelial cells have been shown to liberate mediators such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-8, interferon (IFN)-β, and prostaglandin (PG)E$_2$ upon infection [4, 5, 11–14]. GM-CSF is a 23-kDa haematopoietic growth factor that is able to stimulate *in vitro* survival, proliferation, differentiation and function of myeloid cells and their precursors, particularly PMNs, eosinophils, granulocytes, and monocytes/macrophages [15, 16]. GM-CSF is expressed by several cell types of the respiratory tract, such as epithelial cells, activated T-cells, macrophages, and fibroblasts [15, 16]. Furthermore, GM-CSF plays a critical role in surfactant homeostasis [17] and for stimulating the terminal differentiation of alveolar macrophages (AMs) [18]. The important function of GM-CSF for the pulmonary immune responses has been confirmed *in vivo* by the use of gene knockout mice, demonstrating a pivotal role of this cytokine for host defense function [19]. GM-CSF -/- mice were more susceptible to several pulmonary bacterial infections such as *Pseudomonas aeruginosa* [19], group B *Streptococcus*

spp. [20], and *Pneumocystis jiroveci* [21]. Furthermore, AMs from GM-CSF -/- mice were defective for *Escherichia coli* phagocytosis [18] as well as in the production of tumour necrosis factor (TNF)-α [22] and IFN-γ [23]. The expression of GM-CSF is controlled by a tight regulatory network involving the transcription-factors nuclear factor (NF)-κB and the activator protein (AP)-1 [24, 25]. These transcription factors are activated by complex signalling pathways, including protein kinase C (PKC) [26, 27]. PKCs, a family of serine/threonine kinases, are involved in different biological processes. They participate in the modulation of immune responses by the regulation of gene transcription [28, 29]. This enzyme family includes several isotypes that display different cellular functions in the presence or absence of calcium, diacyl-glycerol, and phospholipids [29]. The PKC isotypes can be categorised into three classes based on structural differences: the conventional isotypes (α, β1, β2, and γ), the novel isotypes (δ, ε, η, μ and θ), and the atypical isotypes (λ and ζ) [29].

Activation of pro-inflammatory pathways in lung epithelial cells, including the PKC, AP-1, and NF-κB pathways, by bacterial infection are suggested to contribute significantly to pneumonia [4, 10, 26]. Although *L. pneumophila* efficiently infects and stimulates lung epithelial cells [4–7] and GM-CSF has been shown as important for bacteria elimination *via* phagocytosis, mechanisms of *L. pneumophila*-induced release of this cytokine are widely unknown.

In the present study, we demonstrate that TLR2, TLR5 as well as PKCα and PKCε controlled the expression of GM-CSF in response to *L. pneumophila* infection of human alveolar epithelium. We showed that after infection of alveolar epithelium with *L. pneumophila*, activities of PKCα and PKCε were increased. Whereas production of GM-CSF by activating NF-κB was promoted by PKCα, its induction by activation of the transcription factor AP-1 (c-Jun) was controlled by PKCε Thus, our results may significantly contribute to the understanding of the pathogenesis of Legionnaires' disease.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle Medium, fetal calf serum, trypsin-EDTA-solution, CA-650, and antibiotics were obtained from Life Technologies (Karlsruhe, Germany). Protease inhibitors, Triton X-100, Tween-20, Pam3CSK4, and flagellin from *Salmonella typhimurium* were purchased from Sigma Chem. Co. (Munich, Germany), TNFα from R&D Systems (Wiesbaden, Germany), and IKK-NBD from Biomol (Hamburg, Germany). Calphostin (Pan-PKC inhibitor), Gö6976 (PKCα inhibitor), PKCε translocation inhibitor peptide (PKCε-TIP), PKCβ inhibitor, Rottlerin (PKCδ inhibitor), PKCη myristoylated pseudosubstrate inhibitor, PKCθ myristoylated pseudosubstrate inhibitor and PKCζ myristoylated pseudosubstrate inhibitor, the JNK inhibitor SP600125 and phorbol-12-myristate-13-acetate (PMA) were purchased from Calbiochem (Darmstadt, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

Cell lines

To study the host–pathogen interaction between *L. pneumophila* and alveolar epithelium *in vitro*, in the present study we used the human primary airway epithelial cells (small airway

epithelial cells (SAEC)), as well as the human alveolar epithelial cell line A549 as cell culture model, closely resembling the *in vivo* state. Primary human SAECs were obtained from Cambrex (Cambrex, Taufkirchen, Germany) and cultured according to the supplier's instructions.

The alveolar epithelial cell line A549 was purchased from DSMZ (Braunschweig, Germany) and cultured in Ham'S F 12 (PAA, Pasching, Austria) with L-glutamine, 10% fetal calf serum without antibiotics. The NF-κB-dependent reporter cell line, A549 6Btkluc, was a kind gift from 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′-AGCTTACAAGGGAC-TTTCCGCTGGGGACTTTCCAGGGA-3′, which contains two copies of the decameric NF-κB binding site driving a luciferase gene [30].

Infection with bacterial strains

L. pneumophila sg1 strain 130b (ATCC BAA-74, kindly provided by N.P. Cianciotto; Northwestern University Medical School, Chicago, USA [31]), strain JR32 (wild type), JR32 ΔdotA deficient in *dot/icm*, encoding a protein essential for the type IVB secretion system (kindly provided by H. Shuman; Dept of Microbiology, Columbia University Medical Center, New York, NY, USA), strain Corby (wild-type), Corby ΔflaA deficient in flagellin (kindly provided by K. Heuner; Robert Koch-Institut, Berlin, Germany), as well as type II secretion system knockout Corby ΔlspDE [7, 32] (also kindly provided by K. Heuner), were routinely grown on buffered charcoal-yeast extract agar for 2–3 days at 37°C before use [33]. A549 cells were infected with *L. pneumophila* with a multiplicity of infection (MOI) of 10 at 37°C and 5% CO_2.

RNA interference in A549 cells

We used for each target gene two different siRNAs which worked well but to enhance the readability of the figures we show example results for one siRNA pro target gene. Control nonsilencing siRNA (sense UUCUCCGAACGUGUCACGUtt, antisense ACGUGACACGUUCGGAGAAtt), siRNA targeting TLR2 (sense GCCUUGACCUGUCCAACAAtt, antisense UUGUUGGACAGGUCAAGGCtt), TLR5 (sense GGAGCA-AUUUCCAACUUAUtt, antisense AUAAGUUGGAAAUUG-CUCCtt), PKCα (sense AAGCACAAGUUCAAAAUCCACtt, antisense GUGGAUUUUGAACUUGUGCUUtt), and siRNA targeting PKCε (sense AAGCCCUAAAGACAAUGAAGtt, antisense CUUCAUUGUCUUUAGGGGCUUtt) were purchased from MWG (Ebersberg, Germany). A549 cells were transfected by using Amaxa Nucleofector™ (Amaxa, Köln, Germany) according to the manufacturer's protocol (Nucleofector™ Solution V, Nucleofector™ program G-16) with 2 μg siRNA per 10^6 cells.

GM-CSF ELISA

A549 cells were infected as indicated, supernatants were collected and processed for GM-CSF-quantification by ELISA, according to manufacturer's instructions (BD Biosciences, Heidelberg, Germany) [11].

Western blot

Western blots were performed to determine siRNA-mediated knockdown of PKCα and PKCε, PKC activation (phosphorylation

of Myristoylated, Alanine-Rich C-Kinase Substrate (MARCKS), PKC isotypes translocation to membrane), as well as NF-κB activation. Briefly, A549 cells were transfected or infected as indicated. 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 target proteins was carried out with specific antibodies against PKCα, PKCε, IκB and actin. All antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) [4]. In all experiments, actin was detected simultaneously to confirm equal protein load.

PKC assay

A549 cells were infected with *L. pneumophila* at an MOI of 1, 5 and 10 for 1 h. Cell extracts, containing activated PKC were collected and processed for PKC activity by PKC activity assay (Stressgen Bioreagents Corp., Ann Arbor, MI, USA) according to manufacturer's instructions [4].

P-c-Jun-assay

The JNK Activity Assay, KinaseSTAR™ from BioVision (Mountain View, CA, USA) was used to detect p-c-Jun (Ser 73) activity. A549 cells were infected with *L. pneumophila*. Cells extracts, containing activated p-c-Jun were collected and processed according to manufacturer's instructions and analysed by western blot.

Chromatin immunoprecipitation

A549 cells were pre-incubated with or without inhibitors and infected with *L. pneumophila* (MOI of 10). Cells were processed for chromatin immunoprecipitation (ChIP) as described elsewhere [12, 34, 35]. The *gmcsf* promoter DNA was amplified by PCR using Hotstart Taq polymerase (Qiagen, Hilden, Germany). PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining of gels. Equal amounts of input DNA were confirmed by gel electrophoresis. For immunoprecipitation, the antibodies used were purchased from Santa Cruz Biotechnology (p65/RelA and polymerase II) or from Cell Signaling (p-c-Jun; Frankfurt, Germany). The following promoter-specific primers for *gmcsf* were used: sense 5'-TGTCGGTTCTTGGAAAGGTT-3' and antisense 5'-GGGCTCACTGGCAAAAGA -3'.

Statistical methods

Data are presented as mean±SEM of at least three independent experiments and were submitted to one-way ANOVA tests. The main effects were then compared by a Newman-Keuls' post-test. A p-value of 0.05 was considered to be significant and indicated; if not indicated otherwise, test was performed *versus* control (*) or stimulated probe *versus* inhibitor treated probe (#).

RESULTS

L. pneumophila induced GM-CSF release in human alveolar epithelial cells

SAECs as well as A549 cells were infected with *L. pneumophila* or stimulated with TNFα for different periods of time. A time-dependent induction of GM-CSF release (fig. 1) was observed in *L. pneumophila*-infected cells.

L. pneumophila-induced GM-CSF release in alveolar epithelial cells is controlled by detection via TLR2 and TLR5 but not dependent on his type II and IV secretion systems

The type II (lspDE) as well as the Icm/Dot type IV secretion system are known to be important virulence factors of *L. pneumophila* [3, 8, 32]. Infection of A549 cells with *L. pneumophila* strains 130b, JR32 and Corby induced comparable GM-CSF release (fig. 2a). Furthermore, no significant difference among the effects of JR32 Δ*dotA* and Corby Δ*lspDE* deletion mutants or wild-type strains with respect to GM-CSF liberation could be observed.

Following our previous and present observations that *L. pneumophila* strongly activates lung epithelial cells, we tested the hypothesis that pattern recognition receptors (PRRs) located on the cells surface or in the cytoplasm are involved in lung epithelial cell responses to *L. pneumophila*.

A549 cells express little or no TLR4 and poorly respond to lipopolysaccharides [36, 37]. We therefore focused on the role of TLR2 and TLR5 for *L. pneumophila*-induced epithelial cell activation. A549 cells were incubated with heat inactivated *L. pneumophila* (TLR2 ligand) or infected with a flagellin-deficient mutant strain (Corby Δ*flaA*). A decrease in GM-CSF release was observed in both groups compare with cells infected with the wild type strains (130b, Corby). To further address this issue, we performed RNAi experiments in A549 cells to inhibit expression of endogenous TLR2 or TLR5 respectively. As shown in figure 2b, both TLR2- and TLR5-specific siRNA but not a non-silencing control siRNA reduced TLR2 as well as TLR5 mRNA levels in A549. Importantly, the siRNA targeting TLR2 as well as TLR5 also significantly blocked the GM-CSF production induced by *L. pneumophila* in A549 cells, but not the TNFα-related cytokine production (fig. 2c). Moreover, siRNA targeting TLR2 and 5 strongly impaired the GM-CSF release induced by the TLR2 ligand Pam3CSK4 and the TLR5 ligand
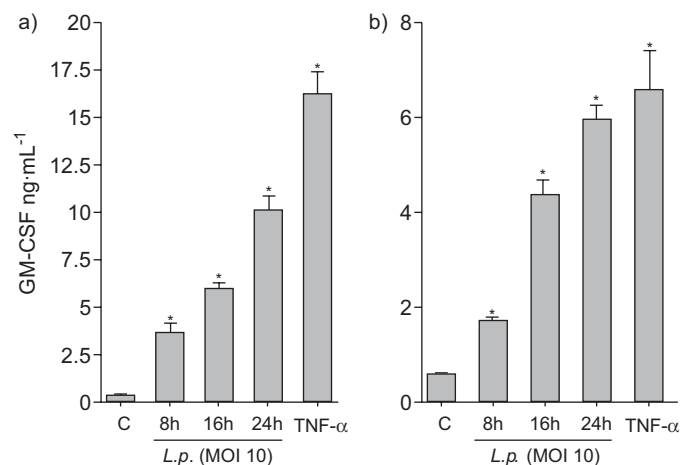
FIGURE 1. Increased granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in *Legionella pneumophila* (*L.p.*)-infected human alveolar epithelial cells. a) A549 cells and b) small airway epithelial cells were infected for 8, 16 and 24 h with *L. pneumophila* 130b (at a multiplicity of infection (MOI) of 10). GM-CSF release was detected by ELISA. Tumour necrosis factor (TNF)-α (50 ng·mL⁻¹) was used as a positive control. The uninfected control (C) was determined by 24 h. Data are presented as mean±SEM of four separate experiments. *: p<0.05 compared with uninfected control cells.
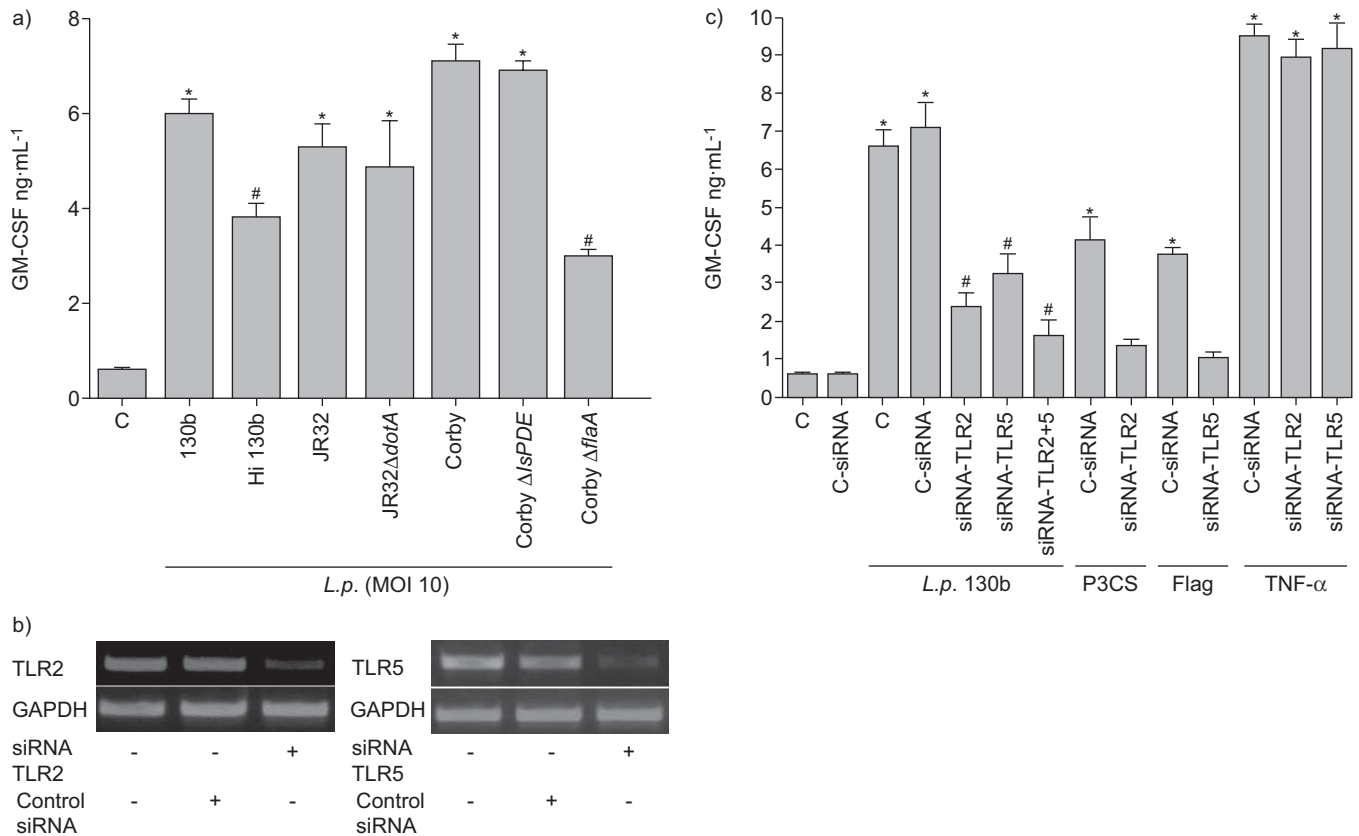
FIGURE 2. *Legionella pneumophila* (*L.p.*)-induced granulocyte-macrophage colony-stimulating factor (GM-CSF) release in alveolar epithelial cells is controlled by toll-like receptor (TLR)2 and TLR5 but not by the types II and IV secretion systems. a) A549 were infected for 24 h with a multiplicity of infection (MOI) 10 of *L. pneumophila* 130b, JR32, JR32ΔdotA, Corby, Corby ΔflaA, Corby ΔlspDE or incubated with heat-inactivated (Hi) 130b. GM-CSF release was analysed by ELISA. b) Furthermore, RNAi experiments in A549 cells to inhibit expression of endogenous TLR2 or TLR5 were performed. Cells were incubated for 72 h with TLR2- and TLR5-specific siRNA as well as a nonsilencing control siRNA (C-siRNA) and RT PCR was performed. Representatives of three independent experiments with similar results are shown. c) Importantly, A549 cells were treated with control siRNA or siRNA targeting TLR2 or TLR5 for 72 h before infection with *L. pneumophila* or incubation with tumour necrosis factor (TNF)-α (50 ng·mL⁻¹) or the TLR2 ligand Pam3CSK4 (P3CS; 1 μg·mL⁻¹) or the TLR5 ligand flagellin (Flag; 10 ng·mL⁻¹) as indicated. GM-CSF release was measured by ELISA (c). ELISA data are presented as mean±SEM of four separate experiments. *: p<0.05 compared with uninfected control cells; #: p<0.05 wild type bacteria *versus* mutant, or control siRNA *versus* target siRNA.

flagellin respectively (fig. 2c). Next, we examined the GM-CSF response in cells which were cotransfected with TLR2 and TLR5 siRNA. A significant additive effect of these two siRNAs was observed in regard to *L. pneumophila*-induced GM-CSF in A549 cells (fig. 2c).

L. pneumophila-*induced PKC activity* via *TLR2 and TLR5 in alveolar epithelial cells*

PKC signalling pathways have been shown to play important roles in the expression of proinflammatory cytokine release [28, 29, 38, 39]. We hypothesised that PKC may contribute to *L. pneumophila*-related proinflammatory activation of lung epithelium. A549 cells were infected with *L. pneumophila* 130b, Corby, Corby ΔflaA, or incubated with heat inactivated 130b, Pam3CSK4 and flagellin. Infection of the cells with *L. pneumophila* wild type strains 130b and Corby lead to an increase in PKC activity whereas the flagellin-deficient mutant strain as well as heat inactivated *L. pneumophila* 130b lead to a reduced PKC activity in these cells (fig. 3a). Moreover TLR2 activation by Pam3CSK4 or TLR5 by flagellin strongly induced PKC activity. The increase PKC activity induced by the *L. pneumophila* wild type strains was

comparable to that induced by PMA, a strong inducer of PKC activity (fig. 3a). Furthermore, we confirmed PKC activation in *L. pneumophila*-infected cells by detection of increased phosphorylation of MARCKS, one major substrate of PKC at Ser 159/163 (fig. 3b).

L. pneumophila-*induced GM-CSF release was dependent on PKC*α *and PKC*ε *activation in alveolar epithelial cells*

To further investigate the contribution of PKC and its different isoforms to *L. pneumophila*-induced GM-CSF release, A549 cells were pre-incubated with the pan-PKC inhibitors calphostin C, and infected with *L. pneumophila*. As shown in figure 4, reduction of GM-CSF release could be demonstrated, suggesting an involvement of PKC in the GM-CSF production in response to *L. pneumophila* infection. Next, the role of different PKC isoforms known to be expressed in alveolar epithelium were studied [40]. Confluent A549 cells were pre-incubated with specific chemical inhibitors blocking PKCα (Gö6976), PKCε (PKCε-TIP), PKCβ (PKCβ inhibitor), Rottlerin (PKCδ inhibitor), PKCη (PKCη myristoylated pseudosubstrate inhibitor), PKCθ (PKCθ myristoylated pseudosubstrate inhibitor)
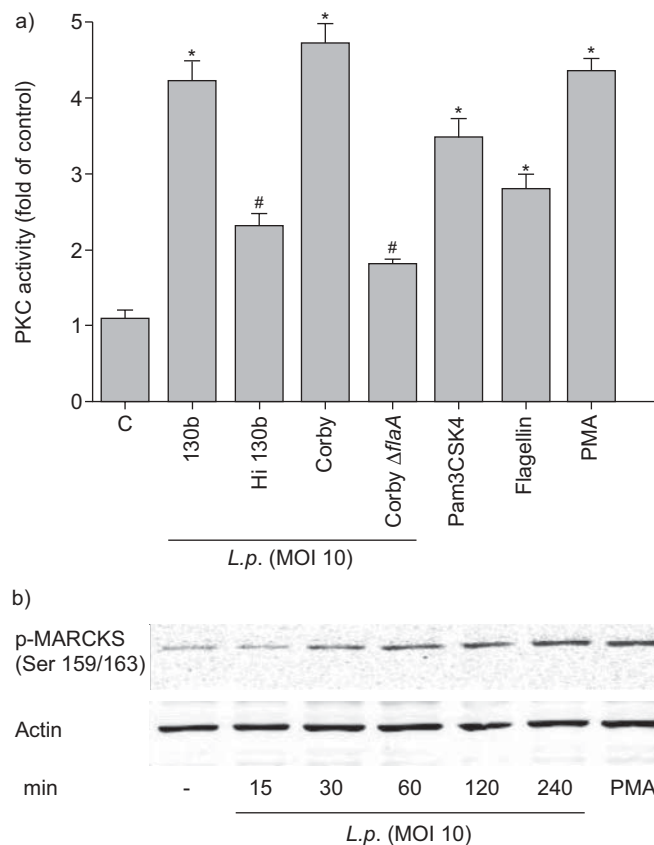
a)

b)

FIGURE 3. *Legionella pneumophila* (*L.p.*) induced activation of protein kinase C (PKC). A549 cells were incubated for 60 min with *L. pneumophila* 130b, Corby, Corby Δ*flaA*, or incubated with heat inactivated (Hi) 130b, Pam3CSK4 (1 µg·mL^{-1}) and flagellin (10 ng·mL^{-1}). a) PKC activity was detected by PKC activity assay. b) Furthermore, phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS), one major substrate of PKC, was assessed *via* western blot in *L. pneumophila* 130b infected lung epithelium (multiplicity of infection (MOI) 10) as indicated. In both experiments phorbol-12-myristate-13-acetate (PMA; 160 nM, 60 min) was used as positive control. Representatives of three independent blots with similar results are shown. PKC activity assay data are presented as mean ± SEM of four separate experiments. *: p<0.05 compared with uninfected control cells; #: p<0.05 wild type bacteria *versus* mutant.

and PKCζ (PKCζ myristoylated pseudosubstrate inhibitor) and infected with *L. pneumophila*. As shown in figure 4, blocking of PKCα as well as PKCε strongly reduced *L. pneumophila*-induced GM-CSF production. Furthermore, a significant additive effect of the two inhibitors was observed in regard to *L. pneumophila*–produced GM-CSF in A549 cells. In contrast, inhibition of other PKC isotypes did not affect GM-CSF release as exemplarily shown for PKCβ, PKCδ, PKCη, PKCθ, and PKCζ (fig. 4). The chemical inhibitors used in these experiments did neither reduce the viability and proliferation of the A549 cells nor they induced morphological signs of cytotoxicity, or alterations of bacterial growth within the time-frame tested (data not shown).

The translocation of PKC isoforms from the cytosol to the cellular membrane has been shown to be an important indicator for their activation [41, 42]. In order to confirm the role of PKCα and PKCε in *L. pneumophila* infection in human
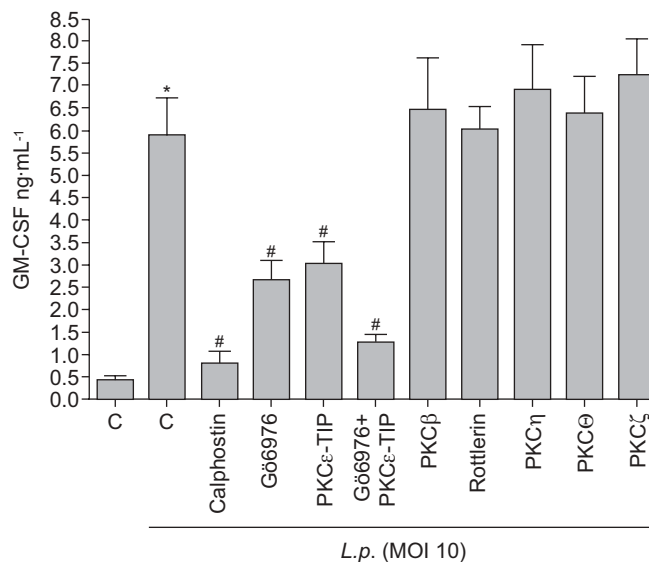

FIGURE 4. *L. pneumophila* (*L.p.*) induced granulocyte-macrophage colony-stimulating factor (GM-CSF) release is dependent on protein kinase C (PKC)α and PKCε. To further investigate the contribution of PKC and its different isoforms to *L. pneumophila* -induced GM-CSF release, A549 cells were pre-incubated for 1 h with the chemical pan-PKC inhibitor Calphostin or PKC isoform inhibitors blocking PKCα (Gö6976, 10 µM), PKCβ (PKCβ inhibitor, 10 nM), Rottlerin (PKCδ inhibitor, 10 µM), PKCε (PKCε-TIP, 10 µM), PKCη (PKCη myristoylated pseudosubstrate inhibitor, 10 µM), PKCθ (PKCθ myristoylated pseudosubstrate inhibitor, 10 µM) and PKCζ (PKCζ myristoylated pseudosubstrate inhibitor, 10 µM) before infection with *L. pneumophila* for 24 h. GM-CSF release was assessed by ELISA. ELISA data are presented as mean ± SEM of four separate experiments. *: p<0.05 compared with uninfected control cells; #: p<0.05 compared with infected cells without inhibitors.

alveolar epithelium, we analysed the translocation of both PKC isoforms to the cell membrane. As shown in figure 5a, PKCα and PKCε were translocated from the cytosol to the cell membrane in the infected cells. Next, the relevance of PKCα and PKCε for *L. pneumophila*-related GM-CSF expression was analysed in more detail. To confirm experimental data obtained by the used inhibitors, we made use of RNAi-induced gene knockdown of PKCα and PKCε. First, we evaluated the siRNAs for their ability to reduce expression of their corresponding genes. PKCα- and PKCε-specific siRNAs inhibited the expression levels of both kinase isotypes (fig. 5b). Moreover, we found that PKCα and PKCε-specific siRNAs significantly reduced the GM-CSF production induced by *L. pneumophila* 130b in A549 cells (fig. 5c). In contrast, non-silencing siRNA (control siRNA) neither reduced PKCα- or PKCε-expression nor inhibited GM-CSF release in these cells (fig. 5c). A significant additive effect of these two siRNAs was observed in regard to *L. pneumophila*-produced GM-CSF in A549 cells.

L. pneumophila-*induced GM-CSF release depended on NF-κB and AP-1 (c-Jun) activation in alveolar epithelial cells*

To further investigate the role of NF-κB-activation for *L. pneumophila*-dependent GM-CSF release, IκB degradation was assessed by immuno-blot. We found a strong *L. pneumophila* induced decrease of IκB in infected alveolar epithelial cells (fig. 6a). Furthermore, blocking of the IκB kinase complex by
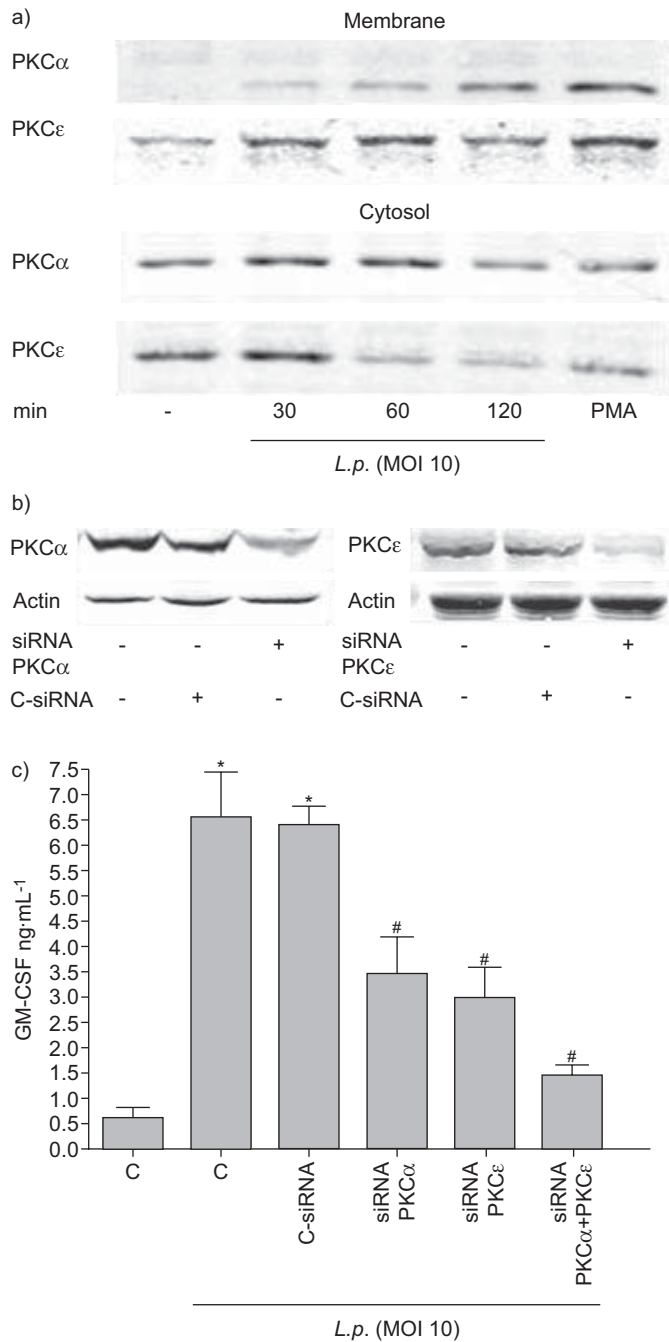
a)

Membrane

PKCα

PKCε

Cytosol

PKCα

PKCε

| min | - | 30 | 60 | 120 | PMA |

L.p. (MOI 10)

b)

PKCα   Actin

PKCε   Actin

| siRNA PKCα | - | - | + |
| C-siRNA | - | + | - |

| siRNA PKCε | - | - | + |
| C-siRNA | - | + | - |

c)

GM-CSF ng·mL⁻¹ (y-axis, 0.0 to 7.5)

Categories: C, C, C-siRNA, siRNA PKCα, siRNA PKCε, siRNA PKCα+PKCε

L.p. (MOI 10)

FIGURE 5. Activation and gene silencing of protein kinase C (PKC)α and PKCε confirmed differential regulation of granulocyte-macrophage colony-stimulating factor (GM-CSF) production in alveolar epithelial cells infected by *Legionella pneumophila* (*L.p.*). To assess activation (translocation) of PKCα and PKCε, lung alveolar epithelial cells were infected with *L. pneumophila* 130b (multiplicity of infection (MOI) 10) for 30, 60 and 120 min (a). Translocated PKCα and PKCε were addressed by western blot. In addition, A549 cells were transfected with control siRNA or specific siRNAs targeting PKCα or PKCε and gene silencing abilities were assessed by western blot (b). Further blockades of PKCα and PKCε prior to *L. pneumophila* infection were performed by targeting both proteins with specific siRNA and GM-CSF production was measured by ELISA (c). Representatives of three independent blots with similar results are shown in parts a and b. ELISA data are presented as mean±SEM of four separate experiments. *: p<0.05 compared with uninfected control cells; #: p<0.05 control siRNA *versus* target siRNA.

a)

| IκB | min | - | 30 | 60 |

| Actin | min | - | 30 | 60 |

L.p. (MOI 10)   *L.p.* (MOI 10)

b)

p-c-Jun Ser 73

| min | - | 30 | 60 | 120 | 240 |

L.p. (MOI 10)

c)

GM-CSF ng·mL⁻¹ (y-axis, 0 to 8)

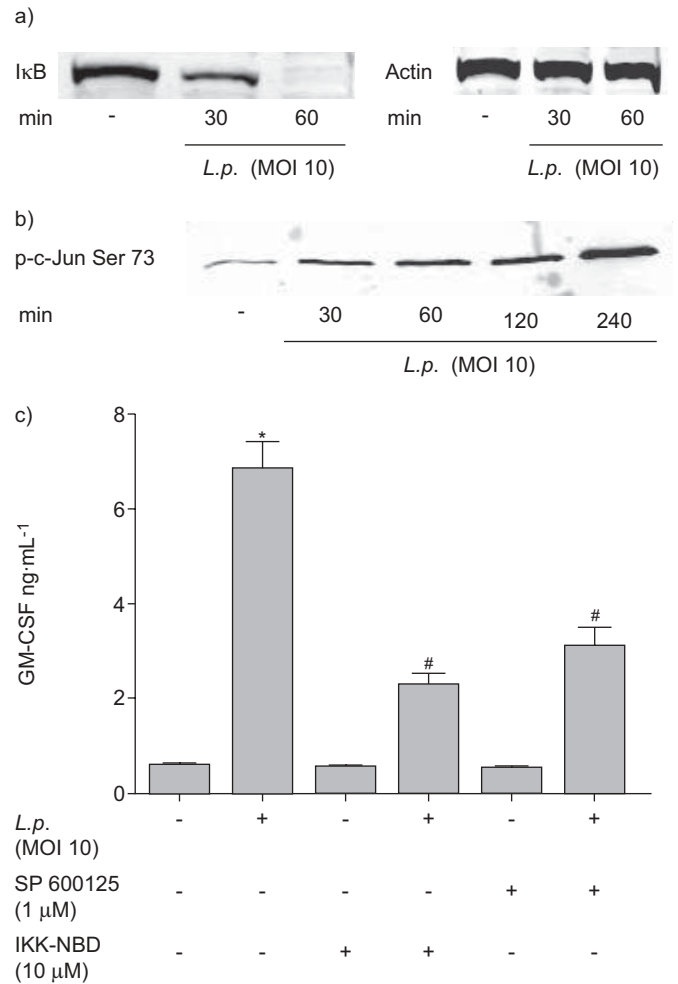| *L.p.* (MOI 10) | - | + | - | + | - | + |
| SP 600125 (1 μM) | - | - | - | - | + | + |
| IKK-NBD (10 μM) | - | - | + | + | - | - |

FIGURE 6. *Legionella pneumophila* (*L.p.*)-induced granulocyte-macrophage colony-stimulating factor (GM-CSF) release depended on nuclear factor-κB and activator protein-1 (c-Jun) in alveolar epithelial cells. A549 cells were infected with *L. pneumophila* as indicated and IκB degradation as well as c-Jun phosphorylation was assessed by western blot (a) or activity assay (b). To address the role of the aforementioned pathways for the *L. pneumophila*-induced GM-CSF release infected alveolar epithelial cells, A549 cells were pre-treated with a specific IκBα kinase inhibitor, IKK-NBD (10 μM), or with a specific JNK inhibitor, SP 600125 (10 μM), for 60 min and infected with *L. pneumophila* 130b for 24 h. Production of GM-CSF was assessed by ELISA (c). Representatives of three independent blots with similar results are shown (a). ELISA data are presented as mean±SEM of four separate experiments. *: p<0.05 compared with uninfected control cells; #: p<0.05 compared with infected cells without inhibitors. MOI: multiplicity of infection.

pre-incubation of A549 cells with the cell permeable peptide inhibitor IKK-NBD significantly reduced GM-CSF release (fig. 6c) in *L. pneumophila*-infected A549 cells. We also observed a time dependently increased phosphorylation of the AP-1-subunit c-Jun at Ser 73 after *L. pneumophila* infection of A549 cells (fig. 6b). Since activation of c-Jun is controlled by JNK, we made use of the specific chemical inhibitor SP600125 to access the impact of c-Jun on GM-CSF expression. We observed a decrease of GM-CSF release in SP600125 exposed *L. pneumophila*-infected cells (fig. 6c). Neither IKK-NBD nor SP600125 altered bacterial growth within the concentration and time frame tested (data not shown). Overall, activation of

NF-κB as well as JNK signaling pathway contributed to *L. pneumophila*-induced GM-CSF release in lung epithelium.

PKCα and PKCε differentially controlled NF-κB and AP-1 (c-Jun) activation in L. pneumophila-*infected alveolar epithelial cells*

To analyse the impact of PKCα and PKCε on NF-κB and AP-1 (c-Jun) controlled GM-CSF expression, both kinases were inhibited in *L. pneumophila*-infected alveolar epithelial cells. To assess NF-κB activity, the NF-κB-dependent reporter cell line A549 6Btkluc was used. As shown in fig. 7a, inhibition of PKCα but not PKCε, reduced NF-κB activation. In contrast, inhibition of PKCε but not PKCα reduced c-Jun phosphorylation in *L. pneumophila*-infected cells (fig. 6c). To further characterise the mechanism by which PKCα and PKCε contribute to *L. pneumophila*-mediated NF-κB and p-c-Jun activation as well as GM-CSF expression, the binding pattern of NF-κB subunit p65, p-c-Jun Ser 73 and polymerase II to the *gmcsf* promoter was evaluated by ChIP assay (fig. 7b and d). A549 cells were pre-incubated with the specific inhibitors Gö6976 (60 min) for PKCα or PKCε translocation inhibitor

peptide (60 min) for PKCε before infection with *L. pneumophila* (MOI 10) for 120 min.

We observed an increase of p65 a subunit of NF-κB, as well as p-c-Jun of the AP-1 to the *gmcsf* promoter. An increase of the RNA polymerase II (POL II) to the *gmcsf* promoter was indicative for the subsequent activation of the *gmcsf* gene (fig. 7b and d) in infected A549 cells. Interestingly, inhibition of PKCα by Gö6976 reduced recruitment of NF-κB to the *gmcsf* promoter (fig. 7b) whereas the binding pattern of p-c-Jun to the promoter was controlled by PKCε (fig. 7d). The enrichment of POL II to the *gmcsf* promoter was inhibited by both inhibitors.

DISCUSSION

In the study presented, we found that *L. pneumophila* induced TLR2 and TLR5-dependent activation of PKC as well as GM-CSF release in human alveolar epithelial cells. The transcription factors NF-κB and AP1 were identified to play a key role for *L. pneumophila* induced GM-CSF release. Moreover, detailed analysis of signal transduction pathways provides evidence that the activation of these transcription factors was differentially regulated by PKC isoforms. Whereas PKCα was involved
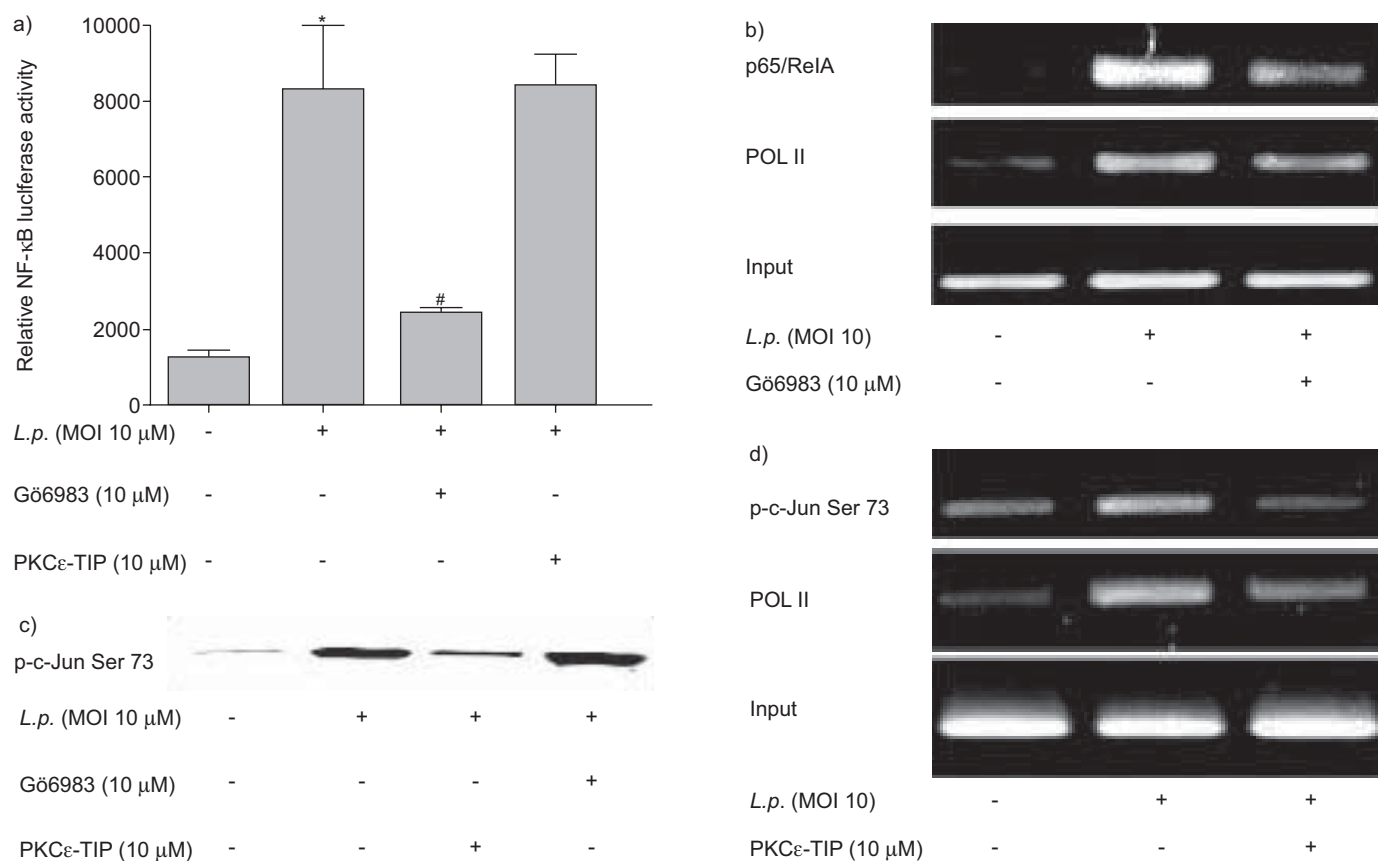

FIGURE 7. Protein kinase C (PKC)α and PKCε differentially controlled nuclear factor (NF)-κB and activator protein-1 (c-Jun) activation in *Legionella pneumophila* (*L.p.*)-infected alveolar epithelial cells. The NF-κB-dependent reporter cell line, A549 6Btkluc was pre-incubated with Gö6976 (10 µM) or PKCε -TIP (10 µM) and infected with *L. pneumophila* 130b for 4 h (a). NF-κB luciferase activity was detected using luciferase assay (a). Furthermore, A549 cells were pre-incubated with Gö6976 or PKCε-TIP before infection with *L. pneumophila* 130b for 2 h and the binding patterns of POL II, p65, p-c-Jun to *gmcsf* promoter were analysed by ChIP (b, d). To address the role of PKCε in c-Jun-activation, cells were pre-incubated with Gö6976 (10 µM) or PKCε -TIP (10 µM) for 1 h and infected with *L. pneumophila* 130b for 4 h before being processed by activity assay (c). Data in (a) are presented as mean±SEM of four separate experiments. Representative gels or blots of three experiments are shown. *: p<0.05 compared with uninfected control cells; #: p<0.05 compared with infected cells without inhibitors.

in NF-κB activation, PKCε- was found to control the activation of the AP1 subunit c-Jun.

Our data showed that *L. pneumophila* infection of pulmonary epithelium led to a strong GM-CSF secretion. Since different isolates of *L. pneumophila* (130b, JR32, and Corby) were found to induce a comparable release of GM-CSF, it is likely that induction of this cytokine is a common phenomenon in *L. pneumophila*-related epithelial cell activation.

Taken together, we assume that GM-CSF might be important for the elimination of invading *L. pneumophila* and thus be important for immune response in Legionnaire' disease.

Pulmonary epithelial cells may detect *L. pneumophila* by TLRs. Accordingly, we demonstrated that recognition of *L. pneumophila* by TLR2 and 5 was essential for the production of GM-CSF. Interestingly, GM-CSF liberation was not reduced in infections with type II or IV secretion system mutant strains, suggesting that recognition of bacterial membrane component *via* TLR2 or flagellin through TLR5 might be the major pathways for *L. pneumophila* induced GM-CSF and thus an early detection system before bacteria cell invasion. In a study published by Shin and co-worker it has been demonstrated that the TLR signaling synergizes with the type IV secretion system to enhance cytokine production in macrophages infected with *L. pneumophila*. Since the type IV secretion system did not play any role in *L. pneumophila*-induced GM-CSF in pulmonary epithelium, a cell type specific response in regard to this virulence factor has to be considered [43].

A complex signaling network regulates the expression of inducible GM-CSF [24, 25]. In *L. pneumophila*-infected lung epithelial cells, we noted a TLR2 and TLR5 dependent activation of PKC. In addition we demonstrate that *L. pneumophila* activated PKCα as well as PKCε in alveolar epithelium and a specific inhibition of both kinases strongly reduced *L. pneumophila*-related GM-CSF liberation. Other PKCs known to be expressed in alveolar epithelium were not involve in *L. pneumophila* induced GM-CSF. In a previous study, PKCα has been shown to be important for COX-2 expression and subsequent PGE$_2$ release in *L. pneumophila*-infected alveolar epithelial cells [4]. Also, we demonstrated that *Moraxella catarrhalis* infection activates PKCα, ε, and θ which differentially regulate interleukin-8 gene transcription in human pulmonary epithelial cells [14]. Since the activation pattern of PKCs as well as their impact on inflammatory immune response depends on the pathogen implicated, the above described mechanism might be specific for a *L. pneumophila*.

In regard to the observed TLR2 and TLR5 dependent activation of PKCα and PKCε, it has been demonstrated that disruption of the PKCε gene in mice leads to compromised innate immunity [44]. PKCε deficient mice had defects in clearance of both Gram-positive (TLR2) and Gram-negative (TLR4) bacterial infections. In an other study, activation of PKC isoforms by TLR5 through flagellin contributes to production of inflammatory cytokines in epithelial cells [45]. Besides, PKCα has been shown to control TLR2 dependent activation of NF-κB [46]. PKCα as well as PKCε are linked to various TLRs through the adaptor protein MyD88 [47]. Thus, the described TLRs-PKCs pathways in this study give more insight into the differential regulation of the TLR2 and 5 dependent immune

response targeting *L. pneumophila*. Since TLRs polymorphism particularly TLR5 have been associated with a weakened innate immune response and an increase susceptibility to Legionnaires' disease, an individual predisposition may impaired the described pathway and therefore severely impact diseases course [48, 49].

Activation of the transcription factors NF-κB and AP-1 is considered to significantly contribute to GM-CSF expression [24, 25]. We therefore demonstrated that *L. pneumophila* induced GM-CSF was controlled by NF-κB and AP-1 (c-Jun). A detail analysis has shown that PKCα was essential for NF-κB activation while PKCε controlled c-Jun phosphorylation.

In conclusion, we found that *L. pneumophila* induced TLR2 and TLR5 dependent GM-CSF release in human alveolar epithelial cells. A TLR2 und 5 dependent activation of PKC has been also demonstrated. Furthermore PKC isoforms differentially controlled GM-CSF induction in *L. pneumophila* infected alveolar epithelial cells. Whereas PKCα controlled production of GM-CSF over NF-κB, PKCε is implicated in the same process through activation of AP-1 (c-Jun). Since control of the immune response is crucial to assure bacterial clearance and to prevent excessive tissue damage in pneumonia, the mechanism described above could be important for the pathogenesis of Legionnaires' disease.

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