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# PKCα and ε differentially regulate *Legionella pneumophila* induced GM-CSF

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**Running Title:** PKCα and ε control *L. pneumophila* induced GM-CSF

**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*, mechanisms of *Legionella pneumophila*-induced release of this cytokine are widely unknown.

In this study we demonstrated a TLR2- and TLR5-dependent release of GM-CSF in *Legionella pneumophila*-infected human alveolar epithelial cells. GM-CSF secretion was not dependent on the bacteria type II or IV secretion system. Furthermore, an increase in Protein Kinase C (PKC) activity, particularly PKCα and ε, was noted. Blocking of PKCα and PKCε activity or expression but not of PKCβ, PKCδ, PKCη, PKCθ, and PKCξ significantly reduced the synthesis of GM-CSF in infected cells. While PKCα was critical for the initiation of an NF-κB-mediated GM-CSF expression, PKCε regulated GM-CSF production via AP1.

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

**Keywords:** GM-CSF, *Legionella pneumophila*, PKC, NF-κB, AP-1, TLR.

## INTRODUCTION

*Legionella pneumophila* (*L. pneumophila*) is the causative agent of Legionnaires' disease, a severe pneumonia with high mortality [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]. Proinflammatory immune responses of the airway epithelium plays 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 (IL-8), IFN-β, and $PGE_2$ upon infection [4, 5, 11-14]. GM-CSF is a 23-kDa hematopoietic 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 knock-out mice demonstrating a pivotal role of this cytokine for host defense function [19]. GM-CSF -/- mice are more susceptible to several pulmonary bacterial infections such as *Pseudomonas aeruginosa* [19], group B *Streptococcus* [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 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-κB (NF-κB) and the Activator Protein-1 (AP-1) [24, 25]. These transcription factors are activated by complex signaling 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-glycerols (DAG), and phospholipids [29]. The PKC isotypes can be categorized 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 is widely unknown.

In the study presented, we demonstrated that TLR2, TLR5 as well as PKCα and ε 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 ε 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

DMEM, FCS, 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 *S. 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 PKCξ myristoylated pseudosubstrate inhibitor, the JNK inhibitor SP600125, and PMA (Phorbol-12-myristate-13-acetate) 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*, we used in the present study the human primary 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 small airway epithelial cells (SAEC) 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% FCS without antibiotics. The NF-κB-dependent reporter cell line, A549 6Btkluc, was a kind gift of R. Newton (Dept of Biological Sciences, University of Warwick, Coventry, UK). These cells contain a stably integrated plasmid with three tandem repeats of the sequence 5'-

AGCTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGA-3', which contains two copies of the decameric NF-κB binding site driving a luciferase gene [30].

**Infection with bacterial strains**

*L. pneumophila* sg1 strain 130b (ATCC BAA-74, kindly provided by Nicholas P. Cianciotto, Northwestern University Medical School, Chicago, USA [31] ), strain JR32 (wild type), JR32 Δ*dotA* deficient in *dot/icm*, encoding a protein essential for the type IVB secretion system (kindly provided by H. Shuman (New York, NY, USA)), strain Corby (wild type), Corby Δ*flaA* deficient in flagellin (kindly provided by K. Heuner, Würzburg, Germany) as well as his type II secretion system knock out Corby Δ*lspDE* [7, 32], were routinely grown on buffered charcoal-yeast extract (BCYE) agar for 2 or 3 days at 37°C before uses [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 exemplary shown the results for one siRNA pro target gene (Fig. 2 and 5). Control non-silencing siRNA (sense UUCUCCGAACGUGUCACGUtt, antisense ACGUGACACGUUCGGAGAAtt), siRNA targeting TLR2 (sense GCCUUGACCUGUCCAACAAtt, antisense UUGUUGGACAGGUCAAGGCtt), TLR5 (sense GGAGCAAUUUCCAACUUAUtt, antisense AUAAGUUGGAAAUUGCUCCtt), PKCα (sense AAGCACAAGUUCAAAAUCCACtt, antisense GUGGAUUUUGAACUUGUGCUUtt), and siRNA targeting PKCε (sense AAGCCCCUAAAGACAAUGAAGtt, 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 instructions (BD Biosciences, Heidelberg, Germany) [11].

**Western Blot**

Western blots were performed to determine siRNA-mediated knock-down 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 as.

**PKC-assay**

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

**P-c-Jun-assay**

The JNK Activity Assay, KinaseSTAR$^{TM}$ 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 analyzed by western blot.

**Chromatin immunoprecipitation**

A549 cells were pre-incubated with or without inhibitors and infected with *L. pneumophila* (MOI 10). Cells were processed for chromatin immunoprecipitation (ChIP) as described elsewhere [12, 34, 35]. *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 shown as means ± SEM of at least three independent experiments. A one-way ANOVA test was used for data of Figure 1; 2A, C; 3A; 4, 5C; 6C, and 7A. The main effects were then compared by a Newman-Keuls´ post-test. $P < 0.05$ was considered to be significant and indicated by asterisks or H-Key. If not indicated otherwise, test was performed vs. control (*) or stimulated probe vs. inhibitor treated probe (#).

## RESULTS

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

SAEC 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 LPS [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 to 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 Fig. 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 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 5 in alveolar epithelial cells.**

PKC signaling pathways have been shown to play important roles in the expression of proinflammatory cytokine release [28, 29, 38, 39]. We hypothesized that PKC may contribute to *L. pneumophila*-related pro-inflammatory activation of lung epithelium. A549 cells were infected with *L. pneumophila* 130b, Corby, Corby Δ*flaA*, 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 PKCα and PKCε 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) and PKCξ (PKCξ 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 PKCξ (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 alveolar epithelium, we analyzed 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. In a next step, the relevance of PKCα and PKCε for *L. pneumophila*-related GM-CSF expression was analyzed 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 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 did alter 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 analyze 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 characterize 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 were evaluated by ChIP assay (Fig. 7B, D). A549 cells were preincubated 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, D) in infected A549 cells. Interestingly, inhibition of PKCα by Gö6976 reduced recruitment of NF-κB to the *gmcsf* promoter (Fig. 7 B)

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

Our data gave evidence 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* und thus be important for immune response in Legionnaire' disease.

Pulmonary epithelial cells may detect *L. pneumophila* by TLRs. In accordance 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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**FIGURE LEGENDS**

**Figure 1:** Increased GM-CSF expression in *L. pneumophila* infected human alveolar epithelial cells. A549 cells (A) and SAEC (B) were infected for 8, 16, and 24 h with *L. pneumophila* 130b [MOI 10]. GM-CSF release was detected by ELISA. TNFα (50 ng/ml) was used as a positive control. The uninfected control was determined by 24 h. Data presented are means ± SE of 4 separate experiments. *, $p<0.05$ compared with uninfected control cells.

Fig. 1

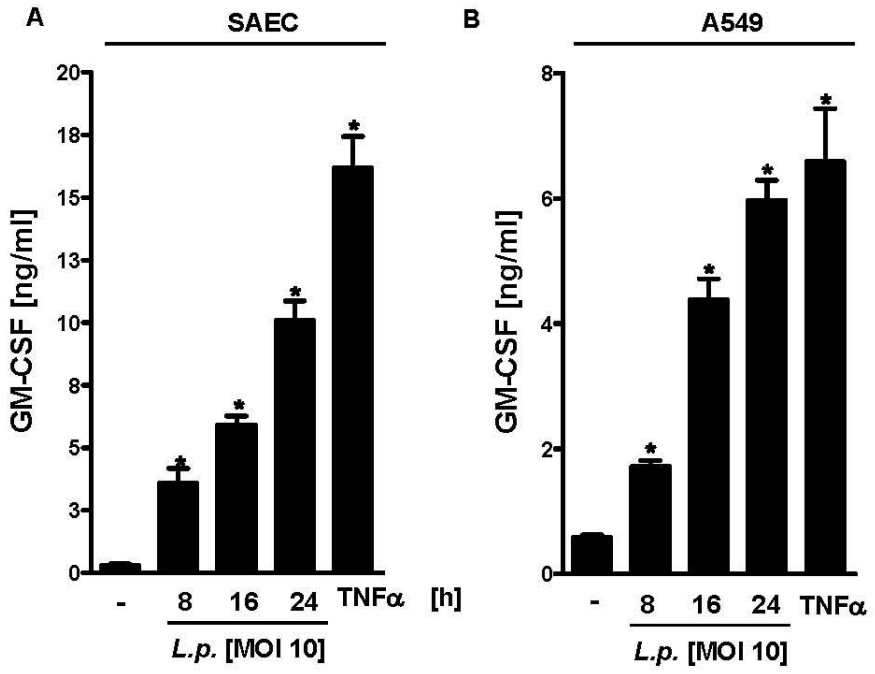



**Figure 2:** *L. pneumophila*-induced GM-CSF release in alveolar epithelial cells is controlled by TLR2 and TLR5 but not by the types II and IV secretion systems.

A549 were infected for 24 h with a MOI 10 of *L. pneumophila* 130b, JR32, JR32 Δ*dotA*, Corby, Corby Δ*flaA*, Corby Δ*lspDE* or incubated with heat inactivated 130b. GM-CSF release was analyzed by ELISA (A). Furthermore RNAi experiments in A549 cells to inhibit expression of endogenous TLR2 or TLR5 respectively were performed. Cells were incubated for 72h with TLR2- and TLR5-specific siRNA as well as a non-silencing control siRNA (C-siRNA) and RT PCR was performed (2B). Importantly, A549 cells were treated with control siRNA or siRNA targeting TLR2 or TLR5 for 72h before infection with *L. pneumophila* or incubation with TNFα (50 ng/ml) or the TLR2 ligand Pam3CSK4 (1 μg/ml) or the TLR5 ligand flagellin (10 ng/ml) as indicated. GM-CSF release was measure by ELISA (2C). Representatives of three independent experiments with similar results are shown (B). ELISA data presented are means ± SE of 4 separate experiments. *, $p<0.05$ compared with uninfected control cells; #, $p< 0.05$ wild type bacteria versus mutant or control siRNA versus target siRNA.

Fig. 2

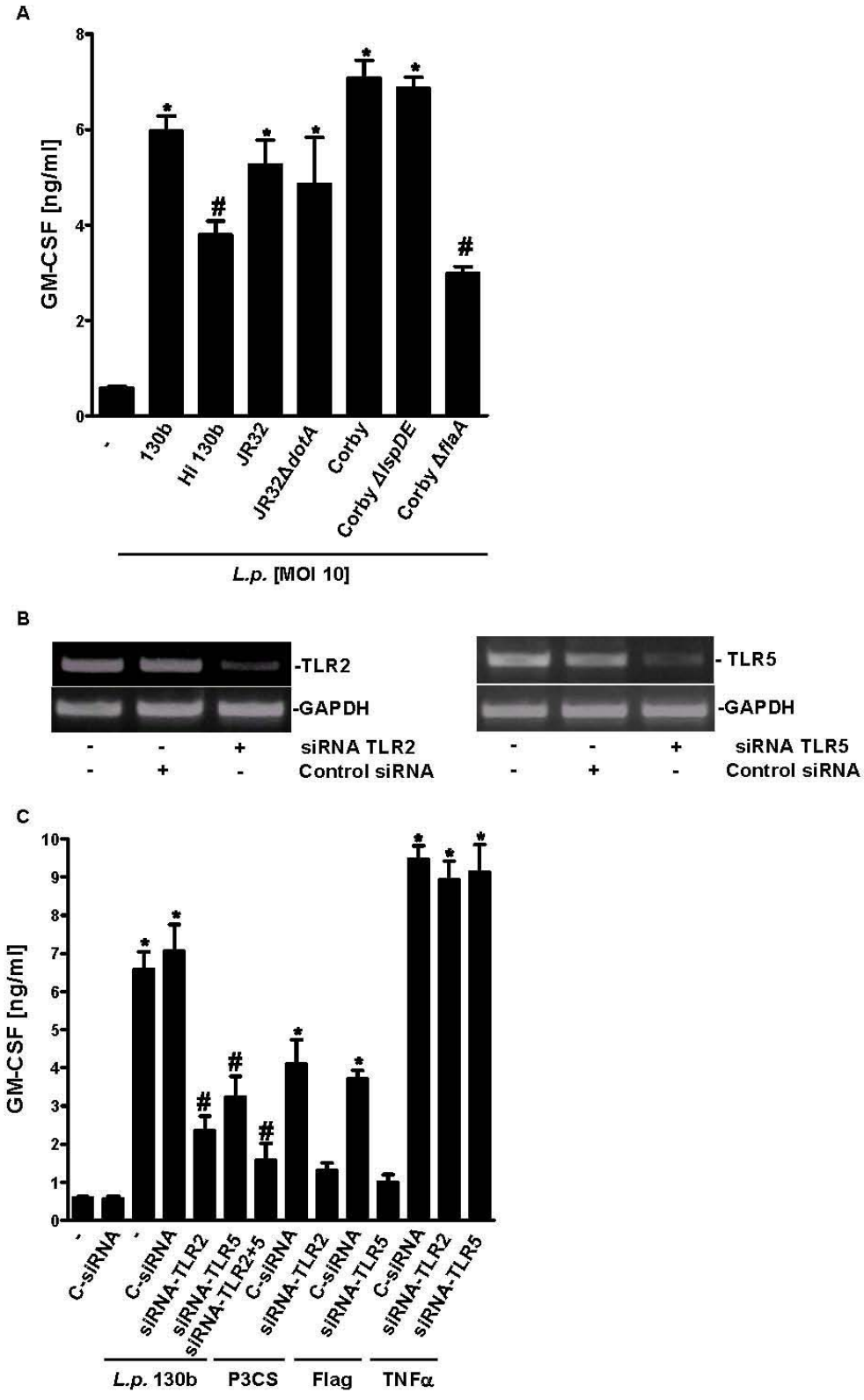

A
GM-CSF [ng/ml]
0
2
4
6
8
-
130b
HI 130b
JR32
JR32ΔdotA
Corby
Corby ΔlspDE
Corby ΔflaA
L.p. [MOI 10]
B
-TLR2
-GAPDH
- - + siRNA TLR2
- + - Control siRNA
- TLR5
-GAPDH
- - + siRNA TLR5
- + - Control siRNA
C
GM-CSF [ng/ml]
0
1
2
3
4
5
6
7
8
9
10
-
C-siRNA
-
C-siRNA
siRNA-TLR2
siRNA-TLR5
siRNA-TLR2+5
C-siRNA
siRNA-TLR2
C-siRNA
siRNA-TLR5
C-siRNA
siRNA-TLR2
siRNA-TLR5
L.p. 130b
P3CS
Flag
TNFα

**Figure 3:** *L. pneumophila* induced activation of PKC. A549 cells were incubated for 60 min with *L. pneumophila* 130b, Corby, Corby Δ*flaA*, or incubated with heat inactivated 130b, Pam3CSK4 (1 μg/ml) and flagellin (10ng/ml). PKC activity was detected by PKC activity assay (A). Furthermore phosphorylation of MARCKS, one major substrate of PKC was assessed via western blot in *L. pneumophila* 130b infected lung epithelium (MOI 10) as indicated (B). PMA (160 nM, 60 min) was used as positive control. Representatives of three independent blots with similar results are shown (B). PKC activity assay data presented are means ± SE of 4 separate experiments (A). *, $p<0.05$ compared with uninfected control cells; #, $p< 0.05$ wild type bacteria versus mutant.

Fig. 3

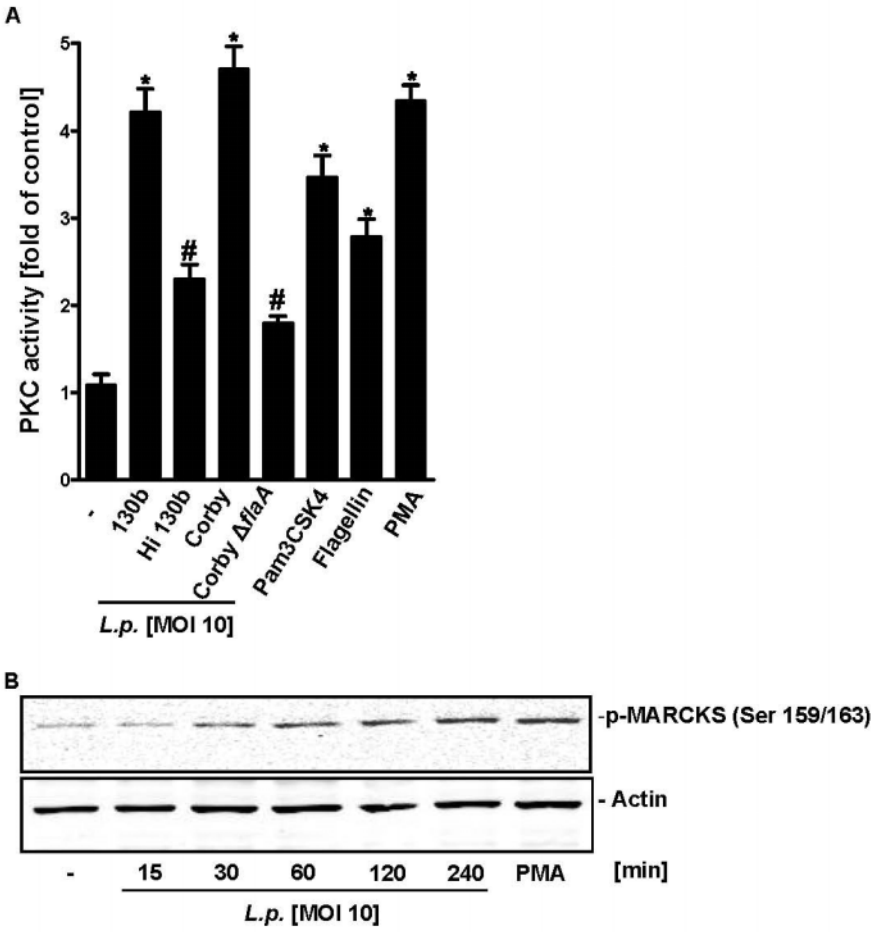



**Figure 4:** *L. pneumophila* induced GM-CSF release is dependent on PKCα and PKCε.

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, 10µM), PKCβ (PKCβ inhibitor, 10nM), Rottlerin (PKCδ inhibitor, 10µM), PKCε (TIP-PKCε, 10µM), PKCη (PKCη myristoylated pseudosubstrate inhibitor, 10µM), PKCθ (PKCθ myristoylated pseudosubstrate inhibitor, 10µM) and PKCξ (PKCξ myristoylated pseudosubstrate inhibitor, 10µM) before

infection with *L. pneumophila* for 24 h. GM-CSF release was assessed by ELISA (Fig. 4). ELISA data presented are means ± SE of 4 separate experiments. *, $p<0.05$ compared with uninfected control cells; #, $p< 0.05$ compared with infected cells without inhibitors.

Fig. 4

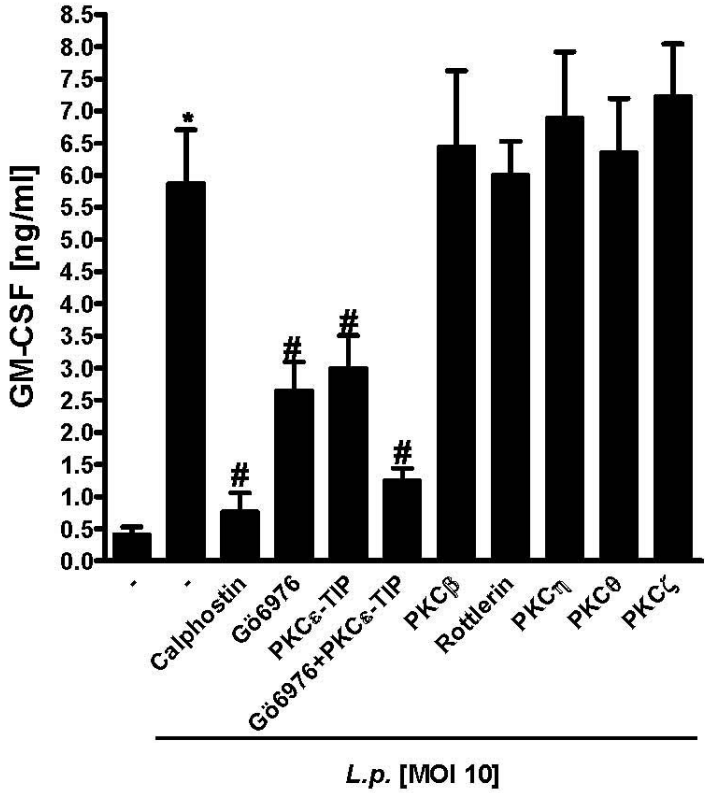



**Figure 5:** Activation and gene silencing of PKCα and PKCε confirmed differential regulation of GM-CSF production in alveolar epithelial cells infected by *L. pneumophila*.

To assess activation (translocation) of PKCα and PKCε, lung alveolar epithelial cells were infected with *L. pneumophila* 130b (MOI 10) for 30, 60, 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 (A, B). ELISA data presented are means ± SE of 4 separate experiments. *, $p<0.05$ compared with uninfected control cells; #, $p< 0.05$ control siRNA versus target siRNA.

Fig. 5

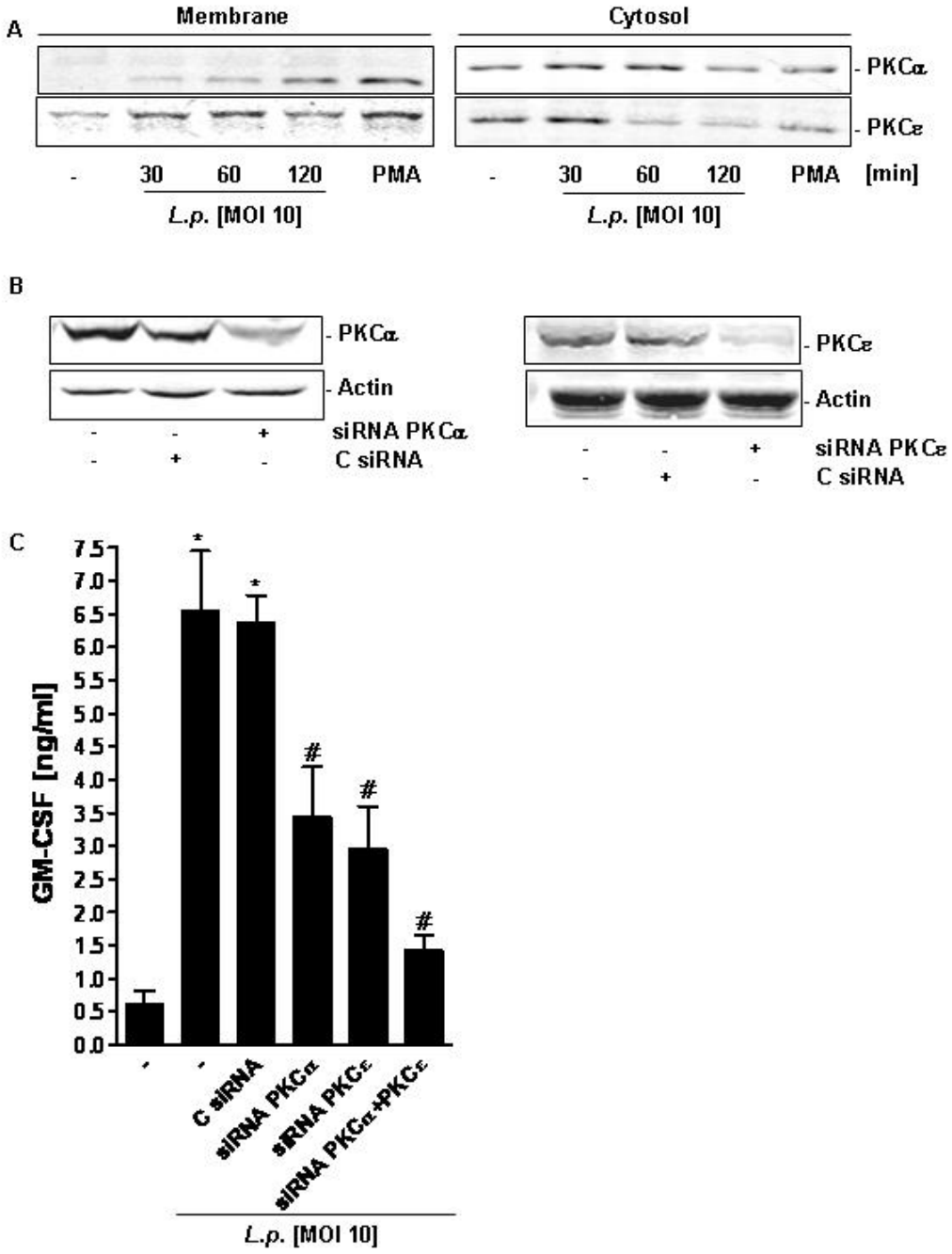



**Figure 6:** *L. pneumophila*-induced GM-CSF release depended on NF-κB and AP-1(c-Jun) in alveolar epithelial cells.

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 above mentioned 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 presented are means ± SE of 4 separate experiments. *, $p<0.05$ compared with uninfected control cells; #, $p< 0.05$ compared with infected cells without inhibitors.

Fig. 6

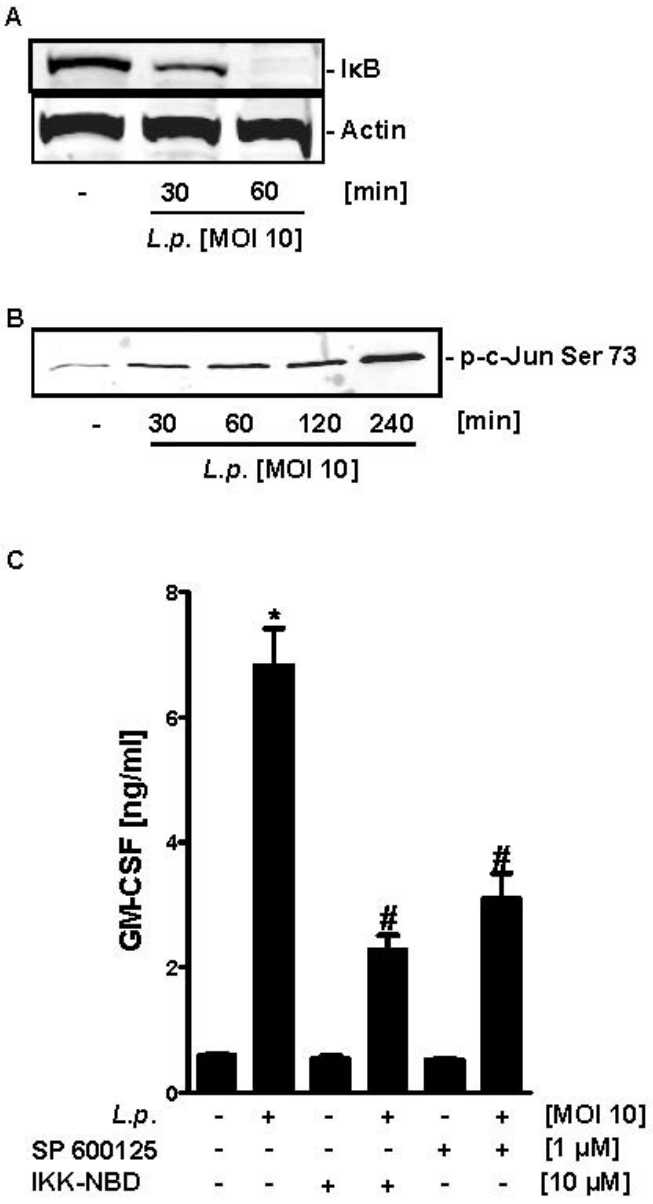
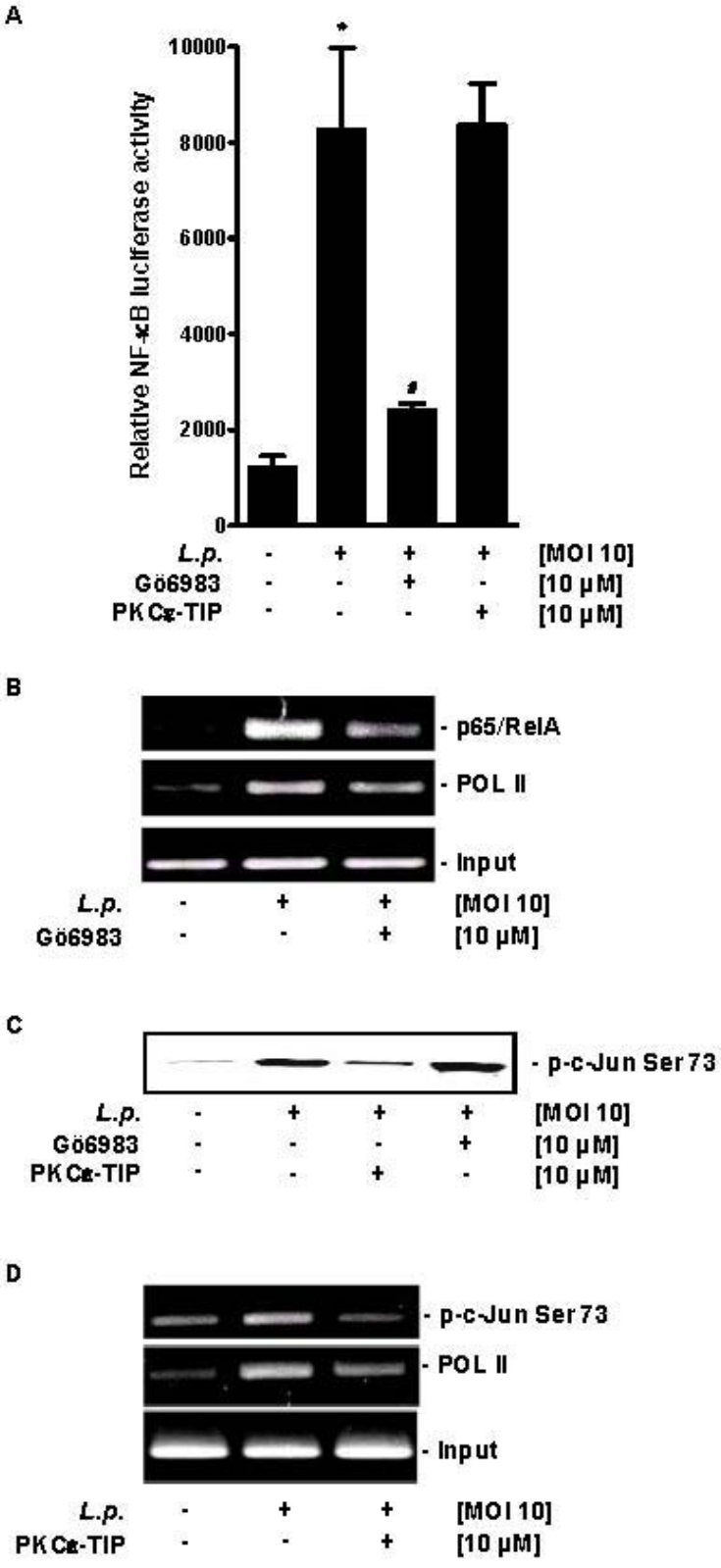



**Figure 7:** PKCα and PKCε differentially controlled NF-κB and AP-1 (c-Jun) activation in *L. pneumophila* -infected alveolar epithelial cells.

The NF-κB-dependent reporter cell line, A549 6Btkluc was pre-incubated with Gö6976 (10μM) or PKCε-TIP (10 μM) and infected with *L. pneumophila* 130b for 4h (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 2h and the binding patterns of POL II, p65, p-c-Jun to *gmcsf* promoter were analyzed by ChIP (B, D). To address the role of PKCε in c-Jun-activation, cells were pre-incubated with Gö6976 (10μM) or PKCε-TIP (10 μM) for 1h and infected with *L. pneumophila* 130b for 4h before being processed by activity assay (C).

Data presented in (A) are means ± SE of 4 separate experiments. *, $p<0.05$ compared with uninfected control cells; #, $p< 0.05$ compared with infected cells without inhibitors. Representative gels or blots out of three are shown.

Fig. 7


A
Relative NF-κB luciferase activity
10000
8000
6000
4000
2000
0
*
#
L.p. - + + + [MOI 10]
Gö6983 - - + - [10 μM]
PKCε-TIP - - - + [10 μM]
B
- p65/RelA
- POL II
- Input
L.p. - + + [MOI 10]
Gö6983 - - + [10 μM]
C
- p-c-Jun Ser 73
L.p. - + + + [MOI 10]
Gö6983 - - - + [10 μM]
PKCε-TIP - - + - [10 μM]
D
- p-c-Jun Ser 73
- POL II
- Input
L.p. - + + [MOI 10]
PKCε-TIP - - + [10 μM]