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# Myeloid RelA regulates pulmonary host defense networks

Christian Hess *, Christian Herr *, Christoph Beisswenger *, Tetyana Zakharkina *, Roland M. Schmid†, and Robert Bals[1, *]

* Department of Internal Medicine, Division for Pulmonary Diseases, Philipps-Universität Marburg, 35043 Marburg, Germany

† Department of Internal Medicine II, Technical University of Munich, Ismaningerstrasse 22, D-81675 Munich, Germany

**Short title**: Function of myeloid RelA in pneumonia

***Correspondence should be addressed to:**

Robert Bals, M.D., Ph.D.

Department of Internal Medicine

Division for Pulmonary Diseases

Philipps-University Marburg

Baldingerstrasse 1

35043 Marburg, Germany

Tel + 49 6421-286 4994, Fax + 49 6421-28 68987

email: bals@staff.uni-marburg.de

# Abstract

The pulmonary innate immune system tract eliminates inhaled pathogens. Several cell types contribute to host defense in a complex network. The aim of this study was to evaluate the role of macrophages during pneumonia and in the regulation of the epithelial response to microorganisms.

We performed lung infection models applying mice lacking myeloid RelA/p65. To study the mechanistic relationships between individual cell types, we applied coculture models composed of airway epithelial cells (AECs) and macrophages.

Mice lacking myeloid RelA/p65 showed significantly decreased bacterial clearance, cytokine expression and neutrophil influx. Also the induction of epithelial KC expression was blunted in these animals. In vitro, AECs were largely insensitive to ligands of Toll like receptor 2 (TLR2) or TLR5. Exposure to secretory products of macrophages results in increased release of proinflammatory cytokines and augmented antimicrobial activity. This was associated with increased expression of TLR genes and surface expression of the proteins. Experiments with blocking antibodies showed that the effect of macrophages depends on secreted mediators including TNF-α.

In conclusion, the present data show that myeloid RelA is critical for pulmonary host defense. One important mechanism is that macrophages induce the sensitivity of AEC's to microbial patterns.



**Key words:** toll like receptor, innate immunity, antimicrobial peptide, chronic obstructive pulmonary disease, asthma

# Introduction

Microorganisms are a potential threat to the integrity of the body and the innate immune system of mucosal surfaces has been evolved largely to protect inner and outer body surfaces. The lung is continuously exposed to microorganisms, however, the pulmonary innate immune system is responsible to maintain a high level of sterility [1]. A breach of pulmonary immune mechanisms result in the development of invasive infection. Pneumonia is a frequent cause of pulmonary morbidity and associated with significant lethality.

The pulmonary innate immune system is composed of multiple components including a number of cell types that fulfill different tasks in host defense [2]. The requirements for the regulation of pulmonary innate immunity are different from other surfaces of the body: Exposure to commensal microorganisms must be handled by a continuous level of protection, while the presence of organisms with pathogenic or invasive potential must induce a local inflammatory and host defense reaction. In the case of invasive infection, the local defense system provides a first line of protection and activates underlying defense mechanisms such as influx of phagocytes and stimulation of the adaptive immune system.

Macrophages are professional host defense cells and involved in the recognition of and response to microorganisms [3]. In the lung, alveolar macrophages comprise the macrophage population. Airway epithelial cells (AECs) are structural cells and constitute a barrier that keeps the environment physically separated from the internal milieu. In addition, AECs actively contribute to innate immunity. AECs express a number of pattern recognition receptors including Toll like receptors [4-6] and nucleotide-binding oligomerization domain

proteins (NOD) [7]. AECs respond to bacteria of different species by activation of inflammatory signaling pathways and release of proinflammatory mediators and antimicrobial compounds [8]. Several studies have addressed the question to which extent different cell types contribute to host defense against infection and to the development of an inflammatory innate immune reaction using chimeric [9-13] or transgenic [14-16] approaches. These data show that both, epithelial / structural cells and professional defense cells, have a role in protecting the lung from infection. It is also evident that different cell types interact in complex ways. Macrophages have been implicated in the regulation of epithelial cell function. Bronchoalveolar lavage fluid (BALF) from pneumonia patients induced the activation of epithelial cells [17]. Conditioned media from activated macrophages has been shown to activate NF-κB in airway epithelial cell lines dependent on TNF- α and IL-1beta [18;19].

The hypothesis of the present study is that macrophages and epithelial cells are involved in pulmonary host defense. While macrophages are sensitive sentinel cells of immunity, AEC are less sensitive to microbial stimulation and represent a physical barrier. Optimal stimulation of AECs requires a second signal in addition to the presence of the microbial compound, a warning signal that unlocks the local innate host defense system to switch from continuous non-inflammatory protection towards an inflammatory activation of host defense. It was the aim of the study to characterize the role of myeloid NF-κB RelA subunit in host defense cells and to test whether the interaction of lung macrophages and AECs is necessary and sufficient for the induction of an epithelial host defense reaction.

# Materials and Methods

## *Cell culture, media and reagents*

Primary airway epithelial cells (pAECs) were isolated from large airways resected during surgery and cultivated as submersed (SM) or air-liquid interface (ALI) cultures as described previously [20]. The protocol was approved by the ethics committee of the University of Munich, and informed consent was obtained from the patients. The human epithelial lung cancer cell line A549 and human monocytic cell line U937 were maintained in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10 % fetal calf serum (FCS) (Invitrogen), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen). For monocytic differentiation, U937 cells were cultured in the presence of 10 nM phorbol-12myristat-13acetat (PMA) (Sigma-Aldrich, Munich, Germany). Differentiation of cells was confirmed by the expression of CD11b and CD14 analyzed by flow cytometry. Primary macrophages were prepared from PBMCs by magnetic cell sorting with anti-CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes adherent to plastic dishes were cultured in RPMI 1640 supplemented with 50 ng/ml M-CSF (Strathmann Biotec, Hamburg, Germany) for 7 days to allow differentiation to macrophages.

## *Stimulation protocols*

Supernatants from U937- and monocyte-derived macrophages were prepared by incubation of the cells with or without 100 ng/ml ultra-pure *E. coli* LPS (Invivogen) for 18 h at 37°C. LPS in this concentration did not induce significant cytokine release from the epithelial cell culture systems used (data not shown). For neutralization assays, the supernatants were pre-treated

with neutralizing mAb (anti-TNF-α, anti-IL-1β, or both) or mouse IgG (all R&D Systems, Wiesbaden-Nordenstadt, Germany) for 60 min. *Pseudomonas aeruginosa* PAO1 was grown to an $OD_{600}$ of 0.8 in Luria-Bertani (LB) medium. *Streptococcus pneumoniae* PN36 was grown to an $OD_{600}$ of 0.3 in soy broth supplemented with 10% FCS. For some experiments bacteria, were heat-inactivated for 30 min at 96°C. Cells were stimulated with the TLR-ligands (Invivogen, San Diego, CA, USA) flagellin (100 ng/ml), PGN (TLR2 ligand, 1 µg/ml), or FSL-1 (200 ng/ml) for 6 hours. To test the effect of macrophage-derived supernatant on the epithelial cells, $1{,}5x10^5$ cells (A549 or pAECs) were seeded per well of a 12-well plate and incubated with the macrophage-derived supernatants for 18 hours. After washes and replacement with fresh medium (not conditioned), heat-inactivated bacteria (*P. aeruginosa* PAO1 or *S. pneumoniae* PN36), flagellin, or PGN were applied for 6 hours. For co-culture experiments, $1x10^6$ epithelial cells (A549 or pAECs) were seeded into each well of a 6-well plate. 24 hrs after plating, $1 \times 10^5$ macrophages (U937 or monocyte-derived macrophages) were seeded on the epithelial cells Six hours after seeding, attachment of the macrophages to the epithelial cell layer was confirmed by microscopy. Co-cultures were stimulated with heat-inactivated bacteria (*P. aeruginosa* PAO1 or *S. pneumoniae* PN36) for 24 hours.

## *FACS analysis*

pAECs were detached with PBS containing 3.7 % EDTA at 37°C. The cells were incubated with phycoerthrin (PE)-conjugated mouse mABs against TLR2 and TLR5 or with their corresponding isotype control (all eBioscience, San Diego, USA) for 30 min at room temperature. To block unspecific binding of the antibodies, the samples were pre-incubated with 20 µg/ml purified mouse IgG (Dako, Hamburg, Germany). Then the cells were washed twice with PBS containing 1% FCS and 0.1% $NaN_3$, resuspended in CellFix (BD

Pharmingen) and analyzed using a FACSort flow cytometer (Becton Dickinson, Heidelberg, Germany).

## *Bacterial survival assay*

Bacterial viability assays were used to determine the effect of macrophage-derived supernatants on the antimicrobial activity of airway epithelium. All experiments were performed using culture medium without antibiotics. After pre-incubation, cells were infected with living *P. aeruginosa* PAO1 or *S. pneumoniae* PN36. 40 µl (corresponding to 5 X $10^4$ CFU) were applied to the apical surface of the differentiated epithelium followed by incubation at 37°C for 6-8 hours. The cultures were then washed three times with 100 µl of PBS and serial dilutions were plated onto LB agar plates. The plates were incubated overnight at 37°C and the number of CFU were counted.

## *Determination of cytokine concentrations*

The concentrations of human IL-6, IL-8, TNF-α, and IL-1β in the cell culture supernatants as well as the levels of murine IL-6, KC, TNF-α and IL-1beta in culture supernatants and cell-free BAL fluid were determined by commercially available sandwich-type enzyme-linked immunosorbent assays (R&D Systems, Wiesbaden-Nordenstadt, Germany).

## *Real Time RT-PCR*

Total RNA was purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany) including treatment with RNase-free DNase set (Qiagen, Hilden, Germany) and 1.5 µg of total RNA was reverse transcribed using a cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany)

applying oligo(dT)18. cDNA was diluted 1 : 5 and 5 µl was used as template in a 25 µl SYBR-Green-PCR mix, according to the manufacturer's protocol (ABgene, Hamburg, Germany). The following PCR primers were used: β-actin, 5'-AGCCTCGCCTTTGCCGA-3'; and 5'-CTGGTGCCTGGGGCG-3'; hBD-2 5'- TCAGCTCCTGGTGAAGCTC -3' and 5'- GGGCAAAAGACTGGATGACA -3'; hBD-3 5'- TATTATTGCAGAGTCAGAGGCG -3' and 5'- CACTCTCGTCATGTTTCAGGG -3'; TLR2 5'- GAGAGTGGGAAATATGGACACCTT -3' and 5'- GCAGTTCCAAACATTCCACG -3'; TLR5 5'- GAAAACCGCATTGCCAATAT -3' and 5'- TGCTGCAAATCGAGAATTT -3' (TIB Molbiol, Berlin, Germany). Specificity of RT-PCR was controlled by no template and no reverse transcription controls and melting curve analysis. Quantitative PCR results were obtained using the ΔΔCT method. Data were normalized to β-actin levels in each sample.

## *Animals and infection models*

Animals were housed in a pathogen-free facility with single ventilated cages and received endotoxin-free diet. All animal experiments were approved by the local Federal authorities (Regierungspräsidium Giessen). To generate a constitutive knockout of RelA/p65 in myeloid cells, $rela^{F/F}$ mice [21] were crossed with LysMCre animals [22] to generate a $rela^{F/F}$LysMCre mouse line. For the experiments sex-matched littermates from $rela^{wt/F}$LysMCre parents were used, the control animals were littermates with $rela^{F/F}$LysMCre or $rela^{wt/wt}$LysMCre. Mice were anesthetized and infected intranasally with viable or heat-inactivated *P. aeruginosa* PAO1 ($5 \times 10^6$ CFU) in PBS or PBS alone as control. 24 (for living bacteria) or 6 hours (for heat-inactivated bacteria) after bacterial infection, the animals were euthanized, the tracheae were cannulated and a bronchoalveolar lavage (BAL) was performed (five times with 1 ml of PBS). Cytopreparations and immunohistochemistry were generated as

described earlier [20]. To determine the bacterial load of the lungs 24 hours after infection, whole lungs were homogenized in 1 ml of PBS, serial dilutions were plated onto LB agar, and colonies were counted after incubation overnight. Murine alveolar macrophages were isolated by BAL and stimulated by LPS. Supernatants were applied to primary murine AECs. After washes, the cells were stimulated with flagellin and the release of KC was measured. Airway epithelial cells were isolated from resected tracheas [23]. Two tracheas were treated with Ham's F-12 (pen/strep, nystatin) containing 1.5 mg/ml pronase E (Sigma-Aldrich GmbH) for 18 h at 4°C and fetal bovine serum was added to a final concentration of 10%. Cells from each six tracheas were pooled and collected by centrifugation at 400 g for 5 min. An incubation in Petri dishes for 2 h was performed to adhere ballast cells. The supernatant was collected and centrifuged at 400 g for 5 min. Primary tracheal epithelial cells were resuspended in culture medium.

*p65 binding activity ELISA*

The DNA binding activity of the NF-κB p65 subunit was measured by using a commercially available p65 ELISA (TransAM p65 Transcription Factor ELISA, Active Motif, Rixensart, Belgium). Whole-cell extracts from murine alveolar macrophages were prepared using a nuclear extract kit from Active Motif (Active Motif, Rixensart, Belgium) and normalized by protein concentration. The activity of p65 is expressed as absorbance at 450 nm.

### *Statistical Analysis*

Values are displayed as mean ± SD. Comparisons between groups were analyzed by t test (two-sided), or ANOVA for experiments with more than two subgroups. Post hoc range tests were performed with the t test (two-sided) with Bon-ferroni adjustment. Results were considered statistically significant for $P < 0.05$.

# Results

Macrophages and epithelial cells are known to contribute to innate immunity of the lung. NF-κB is the prototypic transcription factor of immunity and regulates the expression of macrophage host defense responses [24]. To test whether macrophage activation is necessary for host defense and for epithelial activation in vivo, we evaluated the effect of selected truncation of the *rela* gene in myeloid cells in a murine pneumonia model. To characterize the course of the infection, we determined the numbers of viable bacteria and cells in the lavage. As further outcome we determined the production of KC in epithelial cells. Infection of mice with inactivated myeloid NF-κB resulted in a significant increased number of viable bacterial in the lung homogenates as compared to the wildtype animals (fig. 1a) with suppressed influx of inflammatory cells (fig. 1b). The relative number of individual cell types (macrophages, neutrophils, lymphocytes) was not statistically different between the two groups (data not shown). After inoculation of heat inactivated bacteria, the release of proinflammatory cytokines was largely diminished in the animals with suppressed macrophage function, TNF-α was virtually absent in the investigational group (fig. 1c). In contrast, in animals that were injected with viable bacteria, the higher load of bacterial in *rela* deficient mice resulted in significantly increased levels of cytokines (KO vs. WT: IL-6 (mean (SD)): 343 (152) vs. 31 (23) pg/ml; TNF-a: 46 (15) vs. 13(9) pg/ml). To determine the level of epithelial activation, we used immunohistochemical staining to detect KC in AECs. Mice with deleted myeloid *rela* showed significantly decreased signals of KC immunostaining in the epithelial cells (fig. 1d) as compared to control animals (fig. 1d-e) indicating that the activity of macrophages is necessary to activate epithelial responses to microorganisms. To show that macrophages of mice with deleted myeloid *rela* are less responsive to bacterial stimulation, alveolar macrophages were isolated by lavage and exposed to LPS. The DNA binding activity of p65

was decreased in *rela* deficient cells (fig. 1f). These data show that myeloid RelA is important for host defense during a murine model of pneumonia. Macrophage activation also regulates the response of epithelial cells

## *Macrophages activate AECs to recognize TLR ligands*

AECs form a barrier to the outside world and are continuously exposed to various microorganisms. Under stable conditions, the host defense apparatus provides a basic protection. AECs are intrinsically hyposensitive to PAMPs and barley respond to the exposure to microbes or their structures. These data have been obtained by several laboratories [8;25] and could be replicated in our models. Non-differentiated and in air-liquid interface culture differentiated AECs expressed the message of TLR 1-6, 9, 10 (fig. 2a). We then tested whether AECs respond to prototypical PAMPs that cause a dramatic effect when applied to macrophages. Peptidoglycan and flagellin, the ligands for TLR2 and TLR5, were applied and induced almost no induction of cytokine release by AECs (fig. 2b). Differentiated primary AEC in air liquid interface culture showed increased secretion of cytokines as compared to cell grown submersed in medium (fig. 2b). In contrast, macrophages release abundant IL-8 when stimulated with these molecular patterns (fig. 2b).

The close physical contact between macrophages and epithelial cells in the respiratory tract implies a functional interaction between both cell types. We applied various models to study the reciprocal interaction between these two cell types. A conventional co-culture model of A549 lung epithelial cells and U937 derived macrophages in submersed conditions resulted in a significantly increased release of IL-6 from the epithelial cell line A549 after exposure to

gram-positive or gram-negative heat-inactivated bacteria (fig. 3a). This effect could also been seen in co-cultures using primary monocyte-derived macrophages and primary AECs (fig. 3b). The effect of the co-culture was synergistic as compared to the effect of bacterial application to the individual cells. These data indicate that epithelial cells and professional myeloid host defense cells cooperate during host defense.

## *Soluble factors from macrophages induce TLR signaling in AECs*

AECs can be activated by macrophages to recognize PAMPs and respond with the release of host defense molecules and proinflammatory mediators. To test whether soluble factors in the medium are sufficient to activate AECs, macrophage conditioned medium (mcM) was applied. The response of AECs to PAMPs as measured by the release of IL-8 and IL-6 was significantly stimulated by the preincubation with mcM from macrophages stimulated by LPS, whereas mcM from unstimulated "resting" macrophages has only little or no effect (data not shown) (fig. 4a, b). Of note, the epithelial response without mcM was virtually absent. To determine whether the same mechanisms regulate the expression of epithelial host defense molecules, we determined the transcript levels of the mucosal defensins hBD-2 and hBD-3 in epithelial cells. The application of mcM sensitized the AECs for the production of hBD-2 and hBD-3 (fig. 4c). To test whether alveolar macrophages from *rela* deficient animals mice fail to activate AECs, macrophages were isolated by lavage and stimulated with LPS. The conditioned medium together with the TLR ligand flagellin was used to stimulate primary murine epithelial cells and the secretion of KC was measured. We found that alveolar macrophages from *rela* deficient animals are significantly less capable to stimulate epithelial cells (fig. 4f).

It is clearly established that detection of the PAMPs used in the present study is mediated by TLRs [26]. The induction of gene transcription and the regulation of the protein's surface expression are possible mechanisms to increase the sensibility of a cell to TLR ligands. We tested whether increased availability of these receptors is linked with the activated state of AECs. While the exposure to flagellin and PGN alone did not change the mRNA levels of TLR2 or TLR5, the application of mcM together with the TLR-ligand resulted in increased expression of TLR message (fig. 4d). Exposure to mcM together with the respective ligand also increased surface expression of TLR2 and TLR5 primary airway epithelial cells as determined by FACS analysis (fig. 4e).

Soluble factors secreted from macrophages activate epithelial cells to increase their sensitivity to detect PAMPs. Classical acute phase cytokines that are expressed by macrophages are TNF-α and IL-1β. We tested whether the effects of the mcM could be blocked by neutralizing antibodies that inactivate these cytokines. The induced expression of TLR2 or TLR5 by mcM together with the respective ligand was significantly suppressed by the application of anti-TNF-α, whereas anti-IL-1β had no effect (fig. 5a). The combination of anti-TNF-α and anti-IL1β showed the same effect as anti-TNF-α alone. The exogenous application of TNF-α together with the ligands induced increased expression of the TLRs (fig. 5a). To test whether the principle mechanisms is also relevant in differentiated cells, we exposed AECs in air liquid interface culture with TNF-α and determined the expression of TLR-2 or TLR-5 after the exposure to the respective TLR ligands. Preincubation with TNF-α significantly augmented the expression of TLR-2 and TLR-5 (fig. 5b).

*Macrophages regulate epithelial host defense*

Epithelial cells secrete inflammatory mediators that regulate innate and adaptive host defense. The induction of this activity is largely dependent on the regulation by macrophages. Epithelial cells also provide effective host defense measures by the production of antimicrobial substances such as antimicrobial peptides. To test whether the amount of epithelial host defense depends on macrophage factors, we infected differentiated airway epithelium with bacteria and determined the number of viable microorganisms after 6 h. AECs in air liquid interface culture exhibit significant antimicrobial activity [27]. We found that preincubation of AECs with mcM increased the host defense activity of the epithelium against *P. aeruginosa* (fig. 5c). mcM from LPS-stimulated macrophages had a stronger effect, LPS applied to AECs had no effect.

# Discussion

The main finding of the present study is that myeloid RelA is required for an effective host defense during pneumonia and that macrophages regulate epithelial sensitivity to microbial patterns. Antimicrobial activity, cytokine levels, and neutrophil influx were decreased in animals with defective myeloid RelA. Macrophages sensitize epithelial cells for the detection of bacterial components and subsequent host defense reactions. This effect is mediated by soluble factors released by macrophages and TNF- α appears to be a major mediator of this effect. The study used animal models and further characterized mechanisms in human tissue underscoring the relevance of the identified pathways in humans and model animals.

A number of studies investigated the relative role of epithelial versus professional host defense cells in pulmonary host defense and showed that both cell compartments contribute [9-16]. The present data agree with these observations in that myeloid cells (mainly macrophages and neutrophils) are critical effector cells during lung infection. RelA is part of the canonical NF-κB pathway that is the prototypical immune transcription pathway. RelA and p50 have been identified from nuclear extracts from lungs after bacterial exposure [28]. The present data highlight the role of macrophages as immune sentinels that activate epithelial cells.

Epithelial cells are primarily considered as structural cells. While the formation of a physical barrier likely is a critical function of these cells, epithelial cells are capable to engage in inflammatory and host defense reactions [10;29]. Epithelial cell of the respiratory or the gastrointestinal tract are physiologically hyporesponsive to microbial patterns [8;30;31]. This appears teleologically reasonable because a strong inflammatory reaction to the constant

exposure to inhaled microbial components could cause organ destruction. In the case of microbial invasion and infection, epithelial cells can produce a significant amount of inflammatory mediators, mostly CXC chemokines [9], and produce antimicrobial substances [2]. Macrophages are capable to activate epithelial cells through the release of TNF-α and IL-1β that induce NF-κB [32] and the expression of the defensin hBD-2 [33]. The epithelial host defense reaction is complex and involves numerous factors. In models of acute lung injury [34] and LPS exposure [35], macrophage derived TNF- α was identified as main mediator responsible for the activation of epithelial cells. In the present study, host defense was significantly suppressed in animals with depleted myeloid RelA, likely a consequence of the breach of antimicrobial activity of both, macrophages/neutrophils and epithelial cells. IL-1β and TNF-α levels in the lavage were significantly decreased in the genetically modified animals exposed to heat inactivated bacteria. The blunted induction of epithelial KC underscores the regulator effect that macrophages have on epithelial cells. Macrophage-derived cytokines likely are responsible for stimulation of epithelial cells. Isolated alveolar macrophages from these *rela* deficient animals stimulated AECs significantly less as compared to cells from WT animals. These mechanisms were also identified in the culture model using human material. In our study, TNF-α appeared to have a more prominent role. Animal studies with depletion of macrophages showed that these cells significantly contribute to the production of IL-1β and TNF-α [36;37].

Microbial tolerance of epithelial cells is an intriguing phenomenon, whose mechanism is largely unclear. AECs are under constant exposure to inhaled microorganisms and innate host defense mechanisms result in elimination of inhaled microorganisms without frank inflammation, which would compromise the function of the organ by continuous inflammation and tissue destruction. Our data show that the surface expression of TLR is one

factor that regulates the sensitivity of epithelial cells to microbial compounds. Several other mechanisms have been described that can result in microbial tolerance of epithelial cells. MD2 is necessary for the recognition of TLR4 by epithelial cells and low expression levels might limit the TRL function [25]. Epithelia consist of polarized cells that separate distinct compartments. Interaction with microorganisms from the apical (luminal, outside) surface results in different cellular consequences as compared to the contact at the basolateral side [38]. Our data suggest that a regulation on the transcriptional levels is responsible for the amount of receptor present at the cell surface. The lack of apical surface expression of pattern recognition receptors (PRRs) can also be due to intracellular location of TLRs (TLR2 is localized to intracellular compartments and enriched in caveolin-1–associated lipid raft microdomains presented on the apical surface of airway epithelial cells after bacterial infection [5]) or segregation of receptors to the basolateral membrane compartment [38]. A number of molecules are also known that interact with the TLR signaling including SIGIRR [39] or TRIAD3, or RP105 [40]. Also intracellular proteins such as IRAKM, PI(3)K, Tollip, SOCS1 and sMyD88 downregulate TLR signaling [41]. As shown in the present study, soluble macrophage mediators increase the level of TLR transcript, protein, and surface expression. This increased availability of TLR results in epithelial engagement in inflammation and host defense.

In conclusion, mucosal barrier and host defense functions are regulated within a network composed of different cell types [42]. Under steady-state-conditions, epithelial cells compose a physical barrier and are relatively tolerant to the presence of luminal microbes. Macrophage activation through the canonical NF-κB pathways is critical for an effective immune reaction during pneumonia. In addition to their direct antimicrobial activities, myeloid cells liberate epithelial innate host defense and inflammatory activities. This activation is mediated by soluble mediators and included increased expression of TLRs and sensitive recognition of

microbial patterns with subsequent release of inflammatory mediators and defensin. This network concept allows developing a more detailed insight into mucosal immunity. Disruption of the homeostatic balance between barrier and inflammation likely contributes to the development of lung diseases that are often characterized by a broken epithelial barrier and uncontrolled inflammation. Examples for this situation are common diseases such as asthma, chronic obstructive lung disease, or lung cancer.

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# Figure legends

**FIGURE 1.** Pulmonary myeloid RelA is essential for host defense and activation of epithelial cells. *rela*$^{F/F}$LysMCre (KO) and *rela*$^{wt/wt}$LysMCre (WT) mice were infected with viable (A, B) or heat-inactivated (C-E) *P. aeruginosa* PAO1 (n = 8 animals in each group, significant differences of $P < 0.05$ as compared to the control group are labeled with *). The bacterial load was significantly increased in the macrophage-inactivated animals (KO) (a). Depletion of myeloid p65 (KO) resulted in a decreased total number of cells in the BALs (b). 24 hours after application of heat-inactivated bacteria, the levels of inflammatory cytokines were significantly suppressed in mice that lacked myeloid p65 (c). 24 hours after application of heat-inactivated bacteria, immunostaining for KC revealed significantly decreased expression of this mediator in epithelial cells of animals with depleted RelA in myeloid cells (d) as compared to the wildtype (e). Representative micrographs, 8 mice per group were analyzed, 5 slides / animal. Bar = 150 µm. Data b – e were obtained from the same experiment. (f) Isolated alveolar macrophages from animals with depleted myeloid RelA revealed significantly decreased DNA binding activity of p65 after stimulation with 100 ng ultrapure LPS from *E. coli* as determined.

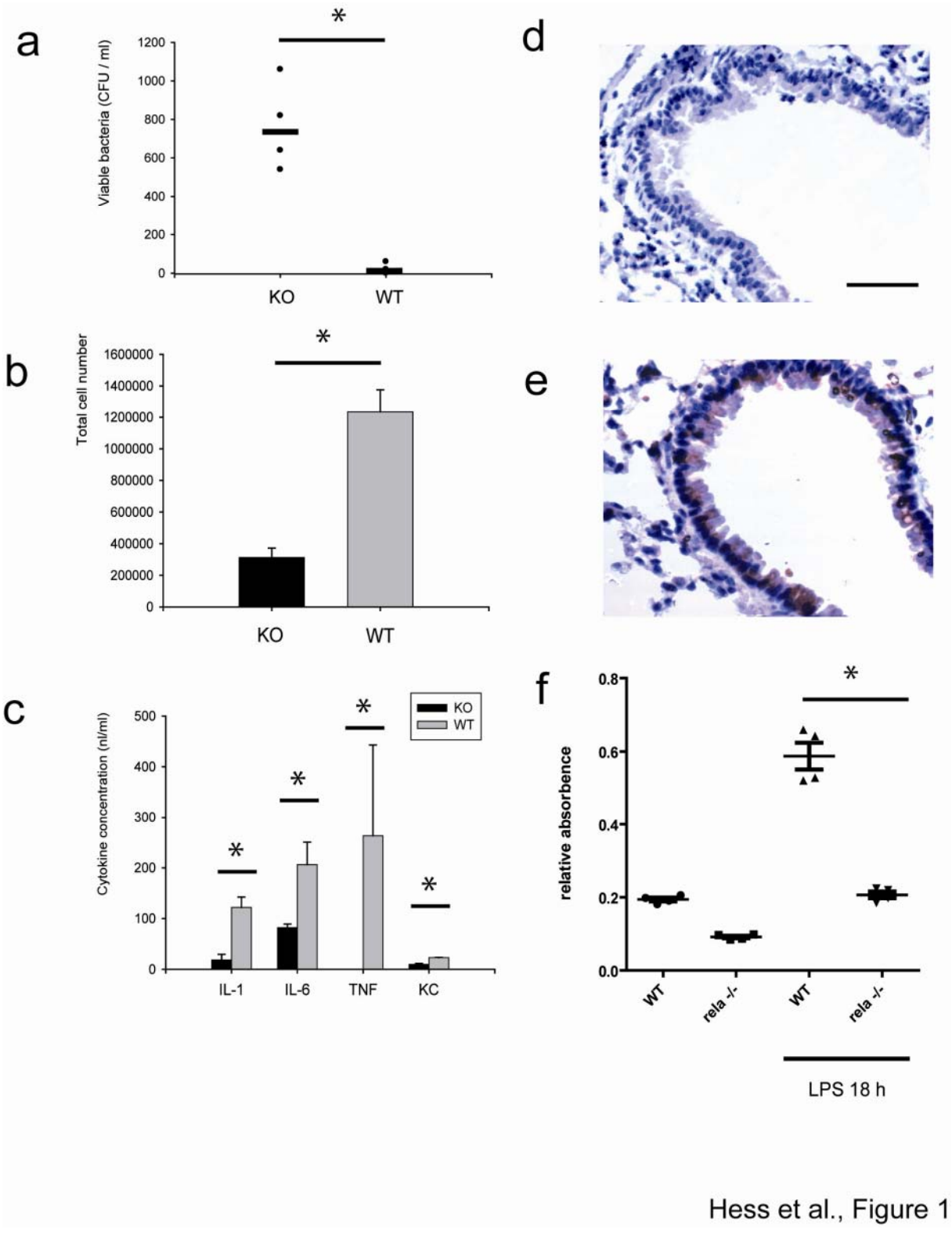



**FIGURE 2.** AECs are hyporesponsive to microbial structures. (a) pAEC expression of TLR1-10 as determined by RT-PCR. pAEC in submersed non-differentiated culture (pAEC) and differentiated primary airway epithelial cells in air liquid

interface culture (pAEC ALI) showed the presence of transcripts of human TLR 1-6, 9, and 10 (representative results out of 5 experiments). (b) pAECs in submersed culture (pAEC), differentiated primary AECs in air liquid interface culture (ALI), and the cell line A549 poorly respond to the exposure to peptidoglycan (P), FSL-1 (F), or flagellin (Fl) whereas macrophages (MA) secrete abundant IL-8. Significant differences of $P < 0.05$ as compared to the control group (-) are labeled with * ($n = 10$).

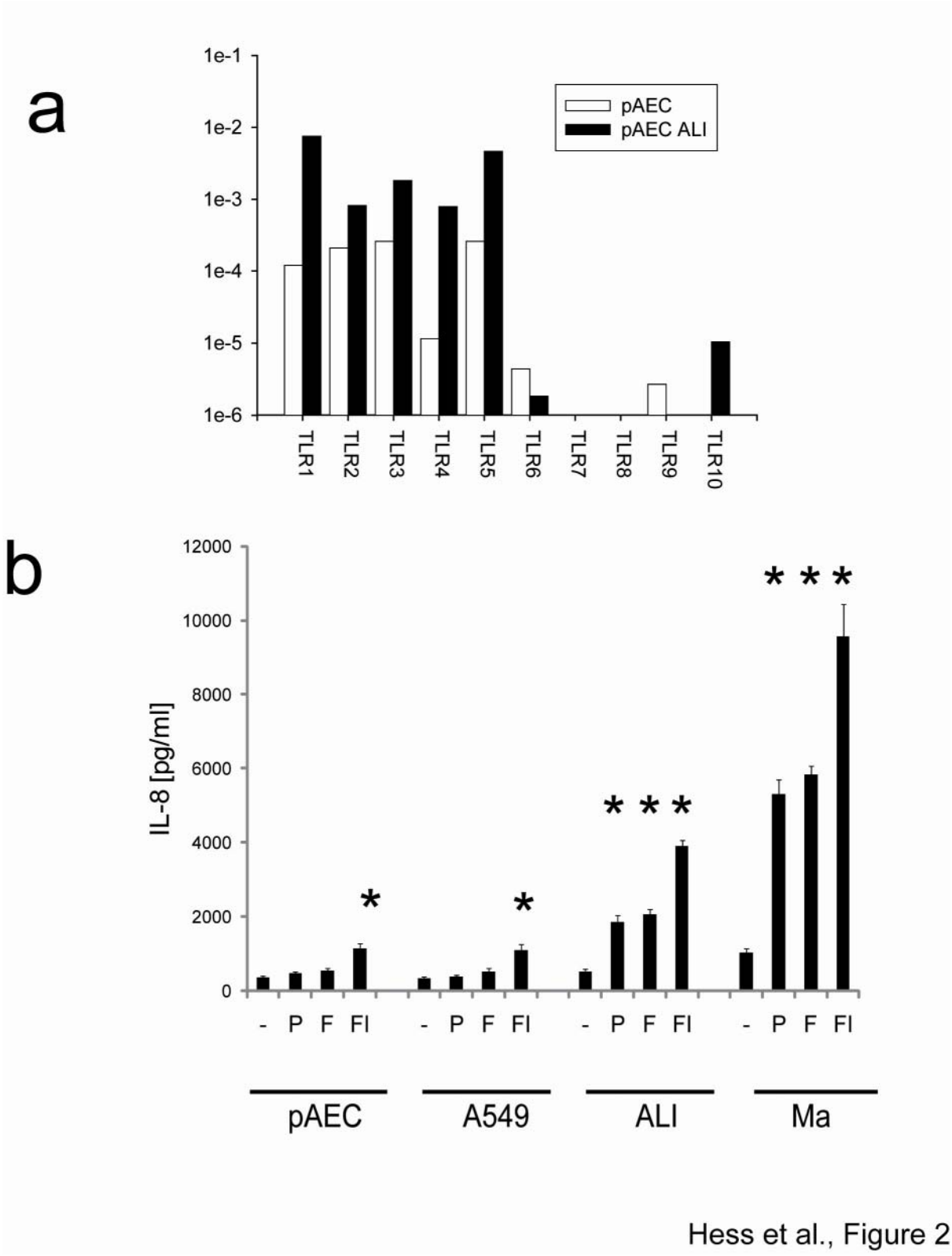
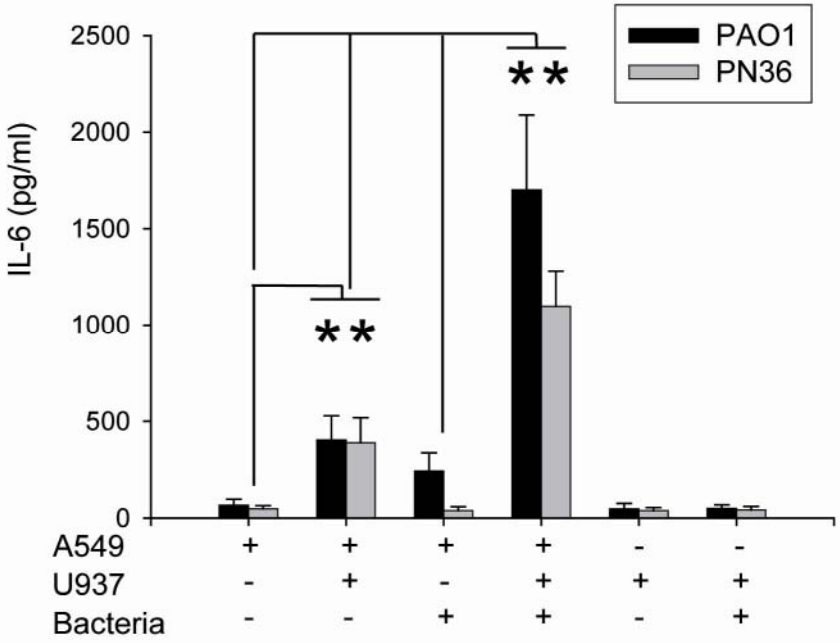



**FIGURE 3.** Macrophages activate airway epithelial cells to respond to microbial structures. (a) Submersed co-culture of the epithelial cell line A549 together with U937-derived macrophages resulted in significantly increased release of IL-6 after

the addition of heat inactivated bacteria (*P. aeruginosa* PAO1 or *S. pneumoniae* PN36). Significant differences of $P < 0.05$ between the indicated groups are labeled with * (n = 4). (b) Stimulation of a submersed co-culture of pAECs and PBMC-derived macrophages with *P. aeruginosa* (PAO1) or *S. pneumonia* (PN36) also resulted in a significantly increase of IL-6 secretion. Significant differences of $P < 0.05$ are labeled with * (n = 4).

a

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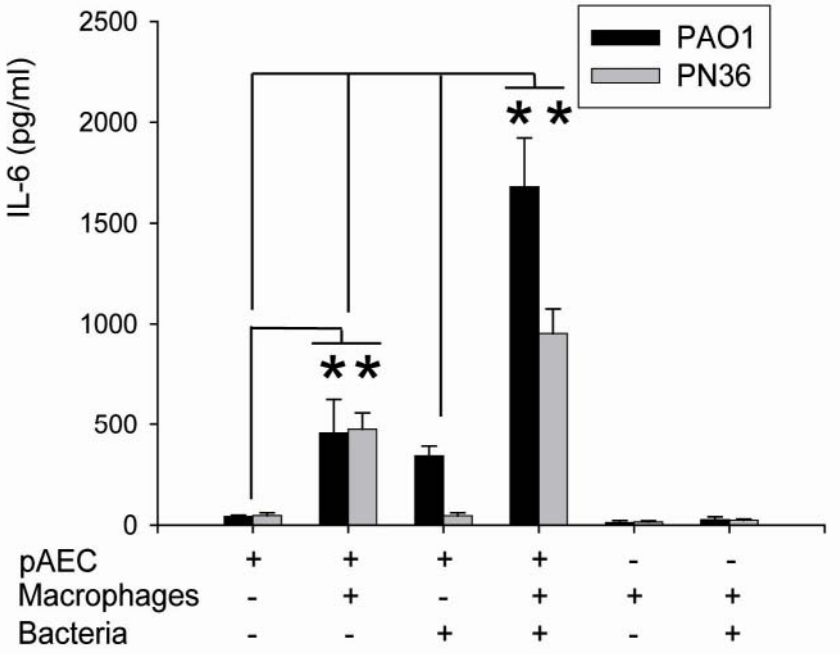
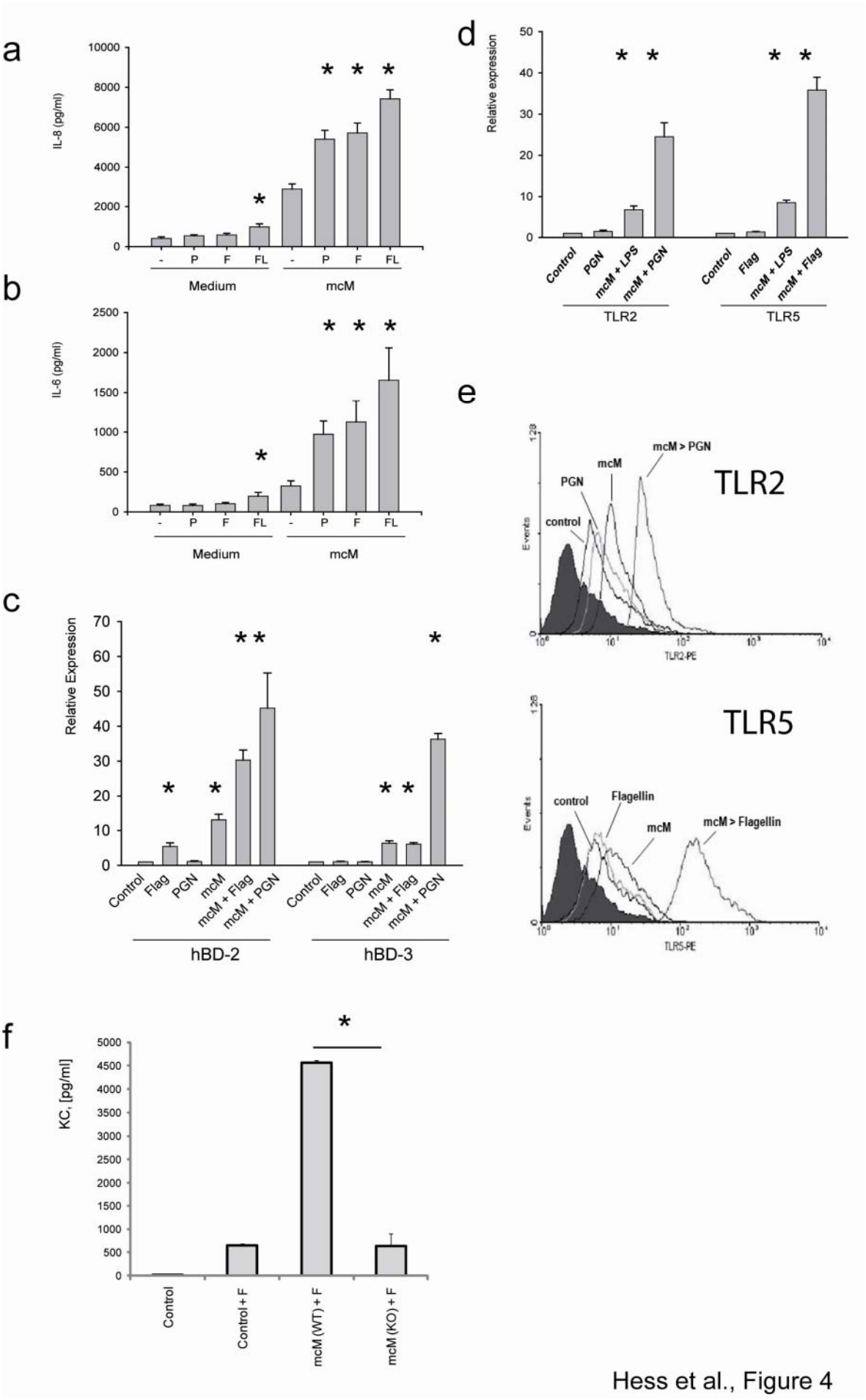


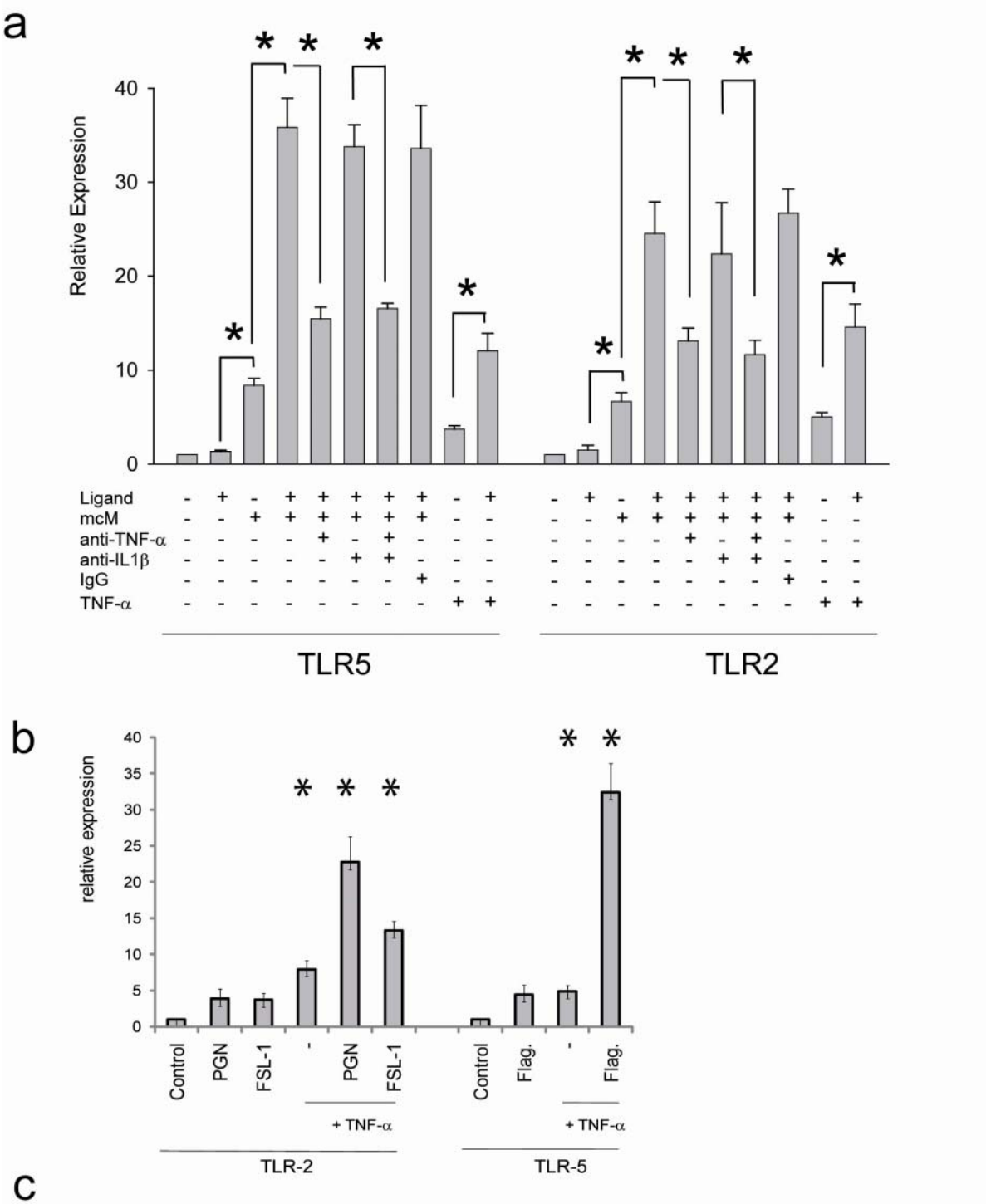

Hess et al., Figure 3

**FIGURE 4.** Interaction between macrophages and AECs depends on soluble factors. (a,b) Pre-incubation with macrophage conditioned medium (mcM) activates AECs in submersed culture as indicated by increased release of IL-8 (a) and IL-6 (b) after stimulation with peptidoglycan (P), FSL-1 (F), or flagellin (Fl). Significant differences of $P < 0.05$ as compared to the control group (-) are labeled with * ($n = 10$). (c,d) The effect of mcM on human beta-defensin and TLR transcript level was measured by quantitative RT-PCR. Expression of hBD-2 and hBD-3 is significantly induced after pre-incubation with mcM and subsequent stimulation with flagellin (Flag) or peptidoglycan (PGN) (c). TLR expression is induced by the presence of both the ligand of the specific receptor and mcM (D). Significant differences of $P < 0.05$ to the control group (for Flag, PGN, and mcM) or to the corresponding group without mcM stimulation (for mcM + Flag and mcM + PGN) are labeled with * ($n = 10$). (e) Surface expression of TLR2 and TLR5 in response to mcM was determined by FACS. Airway epithelial cells were pre-incubated with mcM followed by stimulation with TLR ligands. Pre-treatment with mcM and subsequent stimulation with flagellin or peptidoglycan (PGN) resulted in increased surface expression of the TLR2 or TLR5 as compared to cells treated with mcM or TLR ligand alone. Untreated cells were used as controls. (f) Alveolar macrophages from *rela* deficient animals are significantly less capable to stimulate murine epithelial cells. Epithelial cells were stimulated by macrophage conditioned medium and flagellin (F), KC release was determined by ELISA. Control = AECs without mcM. Significant differences of $P < 0.05$ between the two groups are labeled with * ($n = 4$).




Hess et al., Figure 4

**FIGURE 5.** Effects of macrophages on primary AECs are mediated by TNF- α and are functionally relevant. (a) The expression of TLR2 or TLR5 transcripts by mcM together with the respective ligand is significantly suppressed by the application of anti-TNF- α. External application of TNF- α induces expression of the TLRs. Anti-IL-1β or the control IgG had no additional effect. Significant differences of $P < 0.05$ as compared to the indicated control group are labeled with * (n = 10). (b) Differentiated pAECs in air liquid interface were incubated with TNF-α and then stimulated by PGN, FSL-1, or flagellin. The expression of TLR-2 or TLR-5 was determined by qRT-PCR. Significant differences of $P < 0.05$ as compared to the indicated control group without TNF-α are labeled with * (n = 10). (c) Differentiated airway epithelium (pAECs in air liquid intercafe culture) was used to determine the functional effect of the interaction between macrophages and AECs. The cells were infected with *P. aeruginosa* and the numbers of viable bacteria were determined after 6 h. Coincubation with mcM induced the antimicrobial activity (the significant difference of $P < 0.05$ as compared to the control group is labeled with *, n = 4).




Hess et al., Figure 5