Tyk2 as a target for immune regulation in human viral/bacterial pneumonia

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ABSTRACT The severity and lethality of influenza A virus (IAV) infections is frequently aggravated by secondary bacterial pneumonia. However, the mechanisms in human lung tissue that provoke this increase in fatality are unknown and therapeutic immune modulatory options are lacking.

We established a human lung ex vivo co-infection model to investigate innate immune related mechanisms contributing to the susceptibility of secondary pneumococcal pneumonia.

We revealed that type I and III interferon (IFN) inhibits Streptococcus pneumoniae-induced interleukin (IL)-1β release. The lack of IL-1β resulted in the repression of bacterially induced granulocyte-macrophage colony-stimulating factor (GM-CSF) liberation. Specific inhibition of IFN receptor I and III-associated tyrosine kinase 2 (Tyk2) completely restored the S. pneumoniae-induced IL-1β–GM-CSF axis, leading to a reduction of bacterial growth. A preceding IAV infection of the human alveolus leads to a type I and III IFN-dependent blockade of the early cytokines IL-1β and GM-CSF, which are key for orchestrating an adequate innate immune response against bacteria. Their virally induced suppression may result in impaired bacterial clearance and alveolar repair.

Pharmacological inhibition of Tyk2 might be a new treatment option to sustain beneficial endogenous GM-CSF levels in IAV-associated secondary bacterial pneumonia.

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Introduction
Severe pneumonia causes high levels of mortality worldwide, which have remained almost unchanged since the introduction of antibiotics [1, 2]. Influenza A virus (IAV) and Streptococcus pneumoniae, especially in subsequent co-infections, account for a majority of the fatal outcomes [3]. Considering the fatalities resulting from secondary bacterial pneumonia, the threat of future IAV outbreaks and the increasing bacterial resistance towards antibiotics [4], it is pivotal to apprehend the molecular interplay between viruses, bacteria and pulmonary target cells to enable innovative adjunctive therapies beyond pathogen-directed clinical approaches.

Previous studies in mice indicate that mechanisms accounting for increased severity of secondary bacterial co-infection include IAV-induced alterations of phagocyte functions, epithelial damage which increases bacterial adherence or alteration of immune system components [4]. In particular, IAV-induced type I and II interferons (IFNs) are suspected to modulate innate immune responses such as chemokine (C-X-C motif) ligand [5] chemokines, interleukin (IL)-17-producing γδ T-cells or bacterial clearance in secondary bacterial infections [6, 7]. However, the interference of IAV-induced IFN in the recognition of secondary bacterial pathogens in human lung tissue is completely unknown.

In vitro studies on monocytes and macrophages revealed that IFN may interfere with central proinflammatory cytokine pathways such as the inflammasome, thereby inhibiting IL-1β production [8–10]. In turn, IL-1β seems to be critical for survival in S. pneumoniae infection or in post-IAV Staphylococcus aureus pneumonia in mice [11, 12]. The cellular source, molecular mechanism and its role for induction of subsequent cytokine responses in human lungs are still unknown.

Besides IL-1β, IFN alters the production of another central factor for pulmonary inflammation control, the granulocyte-macrophage colony-stimulating factor (GM-CSF) [13, 14]. In mice infected with IAV (H1N1), GM-CSF enhanced the amount and resistance of alveolar macrophages, as well as the recruitment and activation of dendritic cells, both significantly contributing to survival rates [15–18]. Likewise, in mice mortality rates were improved due to enhanced bacterial killing by alveolar macrophages of S. pneumoniae or group B streptococci, and even infection with Gram-negative Klebsiella pneumoniae showed less apoptosis and alveolar leakage after GM-CSF treatment [19–21]. Moreover, the first clinical trials using GM-CSF for adjunctive treatment in acute lung injury or adult respiratory distress syndrome (ARDS) underscore the therapeutic potential ascribed to this molecule [22, 23].

Therefore, we established an ex vivo human lung co-infection model with IAV and S. pneumoniae to investigate the cellular regulation of these cytokines in the genuine human alveolar compartment. Our study demonstrates on a cellular level how IAV-induced IFN impedes S. pneumoniae-related IL-1β liberation, thereby suppressing GM-CSF production in the human alveolus. Moreover, by inhibition of IFN receptor-associated tyrosine kinase 2 (Tyk2), we offer a potential pharmacological treatment option to restore the virally repressed immune response against secondary bacterial infection.

Materials and methods
Methods
Detailed information is outlined in the online supplementary material.

Human lung tissue
Normal peripheral human lung tissue was freshly obtained from patients suffering from bronchial carcinoma. The study was approved by the ethics committee of the Charité – Universitätsmedizin Berlin (projects EA2/050/08 and EA2/023/07); written informed consent was obtained from all patients. The tissue was stamped into small cylinders (3×8×8 mm) and incubated as described [24].

Isolation of primary human alveolar macrophages and alveolar epithelial cells
Alveolar macrophages were isolated by repeated perfusion of the human lung tissue with Hanks’ balanced salt solution (HBSS) and seeded in six-well plates (1×10⁶ cells) and cultured in RPMI 1640 medium for 2–4 days. Type II alveolar epithelial cells (AECs) were isolated using a modified method described by ELBERT et al. [25]. Briefly, after removal of alveolar macrophages, human lung tissue was finely minced and digested using trypsin type I and DNase. The digested tissue was serially filtered through sterile gauze strainers. Cells were centrifuged and incubated for 1.5 h in 1:1 HBSS:hybridoma–serum-free medium for differential adherence of alveolar macrophages. Afterwards, the cell fraction was collected, centrifuged and layered on a Pancoll (PanBiotech, Aidenbach, Germany) discontinuous gradient. After centrifugation, cells at the interfacial layer were collected, washed with HBSS and seeded in a 24-well plate and cultured in RPMI 1640.
Viral and bacterial strains

The human seasonal IAV strain A/Panama/2007/1999 (Pan/99(H3N2)) was propagated as described by Weinheimer et al. [26]. Two strains of *S. pneumoniae*, the encapsulated strain D39 serotype 2 (NCTC7466) and a clinical isolate serotype 3 (ST3, SN35209) were used and cultured as described [27].

Infection and stimulation of human lung tissue and cells

For infection experiments lung cultures were first inoculated with IAV (1×10^6 PFU) for 24 h as well as for a further 16 h with *S. pneumoniae* (D39 or ST3, 1×10^6 CFU·mL\(^{-1}\)) as indicated. Culture medium containing either recombinant IFN-β, -γ, -λ1, a combination of IFN-β and -γ or a combination of IFN-β, -γ and -λ1 (100 U·mL\(^{-1}\) each), tumour necrosis factor (TNF)-α (100 U·mL\(^{-1}\)), IL-1β (10 ng·mL\(^{-1}\)), anakinra (1 µg·mL\(^{-1}\)) or PRT2070 (cerdulatinib) (1 µM) was injected into lung explants at indicated time points. Control explants received equivalent amounts of culture medium containing PBS. AEC II and alveolar macrophages were infected with *S. pneumoniae* D39 (1 multiplicity of infection (MOI)) for 16 h. Additionally, AEC II were stimulated with IL-1β (5 ng·mL\(^{-1}\)) for 16 h. For alveolar macrophage supernatant experiments, AEC II were pretreated with anakinra (1 µg·mL\(^{-1}\)) for 4 h and supernatant of alveolar macrophages (16 h *S. pneumoniae* D39, 1 MOI), was added and incubated for 20 h.

Results

**Human lung tissue model for ex vivo co-infection of IAV and *S. pneumoniae***

We established a co-infection model using *ex vivo* cultivated human lung tissue inoculated with control medium or Pan/99(H3N2) for 24 h (online supplementary figure S1A and S1B). Additional samples were subsequently challenged with *S. pneumoniae* D39 for a further 16 h (online supplementary figure S1C and S1D). As previously shown, exclusively AEC II were infected by IAV as indicated by pro-surfactant protein C co-localization [26]. *S. pneumoniae* D39 was detected closely attached to the cell surface of AEC I and AEC II as well as alveolar macrophages independent of prior IAV infection (online supplementary figure S1D).

**IAV-induced type I, II and III IFN show unchanged expression during co-infection with *S. pneumoniae***

IFNs are assumed to play a major role in susceptibility to secondary bacterial infection; however, this is still unexamined for human lung tissue. Therefore, we first analysed the secretion of different IFN types after single and co-infection in the lung tissue supernatants. Pan/99(H3N2) significantly induced the release of IFN-α2 and IFN-β (type I), IFN-γ (type II) and IFN-λ1 (type III), whereas *S. pneumoniae* D39 showed no IFN induction at all (figure 1a–d). The secretion pattern of all IFN was not significantly changed in viral and bacterial co-infection, demonstrating that *S. pneumoniae* has no effect on IFN regulation, which might pave the way for a compromised secondary host defence against the bacteria.

**IAV and IFN interfere with the *S. pneumoniae*-induced IL-1β–GM-CSF axis in human lungs**

The induction of cytokines and chemokines plays an important role in the initiation of innate immune responses to control viral and bacterial infections [28]. Accordingly, studies in mice revealed that induction of type I and II IFN during primary nonlethal influenza virus infection complicates the defence against a range of bacterial pathogens [6, 7]. Nevertheless, their role with regard to human lung co-infection is so far unknown. We first examined the influence of IAV on the regulation of typical pneumococcal-induced factors in human lung supernatants. *S. pneumoniae* D39 infection significantly induced all tested cytokines, but no changes were found in co-infection of IL-6, IL-8, IL-10 and TNF-α in the presence of IAV (online supplementary figure S2A–D). However, the preceding IAV infection significantly reduced *S. pneumoniae* D39 induction of IL-1β and GM-CSF (figure 2a and b), which was confirmed for the clinical ST3 isolate (online supplementary figure S2E and F). Since in vitro studies indicate that IFN might interfere with IL-1β and GM-CSF induction, we tested whether IFN in human lung tissue can mimic IAV-induced suppression of IL-1β and GM-CSF [8–10, 13, 14]. Next to single and co-infection with Pan/99(H3N2) and *S. pneumoniae* D39, we replaced IAV with IFN-β and IFN-γ treatment for the same time course and compared the liberated amount of IL-1β and GM-CSF. In line with IAV infection, IFN significantly blocked *S. pneumoniae*-induced IL-1β and GM-CSF synthesis to a similar degree (figure 2c and d). Interestingly, a study by Cakarova et al. [29] showed that expression of alveolar epithelial GM-CSF in mice depends on induction by TNF-α. We observed that compared to IL-1β (figure 2a and c), levels of pneumococcal-induced TNF-α remained unchanged during IAV infection or treatment with IFN (online supplementary figure S3). Hence, we hypothesised that in human lung tissue the expression of GM-CSF is dependent on IL-1β rather than TNF-α. Pretreatment of human lungs with the IL-1 receptor antagonist anakinra (4 h) prior to 16-h *S. pneumoniae* D39 infection or IL-1β stimulation significantly reduced GM-CSF expression (figure 2e). In contrast, TNF-α stimulation of human lungs was not sufficient for induction of GM-CSF (figure 2f). The induction of cyclo-oxygenase-2 in the same lungs served as a positive control for effective TNF-α stimulation (online supplementary figure S4A).
In order to underscore the direct dependency of GM-CSF liberation on IL-1β production we determined the time course of both factors in *S. pneumoniae*-infected human lung tissue. Direct comparison of expression patterns revealed a time-shifted secretion beginning with considerable amounts of IL-1β after 4–6 h followed by GM-CSF after 8–16 h (online supplementary figure S4B). Taken together, these data suggest that the release of GM-CSF in human lungs is mainly dependent on IL-1β rather than TNF-α.

AEC II- and alveolar macrophage-derived IFN blocks IL-1β of alveolar macrophages leading to suppression of AEC II-expressed GM-CSF

Our data indicate that IAV-induced IFNs blocked IL-1β release, finally leading to loss of GM-CSF production in human lungs. To further investigate the underlying cellular interplay of this cytokine regulation in viral and bacterial co-infection, we isolated alveolar macrophages and AEC II from fresh human lung tissue. Phenotype characterisation by immunofluorescence staining showed isolated alveolar macrophages positive for CD68, AEC II for pan-cytokeratin, and pro-surfactant protein C (online supplementary figures S5A and S5B). First, we investigated the release of IFN by IAV-infected AEC II and alveolar macrophages, demonstrating that both cell types produce IFN-α, -β, and -λ1, but IFN-γ is mainly released by AEC II in the human alveolar compartment (figure 3a–d). In contrast, AEC II are negative for release of IL-1β after infection with *S. pneumoniae* D39 (figure 3e), but showed significant release of GM-CSF after IL-1β treatment. In line with stimulation of intact human lung tissue, *S. pneumoniae* failed to directly induce GM-CSF secretion in AEC II (figure 3f). In contrast, *S. pneumoniae* infection of alveolar macrophages led to a strong release of IL-1β, but failed to induce GM-CSF expression (figure 3g and h). Simultaneous treatment of alveolar macrophages with type I and II IFN was sufficient to suppress IL-1β production (figure 3i), while TNF-α was not reduced in the same samples (online supplementary figure S6), mirroring the results obtained in intact lung tissue.
Co-infection with influenza A virus Pan/99(H3N2) (IAV) and treatment with interferons (IFN) inhibits the Streptococcus pneumoniae D39-induced interleukin (IL)-1β–granulocyte-macrophage colony-stimulating factor (GM-CSF) axis in human lungs. Lung explants were ex vivo mock infected or challenged with (a and b) IAV (1 × 10⁶ PFU·mL⁻¹) for 24 h or (c–e) a combination of IFN-β and IFN-γ (100 U·mL⁻¹) each) for 16 h. Afterwards, dedicated specimen were infected with S. pneumoniae (1 × 10⁶ CFU·mL⁻¹). Supernatants were collected 16 h after pneumococcal infection and assayed for release of (a) IL-1β; (b) GM-CSF; (c) IL-1β; (d) GM-CSF. (e) Lung tissue was treated with the IL-1β receptor antagonist anakinra (1 ng·mL⁻¹) or control medium. After 4 h, indicated lung specimen were either mock infected, challenged with S. pneumoniae (1 × 10⁶ CFU·mL⁻¹) or stimulated with recombinant IL-1β (10 ng·mL⁻¹) for 16 h, and subsequently assayed for GM-CSF release. (f) Lung tissue was either mock stimulated or treated with tumour necrosis factor (TNF)-α (100 ng·mL⁻¹) for 16 h in the presence or absence of anakinra (1 ng·mL⁻¹). After 4 h, lung specimens were either mock infected or challenged with S. pneumoniae (1 × 10⁶ CFU·mL⁻¹) for 16 h, and subsequently assayed for GM-CSF release. Data are presented as means±SEM of at least four donors within independent experiments. *: p ≤ 0.05; **: p ≤ 0.01.

Finally, supernatants of alveolar macrophages infected with S. pneumoniae were used for stimulation of GM-CSF in AEC II in the presence or absence of anakinra to provide evidence for the dependence of epithelial GM-CSF on IL-1β released by alveolar macrophages (figure 3j). However, the inhibition of epithelial GM-CSF expression seems not to be restricted to IL-1β suppression in alveolar macrophages alone; instead IFN may also directly inhibit GM-CSF after IL-1β stimulation (online supplementary figure S7).

Tyk2 inhibition restores IAV-induced type I and III IFN-mediated suppression of the IL-1β–GM-CSF axis and reduces bacterial growth

To clarify the cellular level, the IFN type responsible and the mechanism of S. pneumoniae-induced IL-1β suppression after IAV infection, we first used isolated alveolar macrophages from fresh human lung tissue challenged with bacteria in the absence or presence of type I IFN-β, type II IFN-γ or type III IFN-λ1. No differences were found on the transcriptional level for IL-1β in the presence or absence of anakinra (1 ng·mL⁻¹). However, type I IFN significantly suppress pro-IL-1β production and consecutive IL-1β secretion and, to a lower extent so does type III IFN, whereas type II IFN had no suppressive effect, transcriptionally nor translationally (figure 4b and c). In comparison to type II IFN, downstream signalling of IFN type I and III include the IFN receptor I and III-associated Tyk2. Therefore, we used the novel available small-molecule competitive Tyk2 inhibitor PRT2070 in alveolar macrophages and human lungs. Tyk2 inhibition completely restored IFN-β-mediated IL-1β suppression in alveolar macrophages as well as IAV-induced suppression of S. pneumoniae-triggered IL-1β and GM-CSF production in human lungs (figure 5a–c). Moreover, inhibition of Tyk2 shows a promising reduction of bacterial growth in human lung tissue (figure 5d).
Discussion

The induction of cytokines is essential for the initiation of an adequate innate immune response against viral and bacterial infection. However, in subsequent co-infections the antiviral response might strongly compromise antibacterial defence, leading to inadequate bacterial killing, alveolar damage and finally ARDS [4, 7]. Mouse models have indicated virally induced IFNs as important factors for the modulation of antibacterial actions [5–7, 30]. Nevertheless, the influence of IFN on the antibacterial immune response in human lung tissue is unknown so far, and we therefore used an ex vivo human lung tissue model to address this issue. We revealed a cellular communication by which pneumococci lead to upregulation of...
alveolar epithelial GM-CSF via alveolar macrophage-secreted IL-1β and demonstrated that type I and III IFN produced by AEC II and alveolar macrophages after IAV infection strongly interfered with that mechanism on the translational level (figure 6a). By pharmacological inhibition of IFN receptor-associated Tyk2 we were able to fully restore the IL-1β–GM-CSF axis and showed beneficial effects on bacterial growth in human lungs (figure 6b).

Induction of IFN in succession to IAV infection is a conserved and indispensable antiviral response in different species such as mice, ferrets, pigs, horses and humans [31]. Although the cellular antiviral effects seem, in principle, to be similar among species, IFNs induce hundreds of additional genes leading to multifaceted secondary immune reactions, our understanding of which is further complicated by species differences of IFN types, which may potentiate this diversity [32]. Since no species other than human suffers from secondary bacterial pneumonia after IAV infection, it seems that the diverse IFN effects significantly contribute to this fatal superinfection. This is strongly supported by studies in mice infected by adapted IAV strains that become susceptible to secondary bacterial pneumonia mediated by influenza-induced type I and II IFN [3–7, 28, 30].

Therefore, we first analysed the IFN response in human lung tissue after infection with the seasonal IAV strain H3N2. In comparison to mouse data showing type I and predominantly type III IFN responses [33], we found that human lung tissue significantly increased members of all IFN types with an emphasis on type II IFN-γ, which was more than 15 times greater. Interestingly, studies on A549 cells, primary human AECs or even human lung tissue found supporting results for type I and III IFN, but unfortunately neglected the measurement of IFN-γ after IAV infection [34–36]. Current understanding allocates the cellular source of type II IFN-γ to activated T-lymphocytes and natural killer cells rather than AECs, therefore assuming a role in adaptive immune activation instead of innate antiviral activity [37]. However, in the short-term organ culture model used, adaptive immune reactions and activated T-cells are probably absent, and we identified IAV-infected AEC II and not alveolar macrophages as the cellular source of the strong IFN-γ production. The capability of human AEC II to produce IFN-γ is in line with a prior study on interstitial lung disease [38]. Whether IFN-γ directly exerts antiviral effects in human lungs remains unclear, and should be investigated in future studies. In any case, evidence exists that IAV-induced type I and II IFNs facilitate the susceptibility to secondary pneumococcal infection in mice by diminishing

![Graph showing IL-1β expression](https://doi.org/10.1183/13993003.01953-2016)
neutrophil recruitment, IL-17-producing γδT-cells, chemokines such as keratinocyte chemoattractant (KC) and macrophage-inflammatory protein-2 and bacterial clearance [5–7].

Likewise, we postulate that IFN receptor pathways are important in human IAV infection. Unfortunately, recruitment of neutrophils or γδT-cells is currently impossible to measure in the ex vivo model, since systemic blood flow is missing, but cytokine regulation and comparison between species would give important insight. We therefore investigated the effect on the composition of the S. pneumoniae-induced cytokine milieu, which is a prerequisite for the orchestration of an adequate innate immune response. Interestingly, during viral/bacterial co-infection, cytokines such as IL-6, IL-8, IL-10 or TNF-α were unchanged, whereas in mice Smith et al. [28] showed that the same factors (KC as an IL-8 analogue) even increased under co-infection. Additionally, the same effect was found for IL-1β expression, and Hennesset et al. [39] demonstrated upregulation of GM-CSF after IAV challenge. Intriguingly, for the human alveolus
we revealed a difference, since IAV neither increased IL-1β nor GM-CSF, but significantly inhibited these factors under pneumococcal infection, which points towards a species-specific mechanism for the pathogenesis of human lung co-infection.

The importance of IL-1β has been demonstrated in a mouse co-infection model with IAV and *S. aureus* and during pneumococcal pneumonia, but its impact, and in particular its strong downregulation in human lung tissue by IAV infection, is unclear so far [11, 12]. Previous studies have indicated that IFN can inhibit IL-1β production at different levels dependent on species or cell type [8–10, 40, 41]. We therefore replaced IAV by IFN-β and IFN-γ pretreatment and found a similar expression pattern, with significant IL-1β and GM-CSF reduction in human lungs.

In addition to IL-1β, several studies have demonstrated a considerable role of GM-CSF in the outcome of pulmonary infections with IAV or pneumococcal pneumonia and the first clinical trials using GM-CSF as an adjunctive treatment have been introduced [15–17, 19–23, 42, 43]. This emphasises the importance of elucidating its regulation directly in human lung tissue, especially under viral/bacterial co-infection, where a deregulated immune reaction may significantly contribute to fatal outcome.

GM-CSF may be induced by different stimuli, and in mice lungs Cakarova et al. [29] revealed TNF-α to be the responsible mediator after lipopolysaccharide stimulation. However, the regulation of GM-CSF in human lungs requires further investigation.
mouse AECs is not fixed to TNF-α, and seems to depend on cellular origin and culture conditions, determining the phenotypic characteristics. The study by Mir-Kasimov et al. [44] has demonstrated that GM-CSF in *in vitro* mouse AECs can be induced by both TNF-α and IL-1β, demonstrating the importance of validating such results in the human context. In human lungs the cellular source, as well as the driving factor is so far unknown. Either direct pneumococcal Toll-like receptor activation or secondary cytokine-mediated effects may cause GM-CSF expression. Our experiments on intact human lung tissue, isolated primary AECs and alveolar macrophages using anakinra as a specific IL-1 receptor antagonist demonstrated an indirect pneumococcal mechanism in which alveolar macrophages derived IL-1β- rather than TNF-α-fostered AEC II dependent GM-CSF induction. This was further supported by our observation that in co-infection conditions, TNF-α is still present and TNF-α stimulation alone was not sufficient to induce GM-CSF.

In contrast to the induction of GM-CSF, its suppression in AEC II is also of particular interest, since IFNs have been shown to directly inhibit IL-1β-induced GM-CSF in human retinal epithelial or bone marrow cells [13, 45]. We therefore demonstrated that next to IL-1 receptor blockade, IFN treatment in the presence of IL-1β suppressed GM-CSF in lungs, meaning that IFNs act on both alveolar macrophage IL-1β production and on secondary IL-1β-induced epithelial GM-CSF expression.

Our results show a distinct cellular communication mechanism in the human alveolus, where alveolar macrophages are activated by *S. pneumoniae* to produce IL-1β, which then triggers epithelial GM-CSF production. This signalling cascade is arrested by preceding IAV infection. Once more, GM-CSF has shown to be crucial in lung infection [15–21] and is now considered for treatment in acute lung injury and ARDS [22, 23]. Therefore, we aimed to find an immune modulatory intervention strategy to restore the antibacterial GM-CSF response. A systematic analysis was performed to reveal the responsible IFN types as well as the cellular level of IL-1β inhibition, and found that type I and III IFN blocks the translation of pro-IL-1β. Treatment of mice with type I IFN was shown to lead to an increased IAV-induced mortality due to strong proinflammatory immune cell activation [46]. Compared to type II IFN receptors, type I and III receptors signal underinvolvement of Tyk2, which has already been shown to control IL-1β expression in a signal transducer and activator of transcription 1-dependent manner in murine macrophages [47]. Several clinical trials targeting Tyk2 are under way in patients suffering from immunological and inflammatory disorders [48], but it is unknown whether this intervention might be helpful in lung infections, particularly in severe viral/bacterial pneumonia. Here we show that use of the pharmacological Tyk2 inhibitor (PRT2070) fully restored pneumococcal-induced alveolar macrophages IL-1β and finally epithelial GM-CSF after IAV infection in human lung co-infection. This is of particular interest with regard to clinical trials in pneumonia-associated ARDS patients receiving GM-CSF treatment. The systemic administration in the trial by Paine et al. [23] did not reveal significant effects, but a first compassionate inhalative application with a low number of cases by Herold et al. [22] showed positive tendencies of pulmonary host defence modulation. If GM-CSF treatment were successful, compared to IFN receptor pathway inhibition, fewer adverse effects are likely. However, if one single factor such as GM-CSF is not sufficient to restore the entire pulmonary host defence against secondary bacterial pneumonia, an early IFN receptor pathway modulation with, for example, clinically developed Tyk2 inhibitors would offer a further promising approach, since we not only restored endogenous GM-CSF, but also demonstrated a beneficial effect of Tyk2 inhibition on reduction of bacterial growth.

Taken together, our results demonstrated for the first time the human lung tissue model as suitable for the characterisation of innate immune events in IAV and *S. pneumoniae* co-infection. Due to the absence of systemic leukocyte recruitment and adaptive immunity, it becomes possible to discriminate alveolus-specific regulation to model the underlying cellular interplay between alveolar macrophages and AECs controlled by early cytokines.

It has to be stressed that most of the tissue is recruited from lung cancer patients having a history of smoking and chronic obstructive pulmonary disease (COPD) as a comorbidity. Therefore, the peripheral normal lung tissue used cannot be assigned as completely healthy. However, smoking contributes to the major identified risk factors for community-acquired pneumonia (CAP), and patients with COPD have a two- to four-fold higher risk of developing CAP and severe IAV-associated pneumococcal infection [49–51]. This is reflected in large CAP cohorts that demonstrate that a quarter to one-third of hospitalised patients suffer from COPD [52]. Thus, we would not categorise smoking as a limitation of the model, but rather as a very relevant aspect, and we argue that the patients from whom we acquired the tissue are at higher risk of CAP and can be considered a relevant group of interest.

An additional benefit of human lung tissue in comparison to mouse models is that the human lung tissue can be directly infected with viral and bacterial strains isolated from infected patients, which therefore strengthens the relevance of the data. The disclosure of immune differences between mice and humans, as
shown in this study, is of particular importance, since factors such as GM-CSF are already used in clinical trials and considered for immune modulatory treatment in acute lung injury. Moreover, we suggest pharmacological Tyk2 inhibition as an immune modulatory intervention strategy to sustain the crucial endogenous GM-CSF levels in severe viral/bacterial pneumonia.

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References

8. Eigenbrod T, Bode KA, Dalpke AH. Early inhibition of IL-1β expression by IFN-γ is mediated by impaired binding of NF-κB to the IL-1β promoter but is independent of nitric oxide. J Immunol 2013; 190: 6533–6541.