

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

Johanna Berg, Katja Zscheppang, Diana Fatykhova, Mario Tönnies, Torsten T. Bauer, Paul Schneider, Jens Neudecker, Jens C. Rückert, Stephan Eggeling, Maria Schimek, Achim D. Gruber, Norbert Suttorp, Stefan Hippenstiel, Andreas C. Hocke

### **Online Data Supplement**

#### **Methods**

##### **Human lung tissue**

Fresh lung explants were obtained from 78 patients suffering from bronchial carcinoma, who underwent lung resection at local thoracic surgeries. The study was approved by the ethics committee at the Charité clinic (projects EA2/050/08 and EA2/023/07) and written informed consent was obtained from all patients. Tumor-free peripheral lung tissue was first dissected into smaller pieces by scalpel, afterwards stamped into small cylinders (3 x 8 x 8 mm) using a biopsy punch (diameter 8 mm). Finally generated lung tissue pieces were weighted and incubated overnight in RPMI 1640 medium at 37°C with 5 % CO<sub>2</sub> to wash off clinically applied antibiotics. The following infection experiments were done in RPMI 1640 medium supplemented with 0.3 % bovine serum albumin and 2 mM L-glutamine at 37°C with 5 % CO<sub>2</sub> as described [1-3].

##### **Viral and bacterial strains**

The human seasonal influenza H3N2 virus A/Panama/2007/1999 (Pan/99[H3N2]) strain was provided by T. Wolff (RKI, Berlin, Germany) and propagated using MDCK cells as described [3]. Virus stocks were aliquoted, stored at -80 °C and titrated on MDCK cells by a standard plaque assay.

Two strains of *S. pneumoniae* were used, the encapsulated D39 serotype 2 (NCTC7466) (gift from S. Hammerschmidt, University of Greifswald, Germany) and a clinical isolate of *S. pneumonia* serotype 3 (ST3, SN35209) (donated by Dr. Mark van der Linden, National Reference Center for Streptococci, Aachen, Germany). Both bacterial strains were cultured as described [2].

### **Ex vivo infection of human lung tissue**

Lung organ cultures were inoculated with  $1 \times 10^6$  plaque-forming units (PFU) of A/Panama/99(H3N2) further referred to as Pan/99(H3N2) or control medium for 1.5 h. Excess virus was removed by 3 washing steps with PBS and lung cultures were incubated for 24 h (37°C, 5 % CO<sub>2</sub>). Bacterial challenge was performed by injection of *S. pneumoniae*  $1 \times 10^6$  (D39 or when indicated with the clinical isolate ST3) colony-forming units per ml (CFU/ml) or culture medium as control, respectively. Per 100 mg lung tissue 200 µl of infection or control medium were injected using a disposable syringe with needle (0.1 mm x 40 mm) to assure proper stimulation as well as minimal tissue damage. Tissue was processed for analysis after additional 16 h of bacterial infection. Supernatants were assayed for release of cytokines 2, 4, 6, 8 and 16 h following infection. Dilution and infection of Pan/99(H3N2) and *S. pneumonia* were performed using culture medium RPMI-1640 (supplemented with 0.3 % bovine serum albumin and 2 mM L-glutamine).

### **IFN stimulation of lung tissue**

Culture medium containing recombinant interferon  $\beta$ ,  $\gamma$ ,  $\lambda_1$ , a combination of  $\beta$  and  $\gamma$ , or a combination of  $\beta$ ,  $\gamma$ , and  $\lambda_1$  (100 U/ml each) (PeproTech, Hamburg, Germany) was injected into lung explants 16 h before above mentioned pneumococcal infection. Accordingly control explants received control medium 16 h prior to bacterial

infection. The tissue was processed for analysis after additional 16 h of pneumococcal challenging.

### **Stimulation with IL-1 $\beta$ and TNF $\alpha$**

Lung explants received culture medium containing IL-1 $\beta$  (10 ng/ml) or TNF $\alpha$  (100  $\mu$ M) (R&D systems, Wiesbaden-Nordenstadt, Germany) for 16 h before sample processing.

### **Inhibition of IL-1 $\beta$ and IFN signaling**

Lung explants received for IL-1 $\beta$  inhibition culture medium containing either Anakinra (1  $\mu$ g/ml) or equal amounts of PBS. After 4 h the tissue was either challenged with culture medium, *S. pneumonia* D39 (1 x 10<sup>6</sup> CFU/ml) or IL-1 $\beta$  (10 ng/ml) for further 16 h, respectively. For IFN inhibition lung explants received culture medium containing either PRT2070 (Cerdulatinib) (1  $\mu$ M) (Biozol, Eching, Germany) or equal amounts of DMSO. After 1 h they were challenged with 1 x 10<sup>6</sup> PFU of Pan/99(H3N2) for 24 h and with *S. pneumonia* D39 (1 x 10<sup>6</sup> CFU/ml) for further 16 h.

### **Bacterial growth**

Lung explants received culture medium containing either PRT2070 (Cerdulatinib) (1  $\mu$ M) or equal amounts of DMSO. After 1 h they were challenged with interferon  $\beta$  (IFN $\beta$ , 100 U/ml) for 16h and with *S. pneumonia* D39 (1 x 10<sup>2</sup> CFU/ml). After 0, 2, 4 and 6h lungs were homogenized in PBS. Growth of *S. pneumonia* D39 was determined by plating titrated doses of lung homogenates on agar plates as described previously [2].

## **Isolation of primary human alveolar macrophages (AM) and alveolar epithelial cells type II (AEC II)**

AM were isolated by repeated perfusion of the human lung tissue with HBSS. Injected liquid was carefully pressed out of the tissue, collected, centrifuged (340 g, 12 min, RT) and AM were seeded in 6-well plates,  $1 \times 10^6$  cells/well in RPMI 1640 medium. After 4 h of adherence (37°C, 5 % CO<sub>2</sub>) remaining erythrocytes were removed by repeated washing with HBSS. AM were then cultured in RPMI 1640 medium (supplemented with 2 % FCS) for 2 to 4 days. Medium was changed every 48 h.

AEC II were isolated using a modification of the method of Elbert *et al.* [4]. Briefly, after removal of AM human lung tissue (~2 x 5 x 5 cm) was finely minced and digested with trypsin type I (0,25 % in HBSS) (Sigma, Hamburg, Germany) for 45 min at 37°C. After 25 min DNase (350 U/ml) (Applichem, Darmstadt, Germany) was added. After inactivation of enzymes and recovery of the cells using 40 % FCS (GE Healthcare LifeSciences, Freiburg, Germany) the digested tissue was serially filtered through sterile gauze, 100 µm, 70 µm and 40 µm strainers. Cells were centrifuged and incubated for 1.5 h in 1:1 HBSS:Hybridoma-SFM medium (LifeTechnologies, Darmstadt, Germany) at 37°C with 5 % CO<sub>2</sub> for differential adherence of AM. The non-adherent cells were collected, centrifuged and incubated for 45 min in RPMI (supplemented with 10 % FCS and 2 mM L-glutamine) at 37°C with 5 % CO<sub>2</sub> for differential adherence of fibroblasts. Again the non-adherent cell fraction was collected, centrifuged and layered on a Pancoll (PanBiotech, Aidenbach, Germany) discontinuous gradient (1.077 g/ml and 1.040 g/ml). The gradient was centrifuged at 250 g for 20 min, RT. Afterwards cells at the interfacial layer were collected, washed twice with HBSS and finally  $1 \times 10^6$  cells per well were seeded in a 24-well plate and cultured with RPMI 1649 medium (supplemented with 10 % FCS and 2 mM L-

glutamine). The following day dead cells were removed and attached cells grow to confluence within 2 - 4 days. Medium was exchanged daily. The purity of the AEC II population was determined by immunofluorescent staining using antibodies against pro-SPC (AEC II marker), CD-68 (AM marker) and VE-Catherin (endothelial cell marker). Averagely the AEC II population showed a 5% contamination of AM, endothelial cells were detected  $\leq 1$  %. Due to the isolation method, the isolated AM fraction did not contain other cell types. Fibroblast contaminations were rarely and immediately excluded from experimental trials (data not shown).

### **Inhibition of IFN signaling**

Freshly isolated AM received culture medium containing either PRT2070 (Cerdulatinib) (1  $\mu$ M) or equal amounts of DMSO. After 1 h they were challenged with interferon  $\beta$  (IFN $\beta$ , 100 U/ml) for 16 h and with *S. pneumonia* D39 (1 multiplicity of infection (MOI)) for further 16 h.

### **Infection of primary AM and AEC**

AM and AEC II were either mock-infected with culture medium or challenged with *S. pneumonia* D39 (MOI 1). Additionally sets of AEC II were stimulated with IL-1 $\beta$  (5 ng/ml). After 16 h of incubation (37°C, 5 % CO<sub>2</sub>) supernatants were collected and stored at -20°C. Cells were washed twice with PBS, incubated with phosphor-lysis buffer (1xTBS, 150 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 M NaF, 200 mM NaVO<sub>3</sub>, 50 mM Tris-HCL pH7,4, 1 % (v/v) NP-40, 1x Complete protease inhibitor cocktail (Roche, Berlin, Germany)) for 10 min and scraped on ice. The collected cell lysate was centrifuged at 290g for 15 min at 4°C, the supernatant was stored at -80 °C. For viral infection sets of AM and AEC II were either mock infected with culture medium or challenged with IAV Pan/99(H3N2) (MOI 1) for 24 h, afterwards sample processing was performed as

mentioned above. To investigate the effects of IFN regarding pneumococcal induced cytokine release, AM were stimulated with either control medium, recombinant interferon  $\beta$ ,  $\gamma$ ,  $\lambda_1$ , a combination of IFN  $\beta$  and  $\gamma$ , or a combination of IFN  $\beta$ ,  $\gamma$  and  $\lambda_1$  (100 U/ml, each), respectively. After 16 h pneumococcal infection and sample processing was performed as mentioned above.

### **Immunofluorescence**

Primary AM and AEC II were isolated from human lung tissue and seeded on glass coverslips in 24-well plates. The cells were washed twice with PBS, fixed with 3 % paraformaldehyde for 20 min and permeabilized with 1 % Triton-X-100 for 10 min if necessary. Cells were then blocked with 5 % adequate serum and incubated with primary antibodies targeting the specific cell markers CD68 (abcam, Cambridge, UK), surfactant protein C precursor (pro-SP-C) (Millipore, Darmstadt, Germany), and pan-Cytokeratin (Santa Cruz Biotechnology, Heidelberg, Germany). Afterwards, cells were incubated with corresponding Alexa Fluor 488- or 594- conjugated anti-human IgG (H+L) (Invitrogen, Darmstadt, Germany). Nuclear counterstaining was performed using DAPI (Sigma, Hamburg, Germany). Immunohistochemistry of paraffin embedded tissue sections was implemented as described [1]. Antibodies detecting *S. pneumonia* (donation by S. Hammerschmidt), pro-SP-C and Alexa Fluor 488–labeled influenza A virion–specific antibody (Serotec, Puchheim, Germany) were used. Finally cells and tissue sections were mounted in Mowiol, and analyzed using LSM 780 or LSM5 Pascal confocal microscopes (objectives 63x, zoom: 1.9; Plan-Neofluor/oil, NA 1.3, Plan-Apochromat/oil, NA 1.4; Zeiss).

### **Western blot**

For extraction of proteins, human lung explants were transferred into Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA, USA) containing phospho-lysis buffer. Tissue was disrupted in a FastPrep-24 homogenizer (MP Biomedicals, Santa Ana, CA, USA). Protein extracts were separated on a 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (GE Healthcare LifeSciences, Freiburg, Germany). Membranes were blocked with 1:1 PBS:Odyssey blocking buffer (LI-COR Inc., Lincoln, NE, USA) and probed with antibodies against COX-2 and actin (Santa Cruz Biotechnology, Heidelberg, Germany). IRDye 800- and Cy5.5-labelled secondary antibodies were purchased from Invitrogen. Protein quantification was performed using an Odyssey automated infrared imaging system (LI-COR Inc., Lincoln, NE, USA) according to manufacturer's instructions.

## Statistics

Statistics were carried out using PRISM-6 (GraphPad) and data are presented as the mean  $\pm$ SEM of at least three donors within independent experiments. Comparison between groups was established using the Mann-Whitney U-test (non-parametric distribution) or where appropriate the unpaired Student's t test (parametric distribution). Significances are represented as  $*p \leq 0.05$ ,  $**p \leq 0.01$ , or  $***p \leq 0.001$ .

## Supplementary Figure Legends

**Figure S1.** Localization of influenza A virus Pan/99(H3N2) (IAV) and *Streptococcus pneumoniae* D39 (*S. pneumoniae*) in single and co-infected human lung tissue and IAV induced interferon (IFN) expression in single and co-infected human lung tissue. (A) Human lung tissue was either mock infected, (B) challenged with the seasonal IAV for 24 h, (C) *S. pneumoniae* for 16 h, or (D) co-infected with the IAV subsequently followed by *S. pneumoniae*. IAV (green, white arrowheads) typically

replicated in alveolar epithelial type II cells indicated by pro-SP-C (blue, asterisk, *B*, *D*). *S. pneumoniae* closely attached to alveolar epithelial cells (open arrowheads, *C*, *D*) and alveolar macrophages (white arrow, *D*). Lung structure was visualized by differential interference contrast (grey) and nuclei were counterstained using DAPI (orange). Scale bar 10  $\mu$ m.

**Figure S2.** Co-infection with influenza A virus Pan/99(H3N2) (IAV) and *Streptococcus pneumoniae* (*S. pneumoniae*) shows differential cytokine regulation in human lungs. Lung explants were *ex vivo* mock infected or challenged with IAV ( $1 \times 10^6$  PFU/ml) for 24 h. (*A-F*) Afterwards, dedicated specimen were either infected with the *S. pneumoniae* ( $1 \times 10^6$  CFU/ml) (*A - D*) or (*E, F*) the clinical isolate of serotype 3 *S. pneumoniae* ( $1 \times 10^6$  CFU/ml), respectively. Supernatants were collected 16 h after pneumococcal infection and assayed for release of (*A*) IL-6, (*B*) IL-8, (*C*) IL-10, (*D*) TNF $\alpha$ , (*E*) IL-1 $\beta$ , (*F*) GM-CSF as indicated. Data are presented as mean  $\pm$  SEM of six donors within independent experiments. \*\* $p \leq 0.01$ .

**Figure S3.** Influenza A virus Pan/99(H3N2) (IAV) infection or interferon (IFN) treatment of human lung tissue fails to suppress *Streptococcus pneumoniae* D39 (*S. pneumoniae*) induced tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) release. Lung explants were *ex vivo* mock infected or challenged for 24 h with influenza A virus Pan/99(H3N2) (IAV) ( $1 \times 10^6$  PFU/ml) or 16 h pre-treated with a combination of IFN $\beta$  and IFN $\gamma$  (100 U/ml each). Mock or *S. pneumoniae* ( $1 \times 10^6$  CFU/ml) infection followed and supernatants of lung specimen were analysed after additional 16 h for release of TNF $\alpha$ . Data are presented as mean  $\pm$  SEM of six donors within independent experiments. \*\* $p \leq 0.01$ .



**Figure S4.**  $\text{TNF}\alpha$  stimulation induces cyclooxygenase-2 (COX-2) expression and granulocyte macrophage - colony stimulating factor (GM-CSF) expression is time shifted to IL-1 $\beta$  in human lungs. (A) Stimulation with  $\text{TNF}\alpha$  (100 ng/ml) of human lung specimen for 16 h induces pro-inflammatory COX-2. (B) Human lung explants were either mock infected or challenged with *S. pneumoniae* ( $1 \times 10^6$  CFU/ml) for 2, 4, 6, 8, and 16 h to demonstrate time shifted release of GM-CSF in relation to earlier IL-1 $\beta$  liberation. Data are presented as fold of control and mean  $\pm$  SEM of three/four donors within independent experiments.

**Figure S5.** Alveolar macrophages (AM) and alveolar epithelial type II cells (AEC II) were isolated from fresh human lung tissue and seeded on glass coverslips, fixed and phenotyped with different cell markers by immunofluorescence and confocal microscopy. (A) AM, cultured for 2 days, were positive for CD-68 (red). AEC II were cultured for 4 days after isolation. (B) Cells were positive for pan-Cytokeratin (red) and pro-SP-C (green). For nuclear counterstaining DAPI (blue) was used. Microscope: Carl Zeiss LSM780, Plan Apo-Chromat 63x oil/NA 1.4. Scale bar 5  $\mu\text{m}$ .

**Figure S6.** Tumour necrosis factor  $\alpha$  ( $\text{TNF}\alpha$ ) expression was not suppressed by interferons (IFN) in isolated human alveolar macrophages (AM). Isolated AM were cultured for 2 days and either mock challenged or stimulated with a combination of interferon  $\beta$  and  $\gamma$  (100 U/ml each) for 16 h before pneumococcal infection. Afterwards, supernatants were assayed for release of  $\text{TNF}\alpha$ . Data are represented as mean  $\pm$  SEM of four donors within independent experiments.  $*p \leq 0.05$ .

**Figure S7.** IL-1 $\beta$  induced granulocyte macrophage - colony stimulating factor (GM-CSF) release in human lung tissue is suppressed by interferons (IFN). Human lungs were pre-treated with a combination of IFN $\beta$  and IFN $\gamma$  (100 U/ml each) for 16 h and subsequent IL-1 $\beta$  stimulation (10 ng/ml) for 16 h. Supernatants of lung specimen were analysed for release of GM-CSF. Data are presented as mean  $\pm$  SEM of three donors within independent experiments. \* $p \leq 0.05$ .

**Table S1:** IL-1 $\beta$  secretion of alveolar macrophages. Data are presented as mean  $\pm$  SEM of six donors within independent experiments.

	control	IFN $\beta$	S.p.D39	IFN $\beta$ + S.p.D39	IFN $\beta$ + S.p.D39 + PRT2070
IL-1 $\beta$ [pg/ml]	1.0 $\pm$ 1.0	1.2 $\pm$ 1.2	77.9 $\pm$ 36.8	44.7 $\pm$ 26.4	80.0 $\pm$ 37.3

## References

1. Hocke AC, Becher A, Knepper J, Peter A, Holland G, Tonnies M, Bauer TT, Schneider P, Neudecker J, Muth D, Wendtner CM, Ruckert JC, Drosten C, Gruber AD, Laue M, Suttorp N, Hippenstiel S, Wolff T. Emerging human middle East respiratory syndrome coronavirus causes widespread infection and alveolar damage in human lungs. *Am J Respir Crit Care Med* 2013; 188(7): 882-886.
2. Szymanski KV, Toennies M, Becher A, Fatykhova D, N'Guessan PD, Gutbier B, Klauschen F, Neuschaefer-Rube F, Schneider P, Rueckert J, Neudecker J, Bauer TT, Dalhoff K, Dromann D, Gruber AD, Kershaw O, Temmesfeld-Wollbrueck B, Suttorp N, Hippenstiel S, Hocke AC. Streptococcus pneumoniae-induced regulation of cyclooxygenase-2 in human lung tissue. *Eur Respir J* 2012; 40(6): 1458-1467.
3. Weinheimer VK, Becher A, Tonnies M, Holland G, Knepper J, Bauer TT, Schneider P, Neudecker J, Ruckert JC, Szymanski K, Temmesfeld-Wollbrueck B, Gruber AD, Bannert N, Suttorp N, Hippenstiel S, Wolff T, Hocke AC. Influenza A Viruses Target Type II Pneumocytes in the Human Lung. *Journal of Infectious Diseases* 2012; 206(11): 1685-1694.
4. Elbert KJ, Schafer UF, Schafers HJ, Kim KJ, Lee VH, Lehr CM. Monolayers of human alveolar epithelial cells in primary culture for pulmonary absorption and transport studies. *Pharm Res* 1999; 16(5): 601-608.

