

Microenvironmental acidification by pneumococcal sugar consumption fosters barrier disruption and immune suppression in the human alveolus

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Online data supplement

Methods

Human lung tissue

Lung tissue explants were obtained from 103 patients primarily diagnosed with bronchial carcinoma, who had undergone lung resection at local thoracic surgery centres. The study was approved by the ethic committee at the Charité clinic (protocol number EA2/079/13) and the written informed consent was obtained from all patients. Tumour-free peripheral lung tissue was dissected into small pieces by scalpel (5 x 5 x 5 mm, 80 - 200 mg) and incubated overnight in RPMI 1640 medium (PAN-Biotech) at 37°C with 5 % CO₂ to wash off clinically applied antibiotics as described [1-3].

Bacterial strains and PLY

Encapsulated *S.p.* D39 serotype (ST) 2 wild type (NCTC7466; wt), D39 derived mutants Δply [4], $\Delta spxB$ [5], $\Delta ply\Delta spxB$, $\Delta cps\Delta ply$ [6] (friendly gift from S. Hammerschmidt, University of Greifswald, Germany) and Δldh [7] (kindly provided by H. Yesilkaya, University of Leicester, UK) were used. Clinical isolates expressing non-haemolytic PLY variant ST1 (SN35218) and haemolytic PLY variant ST6B (SN33364) were donated by Dr. Mark van der Linden, the National Reference Centre for Streptococci, Germany. Bacterial strains were grown as described previously [2, 3, 8]. *P. aeruginosa* strain PA103 was obtained from ATCC (ATCC29260). We thank Susanne Engelmann (Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany) for kindly providing *S. aureus* Newman (NCTC 10833) and Craig R.

Roy (Yale University School of Medicine, USA) for providing *L.p.* Corby. Bacterial strains were grown as described previously [9-11].

Infection and stimulation of human lung tissue and cells

The following infection experiments were carried out in RPMI 1640 medium blank or supplemented (volume-controlled) with 10 % fetal calf serum (FCS) at 37°C with 5 % CO₂ as described [2, 3, 12]. For infection, tumour-free normal lung tissue was inoculated with culture medium as control (mock infection) or 1x10⁶ CFU·mL⁻¹ *S.p.* (wt, Δply , $\Delta spxB$, Δldh , ST1, ST6B) in the presence and absence of 25 mmol·L⁻¹ HEPES (volume-controlled) for indicated time periods or treated with 1 and 5 µg·mL⁻¹ purified PLY, 1 and 10 mmol·L⁻¹ hydrogen peroxide (H₂O₂, Carl Roth). Applied volumes per lung sample were 600 µl using 27 G needle with insulin syringes with very gentle instillation to avoid tissue damage. For inflammatory activation, 100 ng·mL⁻¹ interleukin (IL)-1 β (R&D Systems) was used. For inhibition of cellular pathways, 50 µg·mL⁻¹ zVAD (Merck), 2 mM 2-DG (Sigma), 0.1, 1 and 10 mM oxamate (MedChemExpress), 20 µM proteasome inhibitor MG-132 (Merck), 10 µM p38 inhibitor SB202190 (Sigma), 10 µM ROCK inhibitor Y-27632 (Tocris), 10 µM hsp90 inhibitors (AUY-922, MedChemExpress; 17-AAG and Ganetespib, Selleckchem), 1 µM TRPV1 inhibitors (AMG 9810 and SB 366791, Bio-Techne) or connexin 43 inhibitors (200 µM gap19, Selleckchem; 150 µM gap26, MedChemExpress) were applied for indicated time points. All inhibitors were pre-incubated on the sample 1 h prior to infection. To assess pH effects on cell junctions, lung explants were exposed to medium at pH 7, 6.5, 6, or 5 (titrated by HCl, Carl Roth) for indicated time points with and without medium exchange. Afterwards, lungs were processed for further analysis. Human umbilical vein endothelial cells (HUVEC), grown to confluent monolayer, were infected with *S.p.* $\Delta cps \Delta ply$ (1 multiplicity of infection (MOI)) for 24 h or incubated in culture medium at pH 7, 6 or 5 for indicated time points with and without medium exchange. For HUVEC supernatant (SN) experiment, lung explants were infected with 1x10⁶ CFU·mL⁻¹ *S.p.* D39 wt in the presence or absence of HEPES for 24 h. Afterwards, SN of human lungs were collected for pH measurement and filtered. Next, HUVEC were stimulated with human lung tissue culture SN for 24 h (see Figure 4a).

For comparison of human pathogens in regard to acidification human lung tissue was infected with 1x10⁶ CFU·mL⁻¹ *Legionella pneumophila* (*L.p.*) or *S.aureus*, *P. aeruginosa* and *L.p.* were

grown in medium (RPMI + 10% FCS) for 24 h. After indicated time point supernatants were collected and pH was measured.

Western Blot

For protein extraction, 80-200 mg shock frozen tissue was transferred into Lysing Matrix D tubes (MP Biomedicals) with RIPA buffer (Thermo Fisher Scientific), containing 1 x complete protease inhibitor cocktail (Sigma-Aldrich). Tissue was disrupted in a FastPrep®-24 homogenizer (MP Biomedicals) applying 3 rounds of tissue lysis at default settings (6 m/s, 30 s). Lysates were subjected to Western blot as described previously [12]. Briefly, protein extracts were separated on a 7.5 % Mini-PROTEAN® TGX™ Precast Protein Gel (Bio-Rad) and transferred to a PVDF membrane (Merck). Gels were loaded with 40 µg protein per lane. Membranes were blocked with Odyssey blocking buffer (LI-COR Inc.) for 1 h at room temperature and probed with antibodies against VE-cadherin, pro-IL-1β, COX-2 (Santa Cruz Biotechnology), occludin, ZO-1, claudin-2, claudin-3, claudin-4, claudin-5, claudin-18 (Invitrogen), PECAM (Novus Bio), GAPDH (Cell Signaling) and β-actin (Sigma) overnight at 4°C and subsequently incubated with corresponding secondary horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. In all experiments β-actin or GAPDH were detected on the same membrane to control for equal protein load. Proteins were detected by incubation with HRP-conjugated IgG antibodies (Santa Cruz Biotechnology) and Amersham ECL Prime Western Blotting System (Cytiva) or Pierce ECL Western blotting Substrate (Thermo Fisher Scientific). The membranes from each experiment were exposed to Carestream Biomax Light Film (Sigma Aldrich) or Amersham Hyperfilm ECL (Cytiva). Densitometry was performed using ImageJ (Image processing and analysis in Java, open platform). Induction or reduction of target protein in the manuscript text was calculated by dividing the normalised signal intensity of the control sample by the median signal intensity value for the corresponding protein.

Isolation of total RNA, cDNA synthesis and qPCR

Human lung tissue explants were stored in RNAlater™ Stabilization Solution (ThermoFisher) at 4°C overnight (to allow the solution to thoroughly penetrate the tissue). For total RNA isolation the RNeasy Mini Kit (Qiagen) was used according to manufacturer's instructions. Human lung tissue was transferred to Lysing Matrix D tubes (MP Biomedicals) in 500 µl RLT

lysis buffer supplemented with β -mercaptoethanol. Tissue was lysed using FastPrep®-24 (MP Biomedicals) applying 3 rounds of tissue lysis at default settings (6 m/s, 30 s). Homogenates were centrifuged and RNA was purified from the supernatant. 0.5 μ g RNA was reverse transcribed and quantitative PCR was performed using TaqMan assays on an ABI 7300 instrument.

Bulk RNA sequencing and analysis

RNA from infected samples was purified as described above. 0.5 μ g RNA was used for RNA-Seq libraries preparation. RNA-Seq libraries were constructed using Illumina TruSeq™ Stranded Total RNA Library Prep Gold kit (New England Biolabs) according to the manufacturer's instruction, with rRNA depletion and 11 PCR cycles in the amplification step. Sequencing was performed on a NextSeq 500 platform using 1x76 cycles single-end sequencing and aiming at 10-20 mln reads per sample.

The pipeline used to process the transcriptomic data is available from Github (<https://github.com/bihealth/seasnap-pipeline/>). Quality control was performed using a variety of tools, including FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), dupradar [13], qualimap [14] and summarised using MultiQC. For adapter trimming, we used the trimadap tool available from <https://github.com/lh3/trimadap>. Reads were mapped to human genome GRCh38, version p7 using the STAR program, version 2.7.8a [15]. Counts were produced by the STAR program using the Gencode GRCh38 annotation version 25. For differential gene expression, the R package DESeq2, version 1.38 was used [16]. Gene set enrichment used the R package tmod, v. 0.50.13 [17], with gene sets included in the MSigDB through the msigdb package, v. 7.5.1 [18]. Gene sets used included Gene Ontology Biological Process gene sets, Hallmark gene sets, REACTOME gene sets and transcriptional modules contained in the tmod package. Results, reports, pipeline output and additional files were stored and managed using the SODAR system [19].

Correction for multiple testing: given that several genes were selected a priori, the p-values obtained for these genes were corrected for multiple testing using the false discovery rate (FDR) Benjamini-Hochberg method within this pre-selected set. FDR for the remaining p-values was calculated within the full set of all genes.

Heatmaps were prepared with the R pheatmap package, version 1.0.12. The expression values were standardized per gene. Heatmaps with gene expression are shown from the selected gene set (GO:0007043, supplementary figure S4c) or the top regulated genes were selected as follow: from each contrast, we have selected 75 top genes (as ordered by p-value, supplementary figure S7i). These resulted in 127 unique genes in total which are shown on heatmap (top-genes heatmap). We divided the genes into three groups according to their function.

The raw data files (counts) from transcriptome sequencing have been submitted to GEO and a GEO ID will be included upon publication. Scripts and programs used to generate the results and the figures are available from the repository <https://github.com/bihealth/manuscript-Microenvironmental-acidification>.

Bacterial RNA isolation and RNA-seq transcriptomics

S.p. were cultivated in supplemented RPMI medium in the presence or absence of 10% FCS, which was added to the +FCS culture at an OD₆₀₀ of 0.3. The +FCS and –FCS cultures were harvested after 2 hours of growth after reaching OD₆₀₀ of 0.3 (Fig. 6E). Bacteria were harvested in ice-cold killing buffer (50 mM Tris pH 7.5, 5 mM MgCl₂, 20 mM NaN₃), centrifuged at 4750 rpm for 10 min at 4°C and the pellets were immediately frozen in liquid nitrogen and stored at –80°C. RNA isolation was performed using an acidic phenol-chloroform extraction protocol [20]. After DNase-I treatment (Zymo Research), the RNA quality was checked by Trinean Xpose (Gentbrugge) and the Agilent RNA Nano 6000 kit using an Agilent 2100 Bioanalyzer (Agilent Technologies). For RNA-seq transcriptomics, Ribo-Zero rRNA Removal Kit (Bacteria) from Illumina (San Diego, CA, USA) was used to remove the rRNA. TruSeq Stranded mRNA Library Prep Kit from Illumina (San Diego, CA, United States) was applied to prepare the cDNA libraries. The cDNAs were sequenced paired end on an Illumina HiSeq 1500 (San Diego, CA, United States) using 70 and 75 bp read length and a minimum sequencing depth of 10 million reads per library. The transcriptome sequencing raw data files are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-13533.

Bioinformatics data analysis, read mapping, data visualization, and analysis of differential gene expression

The paired end cDNA reads were mapped to the *S. pneumoniae* genome sequence (accession number <https://www.ncbi.nlm.nih.gov/nucore/CP000410.2>) using bowtie2 v2.2.7 [21] with default settings for paired-end read mapping [16]. All mapped sequence data were converted from SAM to BAM format with SAMtools v1.3 [22] and imported to the software ReadXplorer v.2.2 [16]. Differential gene expression analysis of 3 biological replicates and normalization was performed using Bioconductor package DESeq2 included in the ReadXplorer v2.2 software [16]. The signal intensity value (A-value) was calculated by log2 mean of normalized read counts and the signal intensity ratio (M-value) by log2 fold-change. The evaluation of the differential RNA-seq data was performed using an adjusted p-value cut-off of $P \leq 0.01$, a signal intensity ratio (M-value) cut-off of ≥ 1 or ≤ -1 and a A-value cut-off of < 8 . Genes with an m-value outside this range and $p \leq 0.01$ were considered as differentially up- or downregulated by FCS addition.

ELISA and LDH assay

Cyto- and chemokines release was quantified by ELISA in tissue-free supernatants according to the manufacturer's instructions (eBioscience; BD Biosciences). Lactate dehydrogenase (LDH) release was quantified by Cytotoxicity Detection Kit according to the manufacturer's instructions (Roche).

Immunohistochemistry, spectral confocal microscopy and live-tissue imaging

Human lung tissue samples were fixed in 4 % paraformaldehyde for 24 h, embedded in paraffin and routinely processed for histology and immunofluorescence staining as described before [12]. Primary antibodies detecting VE-cadherin (Santa Cruz Biotechnology), PECAM-1 (Novus Bio) and *S.p.* (kind donation by S. Hammerschmidt, University of Greifswald, Germany) were incubated overnight at 4°C, followed by labelling with corresponding secondary antibodies Alexa Fluor 488, 555 or 594 (Invitrogen). Nuclei were subsequently counterstained with DAPI (Sigma Aldrich). Immunofluorescence of human lung slices was analysed by spectral confocal microscopy using a LSM 780 [(objective: Plan Apochromat 40x/1.40 oil DIC M27), Carl-Zeiss, Jena, Germany]. Based on a spectral image lambda stack, linear unmixing of tissue autofluorescence and overlapping spectra of fluorochromes were performed using ZEN 2012 software (Carl-Zeiss, Jena, Germany). To reveal lung and cell morphology, images were combined with Differential Interference Contrast (DIC). All image sets were acquired using

optimal configuration regarding resolution and signal to noise ratio. Images were processed using ZEN 2012.

For live tissue imaging of pHrodo red (prepared according to manufacturer's instructions, Invitrogen) were incubated in human lung tissue slices (mock and 1×10^6 CFU·mL⁻¹ *S.p.* for 24 h) and were imaged at 37 °C / 5% CO₂ in RPMI medium supplemented (volume-controlled) with 10% FCS using a LD LCI Plan-Apochromat 25x/0,8 Imm Korr DIC M27 with water immersion. Specific spectra of pHrodo were separated from tissue autofluorescence by spectral imaging and intensity was measured by signal integration and histogram analysis in parallel to pH measurement for correlation.

Blood Gas Analysis

Tissue-free supernatants were centrifuged by 4°C (12000 rpm) and filtered with Filtropur S 0.2 (Sarstedt). 500 µl of the total volume was used for pH, glucose and lactate concentration measurements with the ABL800 FLEX Blood Gas Analyser.

Statistical analysis

GraphPad Prism 9 (Version 9.5.1) software was used for the statistical analysis. Data are presented as means±SD of at least three donors within independent experiments. Mann-Whitney U-test or Wilcoxon test was used for comparison between two groups of at least 4 or 7 experiments, respectively. For multiple comparisons of three or more groups two-way ANOVA with Tukey's post-hoc test was used. Significances are represented as *p < 0.05, **p < 0.01, or ***p < 0.001.

Figure S1. Junctional proteins occludin and VE-cadherin are degraded during pneumococcal infection in human lungs, independent of pneumolysin (PLY) and hydrogen peroxide (H₂O₂) or cell death. Lung explants were stimulated with a) 1 and 5 $\mu\text{g}\cdot\text{mL}^{-1}$ and e) 1 $\mu\text{g}\cdot\text{mL}^{-1}$ purified PLY, (a) 1 and 10 $\text{mM}\cdot\text{L}^{-1}$ H₂O₂ or infected with (c, d) $10^6 \text{ CFU}\cdot\text{mL}^{-1}$ *Streptococcus pneumoniae* wild type (*S.p.* wt) and mutants (b, d, f) Δply , (d) ΔspxB or (g) double mutant $\Delta\text{ply}\Delta\text{spxB}$ for 24 h. b) and f) Lung tissue was challenged with *S.p.* wt as well as *S.p.* Δply alone or treated with pan caspase inhibitor 50 $\mu\text{g}\cdot\text{mL}^{-1}$ zVAD for 24 h. a) and b) Densitometry of occludin and VE-cadherin Western blots are shown. g) Total tissue lysates were analysed by Western blot and representative gel for VE-cadherin is shown. Values represent respective protein expression level relative to control and normalized to β -actin. After indicated time point supernatants were collected and c) LDH or d-f) interleukin (IL)-1 β were measured. Data are presented as mean \pm SD of at least four donors within independent experiments. NS: nonsignificant. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Figure S2. Localization of pH change in human alveoli. Human lung tissue was labelled with pHrodo (yellow) and either mock infected (control, panel I) or infected with $10^6 \text{ CFU}\cdot\text{mL}^{-1}$ *S.p.* wt for 24 h (*S.p.*, panel II). Tissue autofluorescence was measured without pHrodo dye and set as cut off (panel III). The tissue was analysed by spectral confocal microscopy under live cell conditions and intensity of pHrodo was integrated using three images per condition depicted in the histogram (panel IV) and correlated with pH measurement in the supernatants. Erythrocytes were visualized to localize capillaries and small vessels in panel II by spectral imaging (red). Scale bar, 20 μm .

Figure S3. Expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) during pneumococcal infection in human lungs. Lung explants were exposed to pH 6 or infected with $10^6 \text{ CFU}\cdot\text{mL}^{-1}$ *Streptococcus pneumoniae* wild type (*S.p.* wt) alone, or the medium was supplemented (volume-controlled) with 25 $\text{mmol}\cdot\text{mL}^{-1}$ HEPES for 24 h. a) Total tissue lysates were analysed by Western blot and representative gel for PECAM-1 is shown. Values represent PECAM-1 expression level relative to control and normalized to β -actin. Data are presented as mean \pm SD of at least four donors within independent experiments. b) Spectral confocal microscopy illustrates VE-cadherin (green) and PECAM-1 (yellow) expression in alveolar capillaries after 24 h under control as well as *S.p.* infection (red). White arrows indicate either

intact VE-cadherin expression co-localized to PECAM-1 in controls or degraded VE-cadherin after *S.p.* infection with no degradation of PECAM-1. Collagen (white) and cell nuclei (DAPI, blue) are visualized for lung morphology. Scale bar 20 μm .

Figure S4. Differential regulation of cell junctions by pneumococcal infection in human lungs. Lung explants were exposed to pH 6 or infected with $10^6 \text{ CFU}\cdot\text{mL}^{-1}$ *Streptococcus pneumoniae* wild type (*S.p.* wt) alone, or the medium was supplemented (volume-controlled) with $25 \text{ mmol}\cdot\text{mL}^{-1}$ HEPES for 24 h. a) Total tissue lysates were analysed by Western blot and representative gels for ZO-1, claudin-18, claudin-5, claudin-4, claudin-3 and claudin-2 are shown. Values represent respective protein expression level relative to control and normalized to β -actin. Data are presented as mean \pm SD of at least four donors within independent experiments. b) mRNA expression level of occludin, ZO-1, VE-cadherin, claudin-5 and -18 are shown. Values represent respective gene expression level normalized to housekeeping gene and relative to control. Data are presented as mean \pm SD of at least five donors within independent experiments. NS: nonsignificant. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. c) Heatmap with gene expression from the selected gene set (GO:0007043). Up- and down-regulated genes are indicated by red or blue bars respectively.

Figure S5. Acidification during pneumococcal infection with clinical isolates in human lungs leads to pH-dependent reduction of VE-cadherin, which is reversed by HEPES buffering. Lung explants were infected with $10^6 \text{ CFU}\cdot\text{mL}^{-1}$ *Streptococcus pneumoniae* wild type (*S.p.* wt) and clinical isolates, expressing non-haemolytic pneumolysin (PLY) variant serotype (ST) 1 and haemolytic PLY variant ST6B alone or the medium was supplemented (volume-controlled) with $25 \text{ mmol}\cdot\text{mL}^{-1}$ HEPES. a) After 24 h supernatants were collected and pH was measured. b) Total tissue lysates were analysed by Western blot and representative gel for VE-cadherin is shown. Values represent VE-cadherin expression level relative to control and normalized to β -actin. Data are presented as mean \pm SD of seven donors within independent experiments. d) $10^2 \text{ CFU}\cdot\text{mL}^{-1}$ *S.p.* wt was grown in medium (RPMI + 10% FCS) in the absence or presence $25 \text{ mmol}\cdot\text{mL}^{-1}$ HEPES for 24 h. c) and d) After indicated time point colony forming units (CFUs) were counted. Data are presented as mean \pm SD of four independent experiments. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Figure S6. pH threshold for VE-cadherin degradation in human lungs. Lung tissue was incubated at pH 7, 6.5 and 6 for 24 h, followed by Western blot analysis. Linear regression analysis shows VE-cadherin expression level according to pH values relative to control and normalized to β -actin. Data are presented as mean of twenty two donors within independent experiments.

Figure S7. pH effects on inflammatory regulation and stress response in human lungs. Comparison of protein (y-axis) and RNA expression (x-axis) for the same cytokines. Lung explants were exposed to pH 6 (i) or infected with 10^6 CFU \cdot mL $^{-1}$ *Streptococcus pneumoniae* wild type (*S.p.* wt) alone, or the medium was supplemented (volume-controlled) with 25 mmol \cdot mL $^{-1}$ HEPES for 24 h. The cytokine protein data is shown on the log10 scale (y-axis). a) and b) Total tissue lysates were evaluated by Western blot and quantified by densitometry. Values represent respective protein expression level relative to control and normalized to β -actin. c- h) Release of inflammatory cytokine proteins (TNF α , IL-1 β , GM-CSF, IL-6, IL-8) and IL-10 were measured in the supernatants using ELISA. The RNA-seq data is relative and log-normalized (x-axis). e) and f) mRNA expression for the same gene is shown. Points correspond to group median values. Squares show the corresponding interquartile ranges and whiskers show the range of the data of at least six donors within independent experiments. p-values were corrected for multiple testing using the Benjamini-Hochberg method. i) Heatmap with top regulated genes (127 unique genes in total were divided into three groups according to their function). Up- and down-regulated genes are indicated by red or blue bars respectively.

Figure S8. Human lung pathogens induced acidification is dependent on their metabolism. a) pH 6 is optimal for pneumococcal growth. 10^2 CFU \cdot mL $^{-1}$ *Streptococcus pneumoniae* wild type (*S.p.* wt) was grown in medium (RPMI + 10% FCS) with ascending pH for 6 h. After indicated time point colony forming units (CFUs) were counted. Data are presented as mean \pm SD of three independent experiments. b) Fermentative (*S.p.* wt, *S.aureus*) and non-fermentative bacteria (*P. aeruginosa*, *L. pneumophila*) were tested in medium for changing environmental pH during growth. 10^6 CFU \cdot mL $^{-1}$ of bacteria were grown in medium (RPMI + 10% FCS) for 24 h. After indicated time point supernatants were collected and pH was measured. Data are presented as mean \pm SD of three independent experiments. c) *S.p.* wt acidifies the extracellular environment in human lung tissue, whereas *L. pneumophila* does

not. Lung explants were infected with 10^6 CFU·mL⁻¹ of bacteria for 24 h. After indicated time point supernatants were collected and pH was measured. Data are presented as mean±SD of four independent experiments. d), h) and i) Shift from homolactic to mixed-acid metabolites formation leads to stronger acidification, degradation of VE-cadherin and impaired bacterial growth in human lungs. Lung explants were infected with 10^6 CFU·mL⁻¹ *S.p.* wt and mutant Δldh for 24 h. d) After indicated time point supernatants were collected and pH was measured. h) Bacterial growth was assessed by CFUs after 0 and 24 h. i) Total tissue lysates were analysed by Western blot and representative gel for VE-cadherin is shown. Values represent VE-cadherin expression level relative to control and normalized to β -actin. e) and f) Lung explants were exposed to pH 7, 6.5 and 6 or were infected with 10^6 CFU·mL⁻¹ of *S.p.* wt alone, or the medium was supplemented (volume-controlled) with 25 mmol·mL⁻¹ HEPES for 24 h. e) After indicated time point supernatants were collected and concentration of glucose and lactate was measured with Blood Gas Analyzer. f) After indicated time point supernatants were collected, filtered and snap-frozen in liquid nitrogen. Extracellular metabolites were analysed by ¹H-NMR. g) 10^2 CFU·mL⁻¹ *S.p.* wt was grown in medium (RPMI + 10% FCS) supplemented (volume-controlled) with 25 mmol·mL⁻¹ HEPES for 24 h. After indicated time point supernatants were collected and concentration of glucose and lactate was measured with Blood Gas Analyzer. Data are presented as mean±SD of at least three donors within independent experiments. NS: nonsignificant. *: p<0.05; **: p<0.01; ***: p<0.001.

Figure S9. Effect of 2-Deoxy-D-glucose (2-DG) inhibitor on bacterial growth and cell viability of the human lung tissue. a) and d) 10^2 CFU·mL⁻¹ *Streptococcus pneumoniae* wild type (*S.p.* wt) was grown in medium (RPMI + 10% FCS) supplemented (volume-controlled) with 2 mmol·mL⁻¹ 2-DG inhibitor for 24 h. After indicated time point supernatants were collected and a) concentration of glucose and lactate was measured with Blood Gas Analyzer. d) Bacterial growth was assessed by CFUs after 0 and 24 h. b) and c) Lung explants were infected with 10^6 CFU·mL⁻¹ *S.p.* wt or stimulated with 2 mmol·mL⁻¹ 2-DG inhibitor. b) After 24 h supernatants were collected and LDH was measured. c) Bacterial growth was assessed by CFUs after 0 and 24 h. Data are presented as mean±SD of at least four donors within independent experiments. NS: nonsignificant. *: p<0.05; **: p<0.01.

Figure S10. The RNA-seq transcriptome of *S.p.* wt in the absence of human lung tissue (non-host-infection scenario). For RNA-seq transcriptomics, *S.p.* wt was grown in supplemented (volume-controlled) RPMI medium +/-FCS. The transcriptome data are shown as ratio/intensity scatter plot (M/A-plot), which is based on the differential gene expression analysis using DeSeq2 as described [16]. Dark gray symbols indicate significantly induced and repressed transcripts (M-value ≥ 1 or ≤ -1 ; $p \leq 0.01$), while transcripts with light gray symbols are not significantly changed ($p > 0.01$). The most strongly upregulated regulons are color-coded and functionally classified into the carbon catabolite repression/alternative sugar utilization and the competence regulons. The complete transcriptome data and regulon classifications, including the up- and downregulated regulons are listed in Tables S1 and S2.

Figure S11. Effects of proteasome, MAPK, ROCK, hsp90, TRPV1 and connexin 43 inhibitors on VE-cadherin expression after pneumococcal infection or acidic pH challenge. a), c), e) and g) Lung explants were infected with 10^6 CFU·mL⁻¹ *Streptococcus pneumoniae* wild type (*S.p.* wt) for a) 24 h in the media supplemented (volume-controlled) with 10 % FCS or c), e) and g) for 48 h in the media without supplements or b), d), f) and h) stimulated with pH 5 or 6 for 24 h, or a) and b) treated with 20 μ M proteasome inhibitor MG-132, 10 μ M p38 inhibitor SB202190 and 10 μ M ROCK inhibitor Y-27632, or c) and d) treated with 10 μ M hsp90 inhibitors (AUY-922: inh 1; 17-AAG: inh 2; Ganetespib: inh 3), e) and f) 1 μ M TRPV1 inhibitors (AMG 9810: inh 1; SB 366791: inh 2) and g) and h) connexin 43 inhibitors (200 μ M gap19: inh 1; 150 μ M gap26: inh 2). Total tissue lysates were evaluated by Western blot and quantified by densitometry, representative gels for VE-cadherin are shown. Values represent respective protein expression level relative to control and normalized to β -actin. Data are presented as mean \pm SD of at least four donors within independent experiments. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

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