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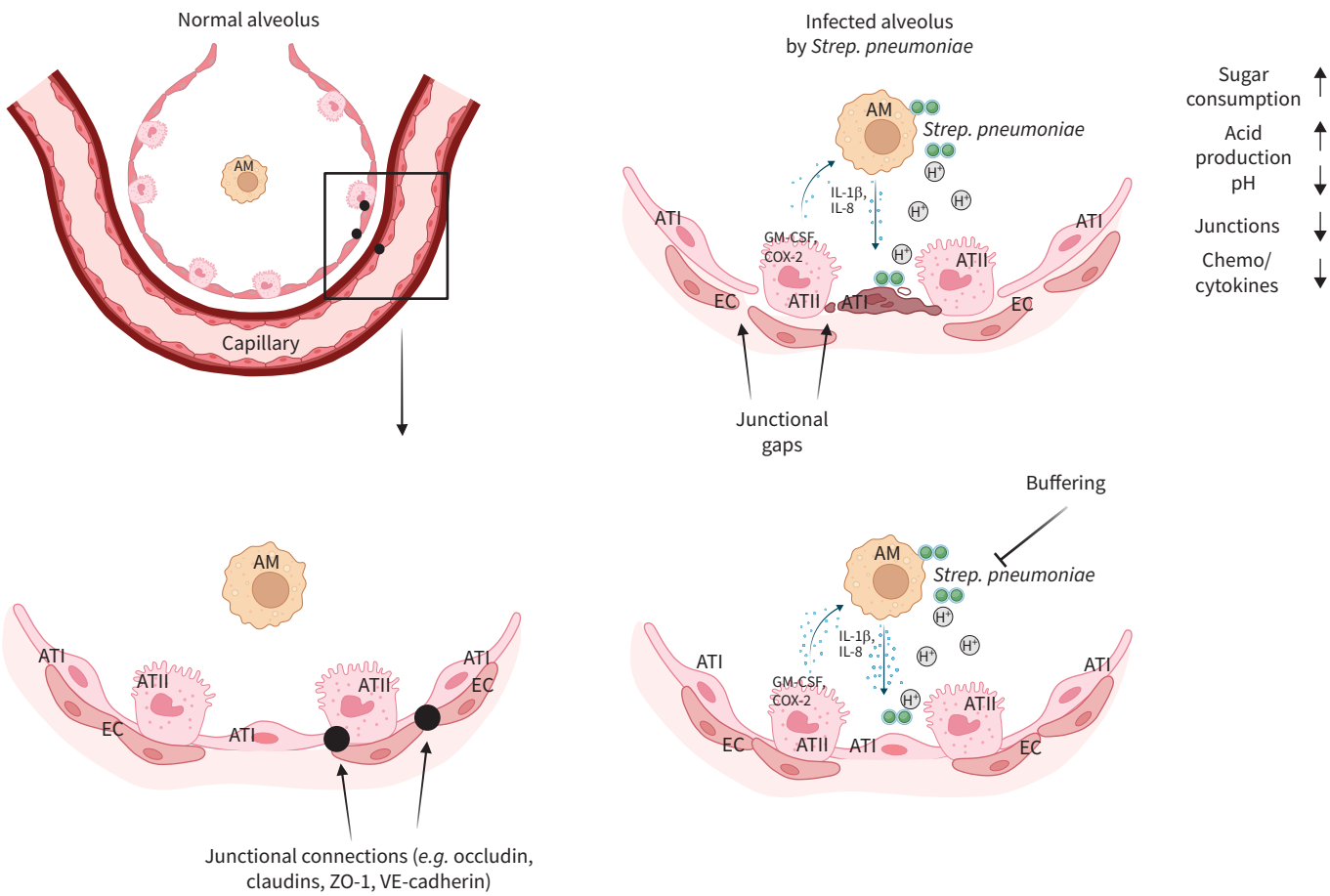

GRAPHICAL ABSTRACT Microenvironmental acidification by pneumococcal sugar consumption fosters barrier disruption and immune suppression in the human alveolus. AM: alveolar macrophage; ATI/II: alveolar type I/II cells; EC: endothelial cells; ZO: zonula occludens protein; VE: vascular endothelial; *Strep. pneumoniae*: *Streptococcus pneumoniae*; IL: interleukin; H^+: hydrogen; GM-CSF: granulocyte–macrophage colony-stimulating factor; COX: cyclo-oxygenase.

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

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Shareable abstract (@ERSpublications)
Sugar catabolism and subsequent lactate production by fermentative bacteria such as *Streptococcus pneumoniae* may serve as an independent virulence mechanism causing alveolar barrier disruption and inflammatory impairment in human lung tissue. https://bit.ly/3Xd8SWN

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Abstract

Streptococcus pneumoniae is the most common causative agent of community-acquired pneumonia worldwide. A key pathogenic mechanism that exacerbates severity of disease is the disruption of the alveolar–capillary barrier. However, the specific virulence mechanisms responsible for this in the human lung are not yet fully understood. In this study, we infected living human lung tissue with *Strep. pneumoniae* and observed a significant degradation of the central junctional proteins occludin and vascular endothelial cadherin, indicating barrier disruption. Surprisingly, neither pneumolysin, bacterial hydrogen peroxide nor pro-inflammatory activation were sufficient to cause this junctional degradation. Instead, pneumococcal infection led to a significant decrease of pH (~6), resulting in the acidification of the alveolar microenvironment, which was linked to junctional degradation. Stabilising the pH at physiological levels during infection reversed this effect, even in a therapeutic-like approach. Further analysis of bacterial metabolites and RNA sequencing revealed that sugar consumption and subsequent lactate production were the major factors contributing to bacterially induced alveolar acidification, which also hindered the release of critical immune factors. Our findings highlight bacterial metabolite-induced acidification as an independent virulence mechanism for barrier disruption and inflammatory dysregulation in pneumonia. Thus, our data suggest that strictly monitoring and buffering alveolar pH during infections caused by fermentative bacteria could serve as an adjunctive therapeutic strategy for sustaining barrier integrity and immune response.

Introduction

Streptococcus pneumoniae is the leading causative bacterium of community-acquired pneumonia [1]. In particular, the disruption of the alveolar barrier is a significant pathophysiological mechanism that contributes to the deterioration of the gas exchange function and a fatal course of disease due to increasing permeability [2–4]. Although promising substances have been tested in clinical studies in recent years that could stabilise the alveolar barrier in pneumonia, acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), no clinically established adjunctive approach is available to date [5–8]. For this reason, further deepening the understanding of the underlying mechanisms of pulmonary barrier disruption is needed to address alternative and possibly preventive approaches.

Regarding *Strep. pneumoniae*, numerous studies suggest that virulence factors such as pneumolysin (PLY) or hydrogen peroxide (H_2O_2), which is generated in high amounts by the pyruvate oxidase, contribute significantly to the induction of alveolar permeability [9–12]. As these studies have demonstrated, the pertinent question is not whether PLY or H_2O_2 can induce endothelial barrier failure, but rather whether these factors are the decisive mechanisms in *Strep. pneumoniae* infection of the human alveolus. This issue is addressed in this study.

Junction proteins such as occludin and vascular endothelial (VE)-cadherin are key factors in the regulation of epithelial and endothelial permeability, and disappearance of these proteins is well suited as surrogate for alveolar–capillary barrier disruption [13, 14]. In a previous study, we showed that infection of human lung tissue with *Strep. pneumoniae* leads to degradation of numerous junction proteins in the alveolar compartment [15], and although the conclusion is thus obvious that either PLY or H_2O_2 might be the driving virulence factors for this effect, we failed in confirming this causal relationship. Rather, we identified significant bacteria-induced acidification of the human alveolar microenvironment causally related to junctional degradation. Studies regarding aspiration pneumonitis, which is accompanied by acid-induced barrier disruption, demonstrated this syndrome as an independent risk factor for ALI/ARDS development with high mortality rates [16–19]. Moreover, lowering of the alveolar pH can, next to increased epithelial/endothelial permeability, lead to inflammatory dysregulation, neutrophilic infiltration and inactivation of pulmonary surfactant [16]. Nevertheless, the mechanisms through which bacteria, such as *Strep. pneumoniae*, may contribute to an acidic alveolar microenvironment remain to be fully elucidated.

In the present study we show that *Strep. pneumoniae* induces loss of alveolar junctional proteins by lowering the pH independently of PLY or H_2O_2. This effect is mainly caused by sugar consumption and fermentative energy metabolism by the bacteria, leading to lactate accumulation [20]. In addition to barrier disruption, the drop in pH also impaired the initial immune response, and both can be restored by simple buffering. Therefore, we have unveiled a previously unnoticed mechanism behind the dysregulation of the alveolar barrier. This mechanism is not attributed to classical virulence factors, but rather to the fermentative energy metabolism of the bacteria. We propose a new virulence mechanism that operates independently, affecting both the alveolar barrier and the immune response. Moreover, bacterially induced acidification of the microcompartment should be considered for other fermentative pathogens of lung and extrapulmonary infections as well.

Methods

Detailed information can be found in the supplementary material.

Human lung tissue

Lung tissue explants were obtained from 103 patients primarily suffering from bronchial carcinoma, who underwent lung resection at local thoracic surgeries, and which were prepared as described previously [21]. The study was approved by the ethics committee at the Charité Universitätsmedizin Berlin clinic (Berlin, Germany; protocol number EA2/079/13) and written informed consent was obtained from all patients.

Bacterial strains and PLY

Encapsulated *Strep. pneumoniae* D39 serotype (ST) 2 wild-type (NCTC7466; wt), D39-derived mutants Δ*ply* [22], Δ*spxB* [23], Δ*ply*Δ*spxB*, Δ*cps*Δ*ply* for *in vitro* experiments [24] (friendly gift from S. Hammerschmidt, University of Greifswald, Greifswald, Germany) and Δ*ldh* [25] (kindly provided by H. Yesilkaya, University of Leicester, Leicester, UK) were used. Clinical isolates expressing the non-haemolytic PLY variant serotype (ST)1 (SN35218) and haemolytic PLY variant ST6B (SN33364) were donated by M. van der Linden (Reference Centre for Streptococci, Aachen, Germany). Bacterial strains were grown as described previously [15, 26, 27]. Production and purification of PLY has been described previously [28].

Infection and treatment of human lung tissue and endothelial cells

The following infection experiments were carried out in RPMI 1640 medium blank or supplemented (volume-controlled) with 10% fetal calf serum at 37°C with 5% carbon dioxide (CO_2) [15, 26, 27]. For infection, tumour-free normal lung tissue was inoculated with culture medium as control or 1×10^6 CFU·mL^{-1} *Strep. pneumoniae* (wt, Δ*ply*, Δ*spxB*, Δ*ldh*, 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} H_2O_2 (Carl Roth). For inflammatory activation, 100 ng·mL^{-1} interleukin (IL)-1β (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 ρ-associated, coiled-coil containing protein kinase (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 the times indicated by the manufacturer. 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 hydrogen chloride (HCl); Carl Roth) for the indicated time with and without medium exchange. Afterwards, lungs were processed for further analysis. Human umbilical vein endothelial cells (HUVEC), grown to a confluent monolayer, were infected with *Strep. pneumoniae* Δ*cps*Δ*ply* (1 multiplicity of infection) for 24 h or incubated in culture medium at pH 7, 6 or 5 for the indicated times with and without medium exchange. For HUVEC supernatant experiments, lung explants were infected with 1×10^6 CFU·mL^{-1} *Strep. pneumoniae* D39 wt in the presence or absence of HEPES for 24 h. Afterwards, supernatants of human lungs were collected for pH measurement and filtered. Next, HUVEC were stimulated with human lung tissue culture supernatant for 24 h.

Western blotting

Shock-frozen human lung tissue samples were lysed in radio-immunoprecipitation assay buffer (Thermo Fisher Scientific), disrupted by the FastPrep-24 homogeniser (MP Biomedicals) and subjected to Western blot [21]. Polyvinylidene fluoride membranes (Merck) were exposed to antibodies for VE-cadherin, pro-IL-1β, cyclo-oxygenase (COX)-2 (Santa Cruz Biotechnology), occludin, zonula occludens protein (ZO)-1, claudin-2, claudin-3, claudin-4, claudin-5, claudin-18 (Invitrogen), platelet endothelial cell adhesion molecule (PECAM)-1 (Novus Bio), GAPDH (Cell Signaling) and β-actin (Sigma) overnight at 4°C and subsequently incubated with corresponding secondary horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology).

Electric cell-substrate impedance sensing

HUVEC were grown on evaporated gold electrodes, connected to an electrical cell-substrate impedance system (Applied Biophysics). All electric cell-substrate impedance sensing experiments were conducted at a frequency of 40 kHz and transendothelial electrical resistance (TEER) values from each microelectrode were continuously monitored and normalised as the ratio of measured resistance to baseline resistance.

Hydrogen-1 nuclear magnetic resonance spectroscopic analysis of extracellular metabolites

Lung explants were exposed to medium at pH 6 or were infected with *Strep. pneumoniae* D39 wt in the presence and absence of 25 mmol·L^{-1} HEPES (volume-controlled) or *Strep. pneumoniae* Δ*ldh* for 24 h. After indicated time points, supernatants were collected, centrifuged at 4°C (13 684×*g*), filtered with Filtropur S 0.2 (Sarstedt), snap-frozen in liquid nitrogen and stored at −80°C before measurement. Samples were analysed for extracellular metabolites using the hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) spectroscopic method, as described previously [29].

Results

Pneumococcal pneumolysin and H_2O_2 as well as pro-inflammatory activation are insufficient for alveolar occludin and VE-cadherin degradation

We have previously demonstrated the localisation and pneumococcal-induced alterations of junctional proteins within the human alveolar–capillary compartment, a characteristic feature of barrier disruption [15]. To elucidate underlying bacterial mechanisms, we tested whether the prototypic virulence factors PLY and H_2O_2 are major drivers of junctional degradation in the human lung. Notably, employing PLY and H_2O_2-depleted bacteria (single and double knockout, *Strep. pneumoniae* Δ*ply*, *Strep. pneumoniae* Δ*spxB* or *Strep. pneumoniae* Δ*ply*Δ*spxB*) resulted in evident junctional degradation (figure 1a, supplementary figure S1a). Conversely, direct stimulation with both factors alone proved insufficient to impact the expression of occludin and VE-cadherin. Except for a rather high concentration of PLY (5 µg), a degradation of occludin and a reduction of VE-cadherin was observed (figure 1b, supplementary figure S1b). Since *Strep. pneumoniae* induces significant IL-1β release by PLY in alveolar macrophages of

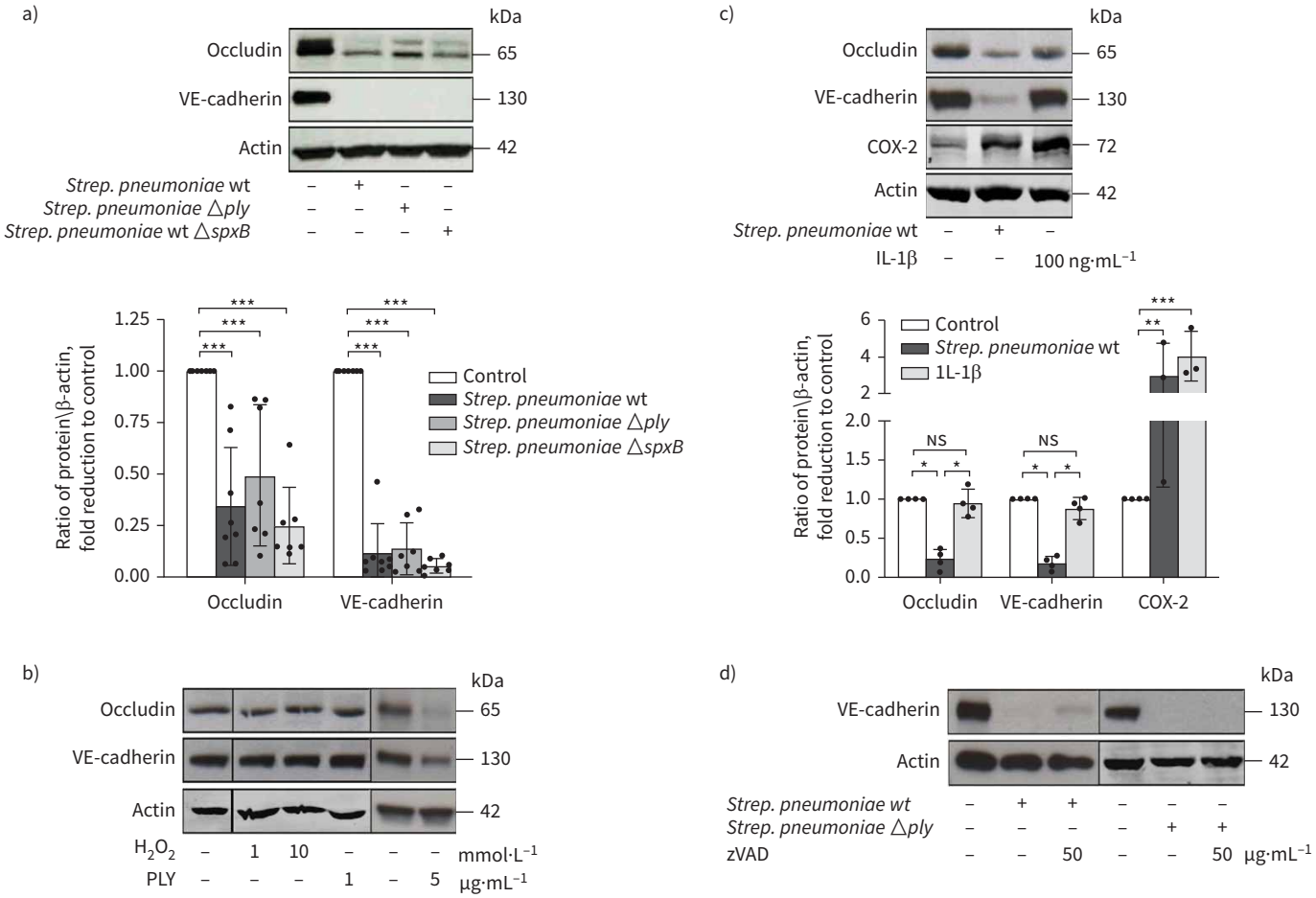

FIGURE 1 Degradation of junctional proteins occludin and vascular endothelial (VE)-cadherin during pneumococcal infection in human lungs is independent of pneumolysin (PLY), hydrogen peroxide (H_2O_2) or interleukin (IL)-1β. Lung explants were infected with a) 10^6 CFU·mL^{-1} *Streptococcus pneumoniae* wild-type (*Strep. pneumoniae* wt) and mutants Δ*ply*, Δ*spxB* or stimulated with b) 1 and 5 µg·mL^{-1} purified PLY, 1 and 10 mM·mL^{-1} H_2O_2 or c) 100 ng·mL^{-1} IL-1β for 24 h. d) Lung tissue was challenged with *Strep. pneumoniae* wt or *Strep. pneumoniae* Δ*ply* alone or treated with 50 µg·mL^{-1} pan-caspase inhibitor zVAD for 24 h. Total tissue lysates were analysed by Western blot and quantified by densitometry. Representative gels for occludin, VE-cadherin and cyclo-oxygenase (COX)-2 are shown. Values represent respective protein expression level relative to control and normalised to β-actin. 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.

human lungs [27, 30] and previous studies on human endothelial cells indicated barrier disruption by pro-inflammatory factors such as tumour necrosis factor (TNF)-α or IL-1β [31–33], we tested if IL-1β stimulation could serve as indirect factor for alveolar junctional degradation. However, no effect on occludin and VE-cadherin expression could be revealed in contrast to *Strep. pneumoniae* infection (figure 1c). COX-2 expression served as positive control for pro-inflammatory activation by IL-1β [26]. To exclude a general junctional degradation process by bacterially induced apoptosis, lung explants were pre-treated with pan-caspase inhibitor zVAD, which did not prevent *Strep. pneumoniae*- or *Strep. pneumoniae* Δ*ply*-induced loss of VE-cadherin (figure 1d, supplementary figure S1c). Likewise, no significant lactate dehydrogenase (LDH) release was observed after *Strep. pneumoniae* infection as an indicator of necrotic tissue damage (supplementary figure S1d). IL-1β production, mainly dependent on PLY-induced inflammasome activation, was measured as a positive control for infection and zVAD treatment (supplementary figure S1e–g).

Pneumococcal infection leads to junctional degradation by acidification

Thus, a distinct mechanism, operating independently of PLY, H_2O_2, pro-inflammatory signalling or apoptosis, seems to be responsible for *Strep. pneumoniae*-induced junctional degradation. Based on previous findings [20, 34], we hypothesised that the bacterium's energy metabolism could result in a reduction of pH levels, which may, in turn, adversely affect the integrity of alveolar occludin and

VE-cadherin. We first measured pH values in human lung tissue supernatants during infection with *Strep. pneumoniae* wt, *Strep. pneumoniae* Δ*ply* and *Strep. pneumoniae* Δ*spxB* mutants deficient for PLY secretion and H_2O_2 generation. This measurement reflects pH in the alveolar space and revealed a significant acidification down to pH 6 for all bacterial strains (figure 2a). Partial compensation (pH 6.5) was achieved by HEPES buffering, which proved effective in preventing the degradation of both junctional proteins (a 2.2-fold reduction for occludin and a 5.6-fold VE-cadherin reduction) (figure 2a and b). The direct acidification of the human lung explant media using HCl demonstrated pH-dependent degradation of occludin and VE-cadherin, even in the absence of bacteria (figure 2c). Spectral confocal microscopy was utilised to reflect pH-dependent morphological changes of the tissue as well as in VE-cadherin expression (figure 2d). Images indicate that, under conditions of infection and pH stimulation, VE-cadherin protein does not redistribute within the alveolo-capillary bed, but instead undergoes degradation, which is prevented by HEPES buffering, while no signs of tissue disintegration were observed (figure 2d). To confirm that acidification does not only take place in the alveolar space, but also extends into the capillary area of the alveolar septs, we analysed relative pH changes using live tissue microscopy. Compared to mock infection (pH 7.2), a pronounced acidification (pH 6.3) can be measured in infected lung tissue after 24 h, which is reflected in a strong increase in pHrodo intensity (indicating acidification) in the entire tissue area (supplementary figure S2). To emphasise that pH-dependent regulation of junctional proteins is a differentiated process that is not based on a general tissue disintegration, we show the acid-stable expression of platelet endothelial cell adhesion molecule (PECAM)-1 (supplementary figure S3a and b). This was additionally supported, as previously shown, by investigation of further junctional proteins [15]. Whereas ZO-1 is regulated by acidic pH, there is no change for claudins 2, 3, 4, 5 or 18 (supplementary figure S4a). Neither quantitative PCR nor bulk-RNA sequencing indicated regulation of junctional proteins at the transcriptional level (supplementary figure S4b and c).

To underscore the clinical relevance of our findings, lung explant infection was conducted using clinical isolates of *Strep. pneumoniae*, which expressed non-haemolytic PLY variant serotype 1 and haemolytic PLY variant serotype 6B, both in the presence and absence of HEPES. Once again, these clinical isolates induced acidification (pH 5.8±0.05 for ST1 and 5.9±0.05 for ST6B), resulting in substantial (11-fold and 15-fold, respectively) decreased levels of VE-cadherin (supplementary figure S5a and b). Similarly, HEPES buffering maintained junctional protein levels (with a 5.9-fold increase for ST1 and a 6.6-fold increase for ST6B) (supplementary figure S5b). No growth-inhibitory effects of HEPES on *Strep. pneumoniae* were observed either in the human lung tissue or in the media alone (supplementary figure S5c and S5d). In order to determine the critical pH value for VE-cadherin degradation, we correlated protein expression levels with pH values after 24 h (supplementary figure S6). The results showed that the maximum reduction (a 16-fold-decrease) in VE-cadherin occurred at pH 5.9±0.25, while a moderate decrease (1.8-fold) was observed at pH 6.25±0.4 (R^2=0.51).

Bacterial acidification hampers host immune and stress response

In addition to examining alterations in the alveolar barrier we investigated whether pneumococcal acidification could also influence the host's inflammatory response. Consequently, we tested key cytokines, revealing a nuanced pattern with a tendency towards secretory inhibition related to acidification compared to HEPES buffering (supplementary figure S7a–h) [26, 27, 30, 35]. Under *Strep. pneumoniae* infection, we found significant increase in mRNA and protein expression of COX-2, TNF-α, (pro-)IL-1β, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-10, while IL-6 and IL-8 showed significant upregulation solely at the mRNA level. HEPES buffering further augmented the induction of mRNA as well as cytokine secretion for TNF-α, pro-IL-1β, IL-10 and, to a lesser extent, IL-8 (supplementary figure S7c–e and h). Bulk-RNA analysis revealed that inflammatory factors, although upregulated by *Strep. pneumoniae* infection, do not appear to be regulated by a decrease to pH 6; however, several stress-response genes, including chaperones, are triggered by both *Strep. pneumoniae* or pathogen-free acidic conditions and are partly responsive to buffering with HEPES (supplementary figure S7i).

Therapeutic-like pH normalisation can partly prevent junctional degradation

To further substantiate our findings regarding the pH-dependent degradation of junctional proteins, we employed a therapeutic-like approach by initially challenging lung tissue with low pH 6/pH 5 followed by timely pH normalisation to pH 7.2 at 4, 8 and 16 h after the pH challenge (figure 3a). All samples were analysed after additional 16 h/8 h of incubation in pH 7.2-normalised media, revealing a reduction of VE-cadherin levels that began at 16 h after pH 6 challenge or at 4 h after pH 5 challenge (figure 3b and c). In line, a partial prevention of VE-cadherin degradation was observed in these samples when media were normalised to pH 7.2. However, particularly at later time points following the pH 5 challenge (8 and 16 h), VE-cadherin loss could no longer be influenced by the therapeutic-like pH neutralisation, suggesting the existence of a pH threshold for junctional degradation.

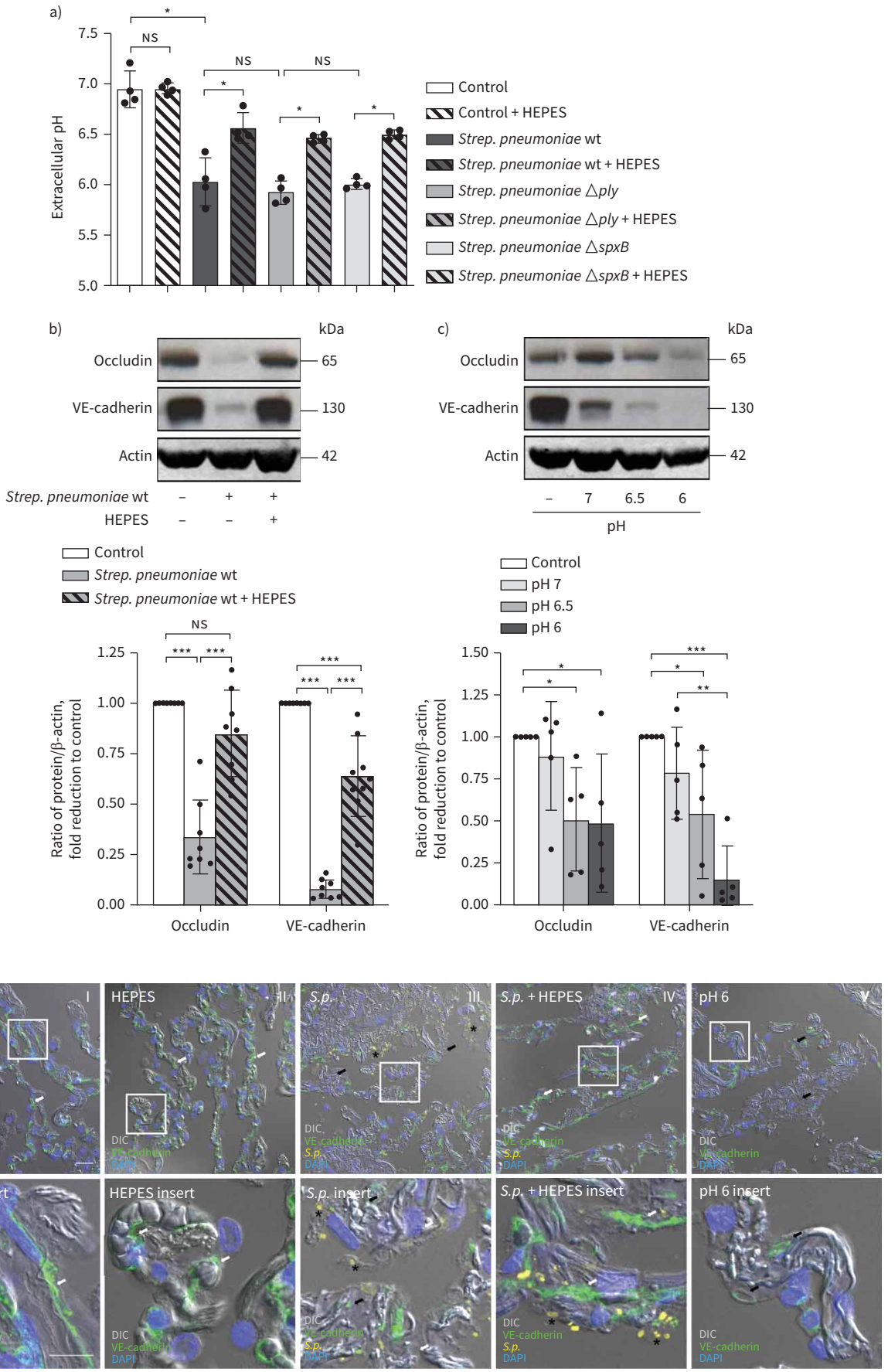
a)
Extracellular pH
Control
Control + HEPES
Strep. pneumoniae wt
Strep. pneumoniae wt + HEPES
Strep. pneumoniae △ply
Strep. pneumoniae △ply + HEPES
Strep. pneumoniae △spxB
Strep. pneumoniae △spxB + HEPES
b)
kDa
Occludin
VE-cadherin
Actin
Strep. pneumoniae wt
HEPES
c)
pH
Control
Strep. pneumoniae wt
Strep. pneumoniae wt + HEPES
pH 7
pH 6.5
pH 6
Ratio of protein/β-actin, fold reduction to control
Occludin
VE-cadherin
d)
HEPES
S.p.
S.p. + HEPES
pH 6
Control insert
HEPES insert
S.p. insert
S.p. + HEPES insert
pH 6 insert
DIC
VE-cadherin
DAPI

FIGURE 2 Acidification during pneumococcal infection in human lungs leads to pH-dependent reduction of junctional proteins occludin and vascular endothelial (VE)-cadherin, which is prevented by HEPES buffering. Tissue was infected with a) and b) 10^6 CFU·mL^{-1} *Streptococcus pneumoniae* wild-type (*Strep. pneumoniae* wt) and a) mutants Δ*ply*, Δ*spxB* alone, or the medium was supplemented (volume-controlled) with 25 mmol·mL^{-1} HEPES. a) After 24 h, supernatants were collected and pH was measured. c) Lung explants were exposed to pH 7, 6.5 and 6 for 24 h. b) and c) Total tissue lysates were evaluated by Western blot and quantified by densitometry, representative gels for occludin and VE-cadherin are shown. Values represent respective protein expression level relative to control and normalised to β-actin. d) Spectral confocal microscopy illustrates VE-cadherin expression (green) in alveolar capillaries after 24 h under control (panel I), HEPES buffering (panel II), *Strep. pneumoniae* infection without HEPES buffering (panels III and IV) as well as pH 6 stimulation (panel V). Related inserts are indicated by white boxes showing high magnification. White arrows indicate intact VE-cadherin expression; black arrows show degraded VE-cadherin. Black asterisks demonstrate *Strep. pneumoniae* distribution in the alveoli. Cell nuclei are visualised by 4′,6-diamidino-2-phenylindole (DAPI) stain (blue). DIC: differential interference contrast. Scale bars=20 µm (panels I and III), 10 µm (panels II and IV). Data are presented as mean±SD of at least four donors within independent experiments. NS: nonsignificant; *: p<0.05, **: p<0.01, ***: p<0.001.

The acidic milieu of infected human alveoli acts on endothelial barrier

Furthermore, we provide evidence that the acidic milieu generated by *Strep. pneumoniae* directly affects the endothelial barrier in the human alveoli. To demonstrate this, we incubated HUVEC with filtered supernatant (without buffering) from infected human lung tissue for 24 h and analysed both pH values and VE-cadherin expression (figure 4a). In line with the observed pH drop (from 7.14±0.2 to 6.3±0.15), we

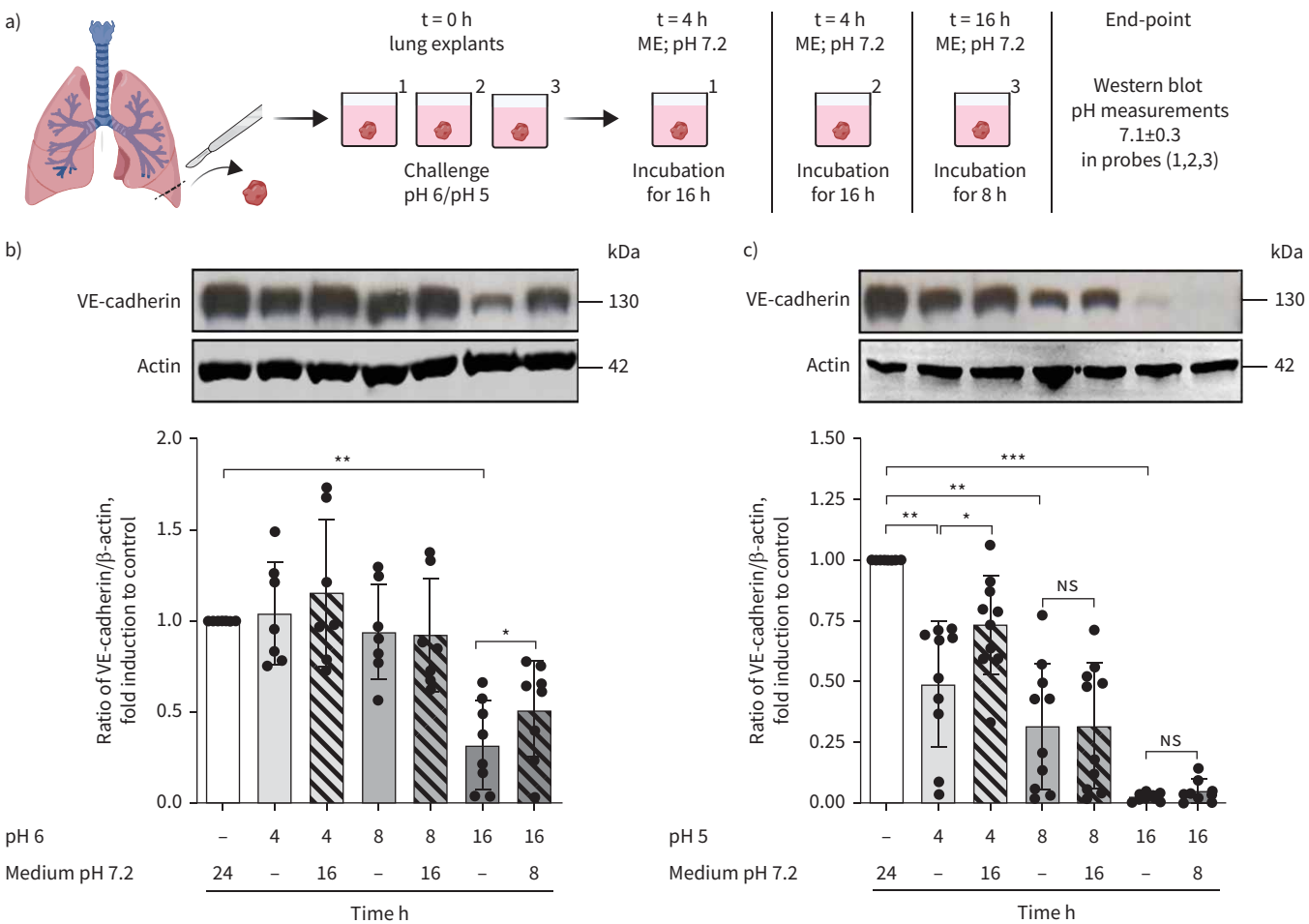

FIGURE 3 pH- and time-dependent degradation of vascular endothelial (VE)-cadherin in human lungs is reverted by pH normalisation. a) The experimental setting. Lung explants were challenged with b) pH 6 or c) pH 5 for 4 and 8 h, followed by medium exchange (ME; pH 7.2) for 16 h; or 16 h, followed by ME; pH 7.2 for 8 h. b) and c) Total tissue lysates were evaluated by Western blot and quantified by densitometry; representative gels for VE-cadherin are shown. Values represent VE-cadherin expression level relative to control and normalised to β-actin. Data are presented as mean±SD of at least seven donors within independent experiments. NS: nonsignificant. *: p<0.05, **: p<0.01, ***: p<0.001.

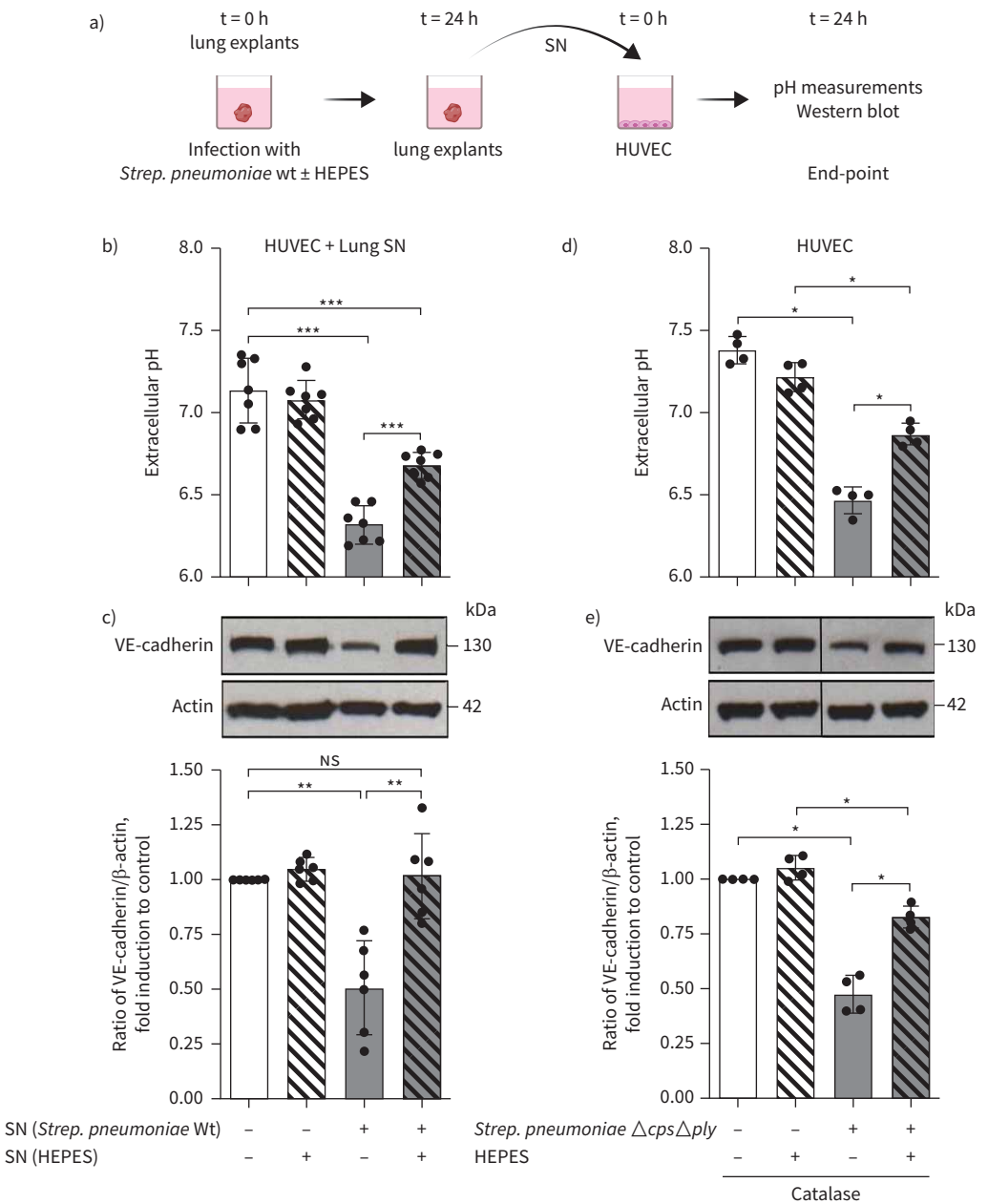

FIGURE 4 Reduction of vascular endothelial (VE)-cadherin expression during pneumococcal infection *ex vivo* and *in vitro* is reverted by HEPES buffering. a) The experimental setting. b) and c) Lung explants were infected with 10^6 CFU·mL^{-1} *Streptococcus pneumoniae* wild-type (*Strep. pneumoniae* wt) alone or the medium was supplemented (volume-controlled) with 25 mmol·mL^{-1} HEPES for 24 h. Afterwards supernatants of human lung tissue (SN) were collected for pH measurement. Next, human umbilical vein endothelial cells (HUVEC), grown to confluent monolayer, were incubated with filtered SN for 24 h. d) and e) HUVEC, grown to confluent monolayer, were infected with *Strep. pneumoniae* Δ*cps*Δ*ply* (1 multiplicity of infection; 24 h) and the medium was supplemented (volume-controlled) with 10 U·mL^{-1} catalase in the presence or absence of HEPES. b) and d) pH measurements of HUVEC SN after 24 h. c) and e) Total tissue lysates were evaluated by Western blot and quantified by densitometry; representative gels for VE-cadherin are shown. Values represent VE-cadherin expression level relative to control and normalised to β-actin. Data are presented as mean±SD of at least four independent experiments. NS: nonsignificant. *: p<0.05, **: p<0.01, ***: p<0.001.

detected a two-fold reduction of the VE-cadherin protein levels (figure 4b and c). Once again, this effect was prevented by HEPES buffering. Similarly, infection of HUVEC with the *Strep. pneumoniae* Δ*cps*Δ*ply* (mutant without capsule (cps) for *in vitro* infection) [24] mutant in the presence of catalase (to exclude

both PLY and H_2O_2 effects) resulted in pH decrease (from 7.4±0.1 to 6.5±0.1) and a 2.1-fold reduction of VE-cadherin (figure 4d and e). In addition, HEPES buffering restored the pH to 6.9±0.1 and increased the protein level 1.8-fold. To assess whether the induced endothelial permeability due to low pH is reversible, we employed a therapeutic-like approach involving medium exchange. Firstly, we demonstrate the pH dependent loss of TEER (figure 5a). Medium exchange at 1 h or 4 h following the pH 5 challenge of HUVEC efficiently prevented TEER loss (figure 5b). Visualisation of VE-cadherin showed thinning of the junctional band after pH 5 stimulation, with the appearance of gaps at 1 and 4 h (figure 5c, panels I–III). Normalisation to pH 7.2 completely reverts this effect at 1 h of acidic treatment, but only partially after 4 h (figure 5c, panels IV–V). Western blot analysis of VE-cadherin expression levels confirmed these findings, showing the prevention of junctional degradation (figure 5d).

Bacterial fermentation of glucose is responsible for acidification of the alveolar microenvironment

As *Strep. pneumoniae* is an aerotolerant anaerobic bacterium relying on fermentative energy metabolism, such as lactic acid fermentation, we hypothesised that the resulting acid formation could be the causative factor for the degradation of junctional proteins in the human alveolar–capillary barrier [20]. Our initial findings indicate that the acidic conditions induced by the bacteria are conductive to *Strep. pneumoniae* growth, with an optimum pH ∼6, which aligns with the pH values observed in infected human lung tissue (supplementary figure S8a). Conversely, lung-pathogenic bacteria lacking fermentative metabolism, such as *Pseudomonas aeruginosa* and *Legionella pneumophila*, do not lower the pH as seen in the case of pneumococci or *Staphylococcus aureus* (supplementary figure S8b and c). To demonstrate that the drop in pH is caused by acidic end-products of the fermentative glucose metabolism of the bacteria, we conducted ^{1}H-NMR to identify extracellular metabolic factors in resting and *Strep. pneumoniae*-infected human lung tissue (figure 6a). As shown, glucose consumption in human lungs alone already leads to lactate production (figure 6a). Infection with *Strep. pneumoniae* wt further increases lactate production, causing a drop in pH from 7.22±0.1 to 6.04±0.02 (figure 6a, supplementary figure S8d). Interestingly, a mere acidic challenge of the lungs by HCl significantly reduced glucose consumption and lactate production of the tissue (supplementary figure S8e), which is opposite to the infection scenario (supplementary figure S8f). To control side-effects probably caused by the buffering, glucose consumption and lactate production was compared between mock and *Strep. pneumoniae* infection (without HEPES) as well as mere acid challenge showing no effect negative influence of HEPES (supplementary figure S8f). Interestingly, slightly reduced lactate production and glucose consumption by *Strep. pneumoniae* was observed when bacteria were grown in buffered media alone, indicating that the pH optimum for bacterial growth changed (supplementary figure S8g). We further applied the *Strep. pneumoniae* Δ*ldh* mutant to illustrate the shift to mixed acid fermentation when lactate production is blocked (figure 6a). Interestingly, the acidification induced by *Strep. pneumoniae* Δ*ldh*, due to formation of acetate and formate, led to a more pronounced decrease of pH to 5.26±0.2 (figure 6a, supplementary figure S8d), which proved to be growth-inhibitory (supplementary figure S8h). However, although self-limited in growth, the induced, strong acidification effect by *Strep. pneumoniae* Δ*ldh* likewise leads to the degradation of VE-cadherin (supplementary figure S8i). To confirm lactate production due to aerobic glycolysis, known as the Warburg effect, in *ex vivo* lung tissue, we stimulated lung explants with glucose analogue 2-deoxyglucose (2-DG), which inhibits glycolysis [36]. The use of 2-DG resulted in reduced glucose consumption and subsequently decreased lactate production in both control and infection conditions in human lungs or media alone (figure 6b, supplementary figure S9a). In turn, this induced weaker acidification (pH 6.65±0.15) compared to infection without inhibitor (pH 6.15±0.15) (figure 6c). Correspondingly, the VE-cadherin expression level was six-fold higher in the presence of 2-DG (figure 6d). Furthermore, 2-DG did not affect cell viability as shown in the LDH assay or bacterial growth in human lungs or media alone (supplementary figure S9b–d). Although acidic stimulation alone already showed that the lung loses ability to consume sugar and produce lactate (supplementary figure S8e), it is important to understand whether the main contribution of lactate during alveolar pneumococcal infection comes from the bacteria or whether a substantial part of the acidification is caused by lactate production in the lung itself. For this purpose, we performed experiments with the human-selective LDH inhibitor oxamate. Oxamate reduces lactate production and glucose consumption in mock-infected lungs in a concentration-dependent manner, but not under infection with *Strep. pneumoniae*, indicating a major contribution of lactate production by the pneumococci (figure 7a) [37]. Oxamate does not lead to a change in pH and thus does not affect the acid-induced degradation of VE-cadherin (figure 7b and c). Control experiments show that oxamate has a slight, nonsignificant effect on bacterial growth and no effect on glucose consumption or lactate production (figure 7d and e).

To validate the activation of bacterial glucose catabolism, we conducted RNA sequencing of *Strep. pneumoniae* transcriptome under infection-like conditions, but without the presence of lung tissue, representing a non-host-infection scenario (supplementary figure S10). The M/A-plot depicts the gene expression profile of significantly upregulated regulons, which can be functionally classified into the

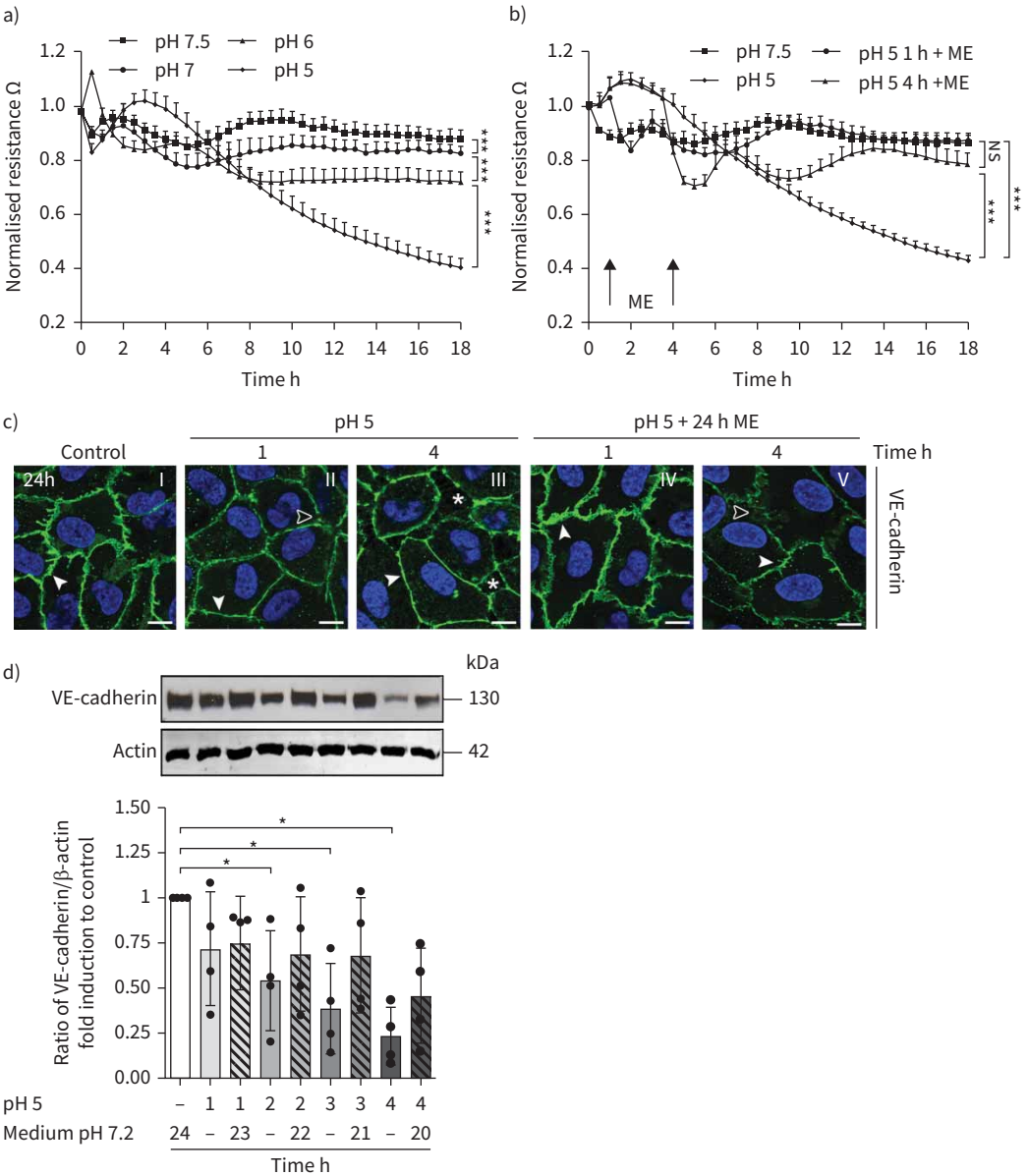

FIGURE 5 Acidic extracellular pH impairs endothelial barrier integrity, vascular endothelial (VE)-cadherin expression and distribution in human umbilical vein endothelial cells (HUVEC), which is reverted by pH normalisation. HUVEC, grown to confluent monolayer, were incubated in culture medium at pH 7, 6 and 5 for 18 h, or challenged with pH 5 for 1 or 4 h, after respective time points followed by medium exchange (ME; pH 7.2) to the end-point of 24 h. a) and b) Electric cell-substrate impedance sensing analysis of pH 7-, 6- and 5-treated HUVEC with or without ME for 18 h. c) Representative immunofluorescence confocal images of HUVEC stained for VE-cadherin (green) and 4′,6-diamidino-2-phenylindole (DAPI; blue). White arrowheads indicate regular structure of VE-cadherin in control (panel I) or after ME (panels IV and V) and linearisation of VE-cadherin in pH 5-treated HUVEC (panels II and III). Open arrowheads point to interruptions in VE-cadherin structure, indicating gap formation (asterisks). Representative figures of three independent experiments are shown. Scale bar=10 µm. d) Total tissue lysates were evaluated by Western blot and quantified by densitometry; representative gel for VE-cadherin is shown. Values represent VE-cadherin expression level relative to control and normalised to β-actin. Data are presented as mean±SD of at least four independent experiments. NS: nonsignificant. *: p<0.05, ***: p<0.001.

carbon catabolite repression (CcpA regulon) and the utilisation of alternative carbon sources, such as fucose, lactose, cellobiose, maltose and ascorbic acid (supplementary figure S10), indicating glucose starvation during entry into the stationary phase. Comprehensive transcriptome data, regulon classifications

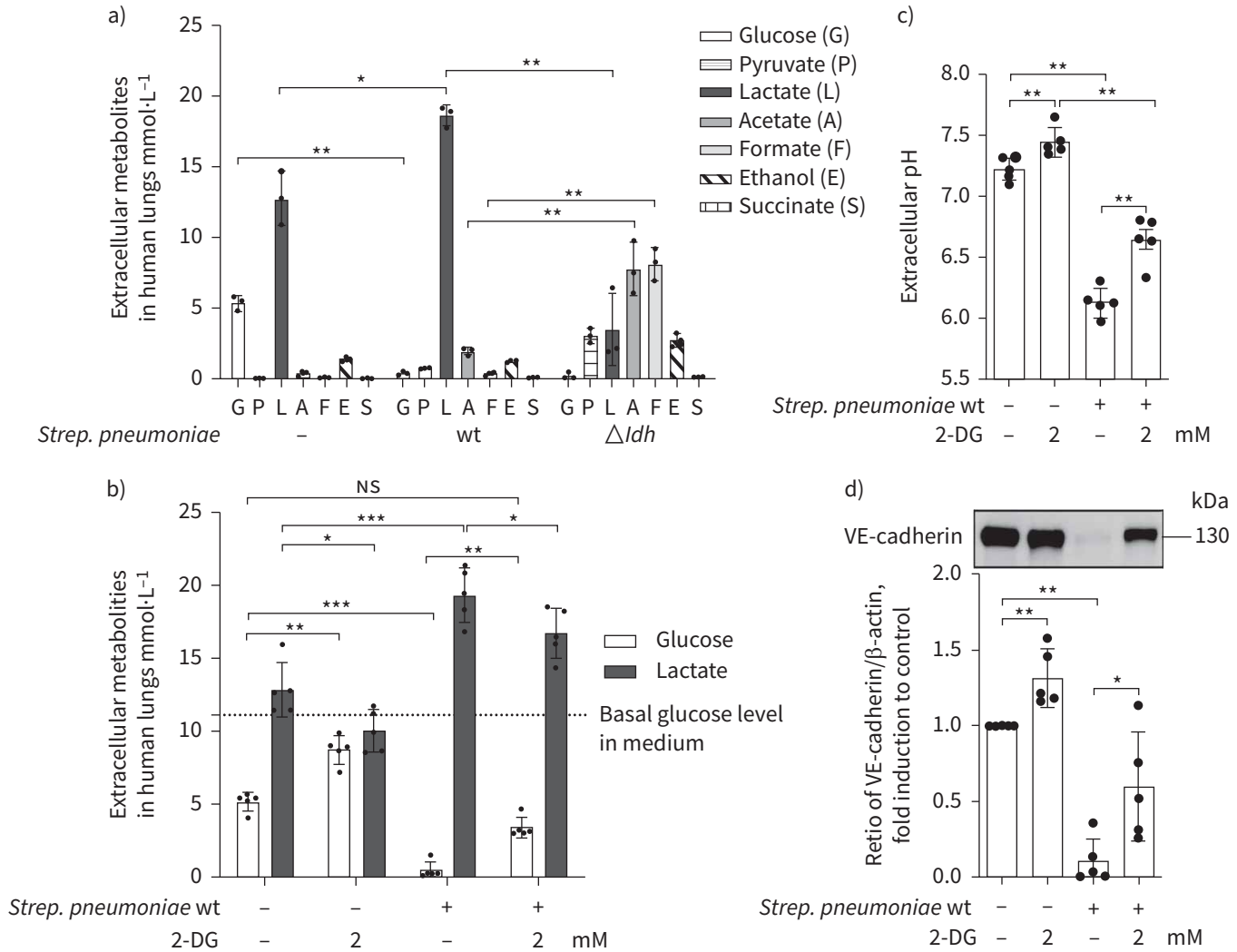

FIGURE 6 Lactate as main fermentation end-product of pneumococcal glucose catabolism leads to acidification in human lungs. a) Lung explants were infected with 10^6 CFU·mL^{-1} *Streptococcus pneumoniae* wild-type (*Strep. pneumoniae* wt) and Δ*ldh* mutant for 24 h. After the indicated time points, supernatants were collected, filtered and snap-frozen in liquid nitrogen. Extracellular metabolites were analysed using hydrogen-1 nuclear magnetic resonance. b–d) Lung explants were infected with 10^6 CFU·mL^{-1} *Strep. pneumoniae* wt in the absence or presence of 2 mM 2-deoxy-D-glucose (2-DG) inhibitor. After 24 h supernatants were collected and b) concentration of glucose, lactate and c) pH was measured using a blood gas analyser. d) Total tissue lysates were evaluated by Western blot and quantified by densitometry, representative gel for vascular endothelial (VE)-cadherin is shown. Values represent VE-cadherin expression level relative to control and normalised to β-actin. 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.

and upregulated and downregulated genes and regulons are listed in supplementary tables S1 and S2 and are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-13533.

Elucidating molecular pathways involved in junctional degradation to acidic pH

To elucidate pH related host signalling pathways that could potentially be targeted for future therapeutic intervention in acid-induced junctional degradation, we tested several inhibitors for related pathways addressing general degradation (proteasome), junctional regulation (mitogen-activated protein kinase (MAPK), ROCK) or pH-related activation (heat shock proteins (hsp), transient receptor potential vanilloid (TRPV), connexins) [38–43]. Firstly, we applied proteasome, MAPK and ROCK inhibitors either during *Strep. pneumoniae* infection or pH challenge, but they showed no effect on VE-cadherin expression (supplementary figure S11a and b). Next, we used different inhibitors for hsp90, TRPV1 or connexin 43 (supplementary figure S11c–h). Interestingly, none of the inhibitors were able to prevent VE-cadherin degradation caused by pneumococci directly (supplementary figure S11c, e and g). However, challenging

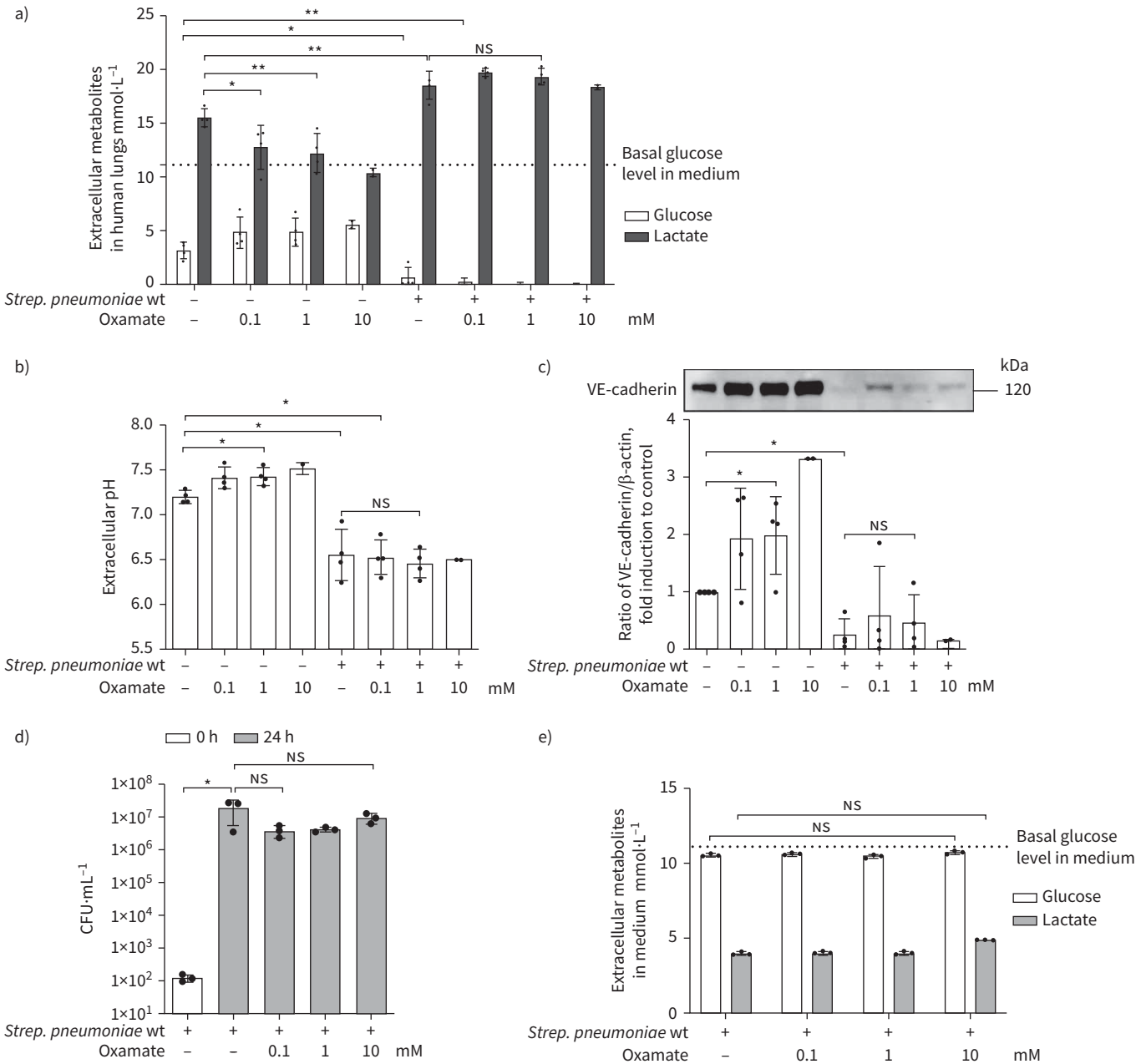

FIGURE 7 Human-selective lactate dehydrogenase (LDH) inhibitor oxamate does not affect lactate production in *Streptococcus pneumoniae*, but reduces lactate production in human lung tissue. a–c) Lung explants were infected with 10^6 CFU·mL^{-1} *Strep. pneumoniae* wild-type (wt) or d) and e) 10^2 CFU·mL^{-1} *Strep. pneumoniae* wt was grown in medium (RPMI+10% fetal calf serum) in the absence or presence of 0.1, 1 and 10 mM oxamate for 24 h. After respective time point supernatants were collected and (a) and (e) concentration of glucose and lactate and (b) pH was measured using a blood gas analyser. c) Total tissue lysates were evaluated by Western blot and quantified by densitometry; representative gel for vascular endothelial (VE)-cadherin is shown. Values represent VE-cadherin expression level relative to control and normalised to β-actin. d) Bacterial growth was assessed in colony-forming units after 0 and 24 h. Data are presented as mean±SD of at least two donors within independent experiments. NS: nonsignificant. *: p<0.05, **: p<0.01.

lungs with acidic pH alone, there was a partial, and, in some cases, partly significant reversion of VE-cadherin expression levels (supplementary figure S11d, f and h).

Discussion

The alveolar barrier disruption causing vascular leakage and oedema is a hallmark in severe pneumonia, ALI and ARDS. With no clinically established adjunctive therapies to stabilise lung barriers, it is crucial to further research and understand causative mechanisms.

In this context, the current study demonstrates how *Strep. pneumoniae* leads to a substantial reduction in the barrier-stabilising junctional proteins occludin and VE-cadherin within the human alveolus, independent of the major pneumococcal virulence factors PLY and H_2O_2. Bacterial sugar fermentation and the resulting secretion of acidic end-products, notably lactate, were found to cause reduction in junctional protein expression. In addition, the pH decrease inhibits the secretion and partly the transcription of central cytokines induced by *Strep. pneumoniae* infection.

Although inhibition of pH-related signalling pathways, such as p38, hsp90, TRPV1 or connexin 43, tended to maintain junctional protein expression after an acid stimulus, inhibition of these pathways had no effect on *Strep. pneumoniae*-induced junctional degradation. Only buffering of the human alveolar microenvironment provided significant protection against loss of junctional proteins and led to increased cytokine secretion.

It is well established that PLY is a potent inducer of hyperpermeability in human lung microvascular endothelial cells *in vitro*, as well as mouse lung, as evidenced by the loss of the surrogate junctional marker VE-cadherin [9, 10, 14]. However, our new data call into question the role of PLY (and H_2O_2) in causing increased permeability during alveolar pneumococcal infection. It is conceivable that PLY-induced permeability requires direct exposure of endothelial cells to initiate pore and subsequent gap formation [9]. However, during the initial intra-alveolar *Strep. pneumoniae* infection, PLY does not directly access the capillaries. Relatively high PLY concentrations would be required as bolus, as previous studies in mice showed by intratracheal toxin administration [10]. Alternatively, pneumococci may form localised microaggregates with high local PLY release, as shown by Hook *et al*. [44] for α-toxin release by *S. aureus*. However, the definitive role of PLY and H_2O_2 in alveolar hyperpermeability during full competency *Strep. pneumoniae* infections remains not fully elucidated [11, 45] and data on the affection of the alveolar barrier in human lungs by *Strep. pneumoniae* are still missing.

To address these gaps, we tested bacterial deletion mutants for PLY and H_2O_2 or the addition of both factors for their effects on junctional protein presence. Our results suggested a PLY- and H_2O_2-independent mechanism for the dissociation of occludin and VE-cadherin at junctions. While clinical isolates of *Strep. pneumoniae* reinforced these outcomes, the lung pro-inflammatory response or bacterially driven cell death seem not to contribute to junctional deterioration [32, 46, 47].

Recent work on acid-induced lung injury [19] guided us to explore bacterially induced acidification in enhancing vascular permeability. Acidic conditions are well documented to cause junctional degradation and increased permeability, a finding consistent across human bronchial epithelial and lung microvascular endothelial cells [34, 41, 42]. Consequently, we put forth the hypothesis that an acidic pH resulting from *Strep. pneumoniae* infection may serve as a central factor responsible for degradation of key junctional proteins of the human alveolar barrier. Indeed, pneumococcal infection leads to notable acidification and the loss of alveolar occludin and VE-cadherin within the first 24 h, a change that can be partially reversed by buffering the microenvironment.

While acid aspiration studies suggest significant effects at extremely low pH of 1–2 within 4–6 h [48], our findings indicate that an average pH drop to 6 over 24 h can also disrupt the alveolar barrier, implying that rather mild pH shifts may promote vascular leakage early in infection. This is supported by an earlier study investigating exhaled breath condensate (EBC) acidification in acute lung injury, demonstrated a low EBC-pH in ventilated patients compared to volunteers (5.85±0.32 *versus* 7.46±0.48; $p<0.0001$). There, a correlation of EBC-pH with markers of local inflammation as well as severity of lung injury (Murray's lung injury severity score) was demonstrated, suggesting that low alveolar pH, in a similar range as observed in this study, may contribute to the observed lung injury [49]. However, the actual mechanism by which an intra-alveolar decrease in pH leads to the loss of capillary endothelial VE-cadherin is unclear and could involve either receptor-independent or -dependent crosstalk between alveolar epithelium and endothelium. Studies in mice have shown that low intra-alveolar pH leads to epithelial calcium influx and the production of reactive oxygen species, which are then transmitted to the endothelial cells [12]. We addressed different signalling pathways acting either on proteasomal degradation or specific junctional regulation, which showed no effect. In addition, inhibition on pH-sensitive signalling pathways such as hsp90, TRPV1 or connexin 43 revealed protecting effects of VE-cadherin expression after HCl treatment, but not under infection with *Strep. pneumoniae* [38–43]. Likewise, our bulk-RNA analysis neither revealed any regulation at the transcriptional level of junctional protein nor further, indirect factors thus indicating (post-)translational effects probably acting in a differentiated manner on junctional proteins. These data show that results from *in vitro* and *in vivo* studies on pH-induced barrier regulation cannot simply be transferred to the human alveolus. As pointed out by Uhlig *et al*. [50], effects on endothelial permeability

can be very similar *in vitro* and *in situ*, but in fact are significantly different in the underlying causes between the applied models. This variation is expected to apply to human lungs as well. While effects observed in HUVEC from infected lung supernatants are similar, and also correlate to results from acid-induced lung injury, a deeper understanding of the exact molecular pathways in human lungs is yet to be elucidated.

The complexity and significance of the bacterial acidification becomes further apparent when considering the cytokine response. As shown in previous studies, pneumococci induce the production of significant amounts of COX-2, TNF-α, IL-1β, GM-CSF or IL-10 in human lungs, which are crucial for the initial immune response [26, 27, 30]. However, under conditions of buffering, this expression is further increased, improving both transcriptional and translational/secretory regulation. Several studies (reviewed in [48]) have shown that even mild acidification is sufficient to inhibit the cytokine response to various pro-inflammatory stimuli in both alveolar macrophages and pulmonary epithelial cells. An acidic environment could potentially impact not only the recruitment of neutrophil granulocytes [51], but also their ability to defend against *Strep. pneumoniae*, for example, by reducing the formation of extracellular traps [52]. In conclusion, we infer that the bacterially induced acidification may lead, next to a disturbed junctional expression, to a delayed cytokine response with probably hampered recruitment of immune cells and bacterial killing.

To complement our understanding of the host response, it's crucial to delve into the metabolic mechanisms at play within the bacteria. First, it is important to recognise that although free glucose in the upper and lower respiratory tract are scarce, aerotolerant anaerobe bacteria such as pneumococci, relying on fermentative energy metabolism, derive sugars from glycocalyx glycans *via* glycosidases [53–55]. This glycan cleavage results in damage to the glycocalyx, potentially diminishing the anti-bacterial adhesion protection, especially in the alveoli. In addition, our metabolomic analysis identified bacterial sugar consumption and lactate production as the primary contributors to acidification leading to impairment of the barrier and the initial immune response. Bacterial transcriptional profiling further corroborated our findings by revealing an upregulation of the CcpA regulon, controlling carbon catabolite repression [20, 56], under infection-like conditions. The de-repression of the CcpA regulon indicates glucose exhaustion upon entry into the stationary phase, leading to the utilisation of alternative carbon sources in the absence of glucose as supported by our transcriptome data. Notably, a mildly acidic environment of pH 6 appears to significantly boost pneumococcal growth by 2 log fold. While this remains speculative at this stage, future studies may shed light on whether the metabolism of different sugars impacts the regulation of bacterial virulence factors, as proposed by others [56]. Such mechanisms could impede the appropriate immune recognition of pneumococci.

Reinforcing our findings, a recent study has shown that an increase in systemic lactate during sepsis directly leads to increased vascular permeability and mortality, a phenomenon linked to the regulation of VE-cadherin [57]. The significant pH drop induced by *Strep. pneumoniae* largely based on sugar consumption is herein associated with a weakening of the alveolar–capillary barrier protein expression. Simultaneously, conditions with elevated blood glucose levels lead to an increased detection of glucose in lower respiratory tract secretions [58] and indicate an elevated risk of pulmonary *Strep. pneumoniae* infection [59]. Taken together, these factors could contribute to the occurrence of severe clinical outcomes in these patient groups.

In summary, our data show that aerotolerant and facultative anaerobic bacteria, such as *Strep. pneumoniae* or *S. aureus*, can acidify the human alveolar microenvironment through their sugar catabolism. Even though the pH decrease is relatively mild when compared to acid aspiration pneumonitis, it exerts an effect that is independent of classical virulence factors. This effect influences both alveolar barrier junction protein expression and the initial immune response. It is conceivable that therapeutic approaches that prioritise early-stage pH stabilisation of the alveolar compartment could positively impact the progression of severe pneumonia. Such approaches would serve to stabilise the barrier and enhance the anti-bacterial immune response.

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Ethics statement: The study using human lung tissue 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.

Author contributions: D. Fatykhova, V.N. Fritsch, K. Siebert, A. Löwa, M. Fischer, M. Mieth, K. Methling, K. Hellwig and D. Frey performed and analysed experiments. J. Weiner, W. Bertrams and T. Busche performed analysis of sequencing data. S. Hammerschmidt and T.P. Kohler constructed *Strep. pneumoniae* double mutant lacking pneumolysin and hydrogen peroxide. J. Kalinowski, D. Beule, H. Antelmann and M. Lalk contributed to data processing and analysis. J. Neudecker, J.C. Rueckert, M. Toennies, T.T. Bauer, M. Graff, H-L. Tran and S. Eggeling provided lung tissue explants. A.D. Gruber performed the processing for histology. D. Fatykhova, S. Hippenstiel and A.C. Hocke wrote the manuscript. S. Hippenstiel and A.C. Hocke conceived and supervised the study.

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