Co-infection with Staphylococcus aureus after primary influenza virus infection leads to damage of the endothelium in a human alveolus-on-a-chip model

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**Abstract**

Pneumonia is one of the most common infectious diseases worldwide. The influenza virus can cause severe epidemics, which results in significant morbidity and mortality. Beyond the virulence of the virus itself, epidemiological data suggest that bacterial co-infections are the major cause of increased mortality. In this context, *Staphylococcus aureus* represents a frequent causative bacterial pathogen. Currently available models have several limitations in the analysis of the pathogenesis of infections, e.g. some bacterial toxins strongly act in a species-specific manner. Human 2D mono-cell culture models often fail to maintain the differentiation of alveolus-specific functions. A detailed investigation of the underlying pathogenesis mechanisms requires a physiological interaction of alveolus-specific cell types. The aim of the present work was to establish a human *in vitro* alveolus model system composed of vascular and epithelial cell structures with cocultured macrophages resembling the human alveolus architecture and functions. We demonstrate that high barrier integrity maintained for up to 14 d in our model containing functional tissue-resident macrophages. We show that flow conditions and the presence of macrophages increased the barrier function. The infection of epithelial cells induced a high inflammatory response that spread to the endothelium. Although the integrity of the epithelium was not compromised by a single infection or co-infection, we demonstrated significant endothelial cell damage associated with loss of barrier function. We established a novel immune-responsive model that reflects the complex crosstalk between pathogens and host. The *in vitro* model allows for the monitoring of spatiotemporal spreading of the pathogens and the characterization of morphological and functional alterations attributed to infection. The alveolus-on-a-chip represents a promising platform for mechanistic studies of host-pathogen interactions and the identification of molecular and cellular targets of novel treatment strategies in pneumonia.
**Introduction**

Pneumonia is the most common infectious disease and also the most serious inflammatory event of the lower respiratory tract. Acute respiratory disease is a major medical concern, particularly for the immunocompromised host [1]. Pneumonia can be caused by a wide variety of microorganisms, including bacteria, viruses, and fungi. In particular, seasonal influenza virus (IV)-associated bronchopneumonia belongs to the infectious diseases with the highest population-based mortality. A major cause of mortality of IV infection consists of the onset of subsequent bacterial superinfection, i.e. with *Staphylococcus aureus* (*S. aureus*) or *Streptococcus pneumoniae* (*S. pneumoniae*). The bacterium *S. aureus* is recognized as a major public health burden and a leading invasive disease such as pneumonia 30% of the human population, and published data indicate that colonization is a known risk factor for an invasive disease such as pneumonia [2]. The lung is a preferred entry point for pathogens, and infections can be associated with a depressed immune cell function [3].

To date, the majority of studies investigating microbial infections of the lung rely on the use of animal models such as mice. However, lung anatomy and physiology, as well as the immune response, significantly differ between mice and men [4]. In particular, significant differences in the composition of innate and adaptive immune cells, including the expression of toll-like receptors, chemokines, and the differentiation of Th1/Th2, were described [5]. The interactions of pathogens and the immune system are partly based on species-specific effects [6]. The pathogenic mechanisms of *S. aureus* are largely dependent on a panel of species-specific virulence factors. Hence, the activity of toxins plays a major role in acute respiratory failure in humans [7]. Thus, reliable *in vitro* models closely resembling physiologically relevant conditions that reflect the human situation are required for the identification of novel targets in the interaction between microbial communities and their host.

The human alveolus, as a prominent part of the air-blood barrier, is mainly composed of type I and type II alveolar epithelial cells, endothelial cells and alveolar macrophages. This composition of various cells leads to the development of a solid permeability barrier of the human alveolus [8]. Epithelial cells function as oxygen and carbon dioxide exchange systems, but in addition, their immunological tasks are highly relevant. However, human alveolar cells often fail to maintain the differentiation and expression of lung-specific functions due to static mono-cell culture conditions. We generated a human alveolus model that provides immune-responsive cellular interphase between a vascular endothelium and an epithelial layer exposed to an air phase. The TEER-measurement as well as the permeability assays is able to show a high barrier formation of the human-alveolus-on-a-chip after 14 d of cultivation demonstrating the optimal comparability to the human alveolus. The coculture of epithelial, endothelial and myeloid THP-1 cells to recreate a lung barrier model was already proposed by Dohle et al [9]. This approach was extended by us to a triple cell co-culture system comprising human monocyte-derived macrophages as THP-1 cells are known to possess limitation in their inflammatory response to stimulation with bacteria derived molecules and therefore do not fully reflect the human monocyte/macrophage biology *in vitro* [10]. In addition, the culture approach was improved by perfusion of the endothelial cell layer to recreate more physiological conditions and to prevent endothelial dedifferentiation that is often associated with static culture conditions [11]. By integrating tissue-resident immune cells and monitoring their function, biochips represent a valuable tool to perform complex analysis of host-pathogen interactions in an immune competent environment *in vitro*. In a model of human endotoxemia caused by LPS and in infection models with IV and *S. aureus*, we demonstrated that central pathophysiological aspects of pneumonia can be emulated in the system. The alveolus model has been used to characterize the spatiotemporal spreading of *S. aureus* in mono- and co-infection conditions with IV and to identify endothelial dysfunction as a potential therapeutic target in the course of superinfection within the lung.

### 1. Materials and methods

#### 1.1. Composition of the human alveolus-on-a-chip model

Multiorgan tissue flow (MOTiF) biochips were manufactured by microfluidic ChipShop GmbH (Jena, Germany). Briefly, MOTiF biochips consist of two chambers separated by a porous membrane (figure 1) [11]. The membrane has a thickness of 12 μm and is made of polyethylene-terephthalate. It contains pores randomly distributed with a diameter of 8 μm and a median pore density of \( 1 \times 10^5 \) pores cm\(^{-2} \).

The volume of the epithelial chamber is 220 μl (including the afferent and efferent channels), while the volume of endothelial chamber is 120 μl (including the channels). The channel and chamber structures are covered by a gas permeable polystyrene (PS) bonding foil. The biochip was perfused by peristaltic pumps (Ismatec REGLO digital MS-CA-4/12-100, Wertheim, Germany), silicon tubing (Ismatec), and connectors and plugs (microfluidic ChipShop). Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described previously and seeded into the biochip (vascular cavity) at a density of \( 2.7 \times 10^5 \) HUVECs cm\(^{-2} \) in endothelial cell growth medium (PromoCell, Heidelberg, Germany) [12]. Cells were cultured for 48 h
with a daily medium exchange. NCI-H441 cells (ATCC, Manassas, USA) were cultured in RPMI-1640 (Lonza, Cologne, Germany) and 10% fetal calf serum (FCS, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and were seeded on the opposite side of the membrane (epithelial chamber) with a density of $2.7 \times 10^5$ NCI-H441/cm$^2$. Cells were cultured for 2-3 d until confluency with a daily media exchange. Subsequently, NCI-H441 medium was supplemented with 1 μM dexamethasone ( Sigma-Aldrich, Munich, Germany). After 1 week of culture, monocyte-derived macrophages were seeded on top of the NCI-H441 cell layer with a density of $0.9 \times 10^5$ macrophages cm$^{-2}$. Beside the epithelial cell line NCI-H441, we used human primary epithelial cells (NHBE, human bronchial epithelial cells, Lonza Group AG, Basel, Switzerland) to verify the key-findings of our model. For this, cells were seeded with a density of $3 \times 10^5$ NHBE cm$^{-2}$ at the epithelial side of the human alveolus-on-a-chip. The cells were cultured for 7 d and afterwards, monocyte-derived macrophages were seeded on the chip as described above.

After the integration of macrophages, the cells were cultivated until day 9 with media in both chambers and without connection to the pump system. From the next day on, the system was cultivated under flow condition on the endothelial side of the membrane (dynamic condition). To determine the effect of this flow condition, we have compared the parameters with the chip model which was not connected to the peristaltic pump (static condition).

The medium composition for the epithelial cavity was adjusted to RPMI-1640 containing 20% autologous human serum, 10 ng ml$^{-1}$ human granulocyte macrophage colony-stimulation factor (GM-CSF, Peprotech, New York, USA), 1 μM dexamethasone ( Sigma-Aldrich, Munich, Germany) penicillin/streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After one day of macrophage attachment, chip perfusion was initiated in the vascular cavity with a flow rate of 21 μl min$^{-1}$ corresponding to a shear stress of 0.23 dyn cm$^{-2}$. Flow rates and shear stress values were calculated as recently described [111]. One day later, the air–liquid interface

![Figure 1. Overview of the human alveolus-on-a-chip setting perfused with a pump system. The microfluidic chip system consists of an upper and a lower chamber divided by a porous membrane. The upper chamber with alveolar epithelial cells (NCI-H441) and cocultured macrophages (Monocyte-derived macrophages) after 14 d with air phase and the lower chamber consisting of endothelial cells (HUVECs) with medium. The perfusion of the cell culture medium from a reservoirs placed on top of the chip is enabled by use of a peristaltic pump and biochip integrated microchannels.](image-url)
(ALI) was established in the alveolar cavity and the vascular side was nourished by phosphate buffered saline (PBS), and 1 μM dexamethasone. During the maintenance of the alveolar biochip cell culture, a medium exchange was performed on a daily basis in both cavities with the respective media. As soon as the epithelial cavity was filled with air, medium exchanges ceased in this cavity, and cells were nourished by the vascular side (supplementary figure 1 is available online at stacks.iop.org/BF/12/025012/mmedia).

1.2. Infection and stimulation

The human alveolus-on-a-chip model was perfused for a total of 7 d. The infection with IV and/or S. aureus was subsequently performed under static conditions. All infection scenarios were performed in a complete human alveolus-on-a-chip model with integrated macrophages. The IV strain PR8 (H1N1) was passaged on Madin-Darby canine kidney cells (MDCK) and stored at −80 °C until infection. The methicillin-resistant S. aureus (MRSA) strain USA300 was grown in brain heart infusion-medium at 37 °C with shaking. For the determination of the multiplicity of infection (MOI), the colony forming units (CFUs) were ascertained weekly.

For infection, we simultaneously performed single bacterial and viral infection as well as co-infection consisting of a primary virus and a secondary bacterial infection [13]. NCI-H441-cells or human primary epithelial cells (NHBE) were infected with IV (MOI 1) or S. aureus (MOI 5) or superinfected with both pathogens. For the control, cells were mock treated. Briefly, cells were incubated with the virus for 30 min. Subsequently, the media were changed, and the cells were superinfected with S. aureus. Extracellular bacteria were removed by lysostaphin treatment (6 μg ml⁻¹, 20 min) 90 min after bacterial infection [13]. Subsequently, lysostaphin was rinsed with phosphate buffer solution (PBS), and cells were supplied with fresh medium. Chambers were incubated for 3 h after a lysostaphin washing step to analyze the cytokine production.

Furthermore, the alveolus model was cultured under perfused conditions for 7 d and subsequently stimulated with 100 ng ml⁻¹ LPS (from Escherichia coli (E. coli) strain E0111:B4; Sigma-Aldrich, Munich, Germany) for additional 24 h.

Supernatants of the epithelial and endothelial chamber were stored at −80 °C until analysis. The verification of the infection with S. aureus (MOI 5) and with both pathogens (co-infection) was performed by collecting the supernatants of the epithelial and endothelial layer of the human alveolus-on-a-chip model. Supernatants were collected and promptly plated on agar plates in 3 different dilutions. After 24 h of growth, CFUs were counted.

1.3. Monocyte isolation and cell culture

Primary monocytes were isolated from whole peripheral blood of healthy donors. The blood was diluted in an equal volume with phosphate buffer saline (PBS), 2 mM ethylenediaminetetraacetate (EDTA, Sigma-Aldrich, Germany), and 0.1% bovine serum albumin (BSA, Carl Roth GmbH and Co. KG, Karlsruhe, Germany) and gently transferred on top of a Biocoll separation solution (Biochrom, Berlin, Germany). Peripheral blood mononuclear cells (PBMCs) were separated from blood cells by centrifugation without brakes. Cells were washed three times with PBS, 2 mM EDTA, and 0.1% BSA with centrifugation steps in between. For alveolar chip generation, 1 × 10⁷ PBMCs/well were cultured in 6-well dishes in X-VIVO 15 medium supplemented with 10% autologous human serum, 10 ng ml⁻¹ human GM-CSF (PeproTech, Hamburg, Germany), 10 ng ml⁻¹ macrophage colony-stimulation factor (M-CSF) (PeproTech, Hamburg, Germany), and penicillin/streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The cells were incubated for 2 h at 37 °C and 5% CO₂ and subsequently washed twice with X-VIVO 15 (Lonza, Cologne, Germany) and cultured in X-VIVO 15 with supplements [14]. Cells were cultured for 1 d and subsequently used for seeding in the human alveolus-on-a-chip model (supplementary figure 1).

1.4. Quantification of cytokines

Measurement of cytokines was performed by using multiplex bead-based immunoassays (LEGENDplex, BioLegend, San Diego, CA, USA) [15]. Briefly, supernatants from both chambers were collected 3 h post infection (p.i.). Experiments were repeated at least three times, and all samples were measured in duplicate within a 96-well plate by using the BD Accuri flow cytometer (BD, Heidelberg, Germany). Data analysis was performed with LEGENDplex™ data analysis software from BioLegend (San Diego, CA, USA).

1.5. Quantitative real-time PCR

The isolation of total RNA from all cells of the human alveolus-on-a-chip model was performed by using QIAshredder homogenizers (Qiagen, Hilden, Germany). The RNA was isolated with RNasy Micro Kit (Qiagen, Hilden, Germany) following manufacturer’s directions. The gene expression of indicated genes was determined by quantitative RT-PCR on a Thermo Scientific™ PikoReal™ Real-Time PCR System using the QuantiTect SYBR Green kit (Qiagen) and gene-specific primers. For this we use GAPDH(fwd) 5′-CTCTGCTCCTCCTGTTCGAC-3′ and GAPDH(rev) 5′-CAATACGACCAAATCCGTGC-3′ and for the
detection of IV we use IV(fwd) 5'-GACCAATCCTGT-CACCTCTGAC-3' and IV(rev) 5'-AGG GCA TTT TGG ACA AAG CGT CTA-3'. For the detection of the surfactant protein A (SP-A) mRNA we use the following primer set SP-A(fwd) 5'-GAT GGG CAG TGG AAT AAT GAA GTG GCT AAG GGT G-3'. All primers were synthesized by metabion international AG (Planegg, Germany).

1.6. Permeability assay
To test the permeability of the epithelial and endothelial barrier, 1 mg ml⁻¹ of 3–5 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, Germany) in phenol-red free DMEM/F12 medium (Sigma-Aldrich, Germany) was injected into the upper chamber of the chip. The lower chamber contained only phenol red free DMEM/F12. The ALB model was incubated for 60 min under static conditions. Afterwards, the media from the lower and upper chambers were collected, and fluorescence intensity (exc. 488 nm; em. 518 nm) was measured in a 96-well Clear black plate (Greiner BioOne, Frickenhausen, Germany) by a Tecan Infinite M200 Pro microplate reader (Tecan Group Ltd, Maennedorf, Switzerland). The permeability coefficient (P_app) was calculated according to P_app (cm s⁻¹) = (dQ/dt)(1/ACo). For this, dQ/dr represents the steady-state flux (g s⁻¹), A the culture surface area (cm²) and C₀, the initial concentration (mg ml⁻¹) [16].

1.7. Transepithelial/transendothelial electrical resistance (TEER)
To determine the integrity of the barrier, we performed TEER measurements. For this, indium tin oxide (ITO)-covered glass slides (Sigma-Aldrich) were attached with adhesive foil to the top and bottom of MOTIF biochips facing the cell seeded membrane. Slides were pressed for 24 h to the chips to achieve fluid impermeable ‘bonding’. Cell seeding was performed according to the ‘Composition of the human alveolus’ section. Every 24 h transepithelial resistance measurements were performed until the establishment of an ALI. Subsequently, resistance was measured on days 1, 3, 5, and 7. For these measurements, the air-containing channel was carefully filled with medium allowing current flow between the fixed ITO slides. After the measurement, the channel was emptied by aspirating the medium. For TEER monitoring, the AC resistance of the biochip chamber was measured. Therefore, the ITO-covered slides were connected by copper clamps and supplied with a constant amplitude AC voltage of 0.87 V_RMS at a frequency of 1.6 kHz. The resulting AC current was measured by means of a U1251A digital multimeter (Agilent, Santa Clara, USA). The maximum possible AC current of the given structure of the biochip filled with growth medium and without cells was found to be 1.7 mA_RMS, which corresponds to a resistance of 512.

1.8. Immunofluorescence staining
The membranes of the chip with attached cell layers were carefully excised 3 h p.i. and washed in PBS containing Ca²⁺ and Mg²⁺ (Lonzar). Subsequently, membranes were fixed in 4% paraformaldehyde at room temperature (RT) for 10 min or 100% methanol (at −20 °C for 10 min). Further, membranes were washed three times with PBS containing Ca²⁺ and Mg²⁺ and then blocked in PBS with 3% normal donkey serum (Dianova) 0.1% Triton X-100 (Sigma-Aldrich, Germany) or PBS with 3% normal donkey serum and 0.1% saponin (Sigma-Aldrich, Germany). Subsequently, membranes were incubated overnight by combinations of the following primary antibodies against aquaporin-5 (Abcam, Cambridge, UK), von Willebrand Factor (VWF) (DAKO, Hamburg, Germany), vascular endothelial cadherin (VE-cadherin) (BD Biosciences, Heidelberg, Germany), zonula-occludens-1 (ZO-1) (Thermo Fisher Scientific, Wallingford, Massachusetts, USA), endothelial cadherin (E-cadherin) (BD Biosciences, Heidelberg, Germany), SP-A (Santa Cruz Biotechnology Inc., Heidelberg, Germany), CD68 (BD Biosciences, Heidelberg, Germany), and IV (Thermo Fisher Scientific, Wallingford, Massachusetts, USA). Following three washing steps in PBS or PBS/0.1% saponin, membranes were incubated for 30 min at RT with DAPI (Life Technologies) and the following secondary antibodies: donkey immunoglobulin (Ig)G anti-mouse IgG-Cy3, donkey IgG F(ab')₂ anti-rabbit IgG-AF488 and donkey anti-goat IgG-Cy5 (all obtained from Dianova, Hamburg, Germany). Membranes were washed 4 times and finally mounted in fluorescent mounting medium (DAKO). Finally, images were recorded using an AXIO Observer Z1 fluorescence microscope with an Apatome 2 extension (Carl Zeiss AG, Jena, Germany).

1.9. Infection kinetics
To investigate the replication of IV within epithelial and endothelial cells, we performed infection kinetics testing. For this, cells were infected with different MOIs (0.01, 0.1, 1 and 0.05, 0.5 and 5) and fixed after 1 h, 6 h, 24 h and 48 h of incubation with ice-cold methanol. Subsequently, the immunocytochemical staining of IV nucleoprotein (monoclonal antibody: Acris GmbH, Hiddendenhausen, Germany) was performed according to the manufacturers protocol (DAKO, Hamburg, Germany). The detection of the virus-infected cells was carried out by microscopy (AxioCamERc 5s, Carl Zeiss Jena, Germany). The experiment was performed three times per time point.
1.10. Scanning electron microscopy (SEM)
The cell structures were fixed inside the biochip by switching the flow of the medium to the fixative solution (2.5% glutaraldehyde in cacodylate buffer) for 90 min. Afterwards, the flow was switched again to fresh cacodylate buffer for 30 min to wash out the fixative. Then, the populated membranes were removed carefully, cut into two halves and needle-pinned onto pieces of cork, with one of the halves flipped to offer access to both sides. Next, the samples were dehydrated in ascending ethanol concentrations (30%, 50%, 70%, 90% and 100%) for 15 min each. The samples were critical-point dried using liquid CO2 and sputter coated with gold (thickness approx. 2 nm) using an SCD005 sputter coater (BAL-TEC, Liechtenstein) to avoid surface charging. Finally, the specimens were investigated with a field emission SEM LEO-1530 Gemini (Carl Zeiss NTS GmbH, Oberkochen, Germany).

1.11. Image acquisition
Images were acquired on the structured-illumination system ‘Aptome.2’ from Zeiss (Carl Zeiss Ag, Oberkochen, Germany) built in the inverted configuration on a Zeiss Observer.Z1 microscope. Illumination was provided by an HPX 120C external unit. For the overview experiments a 10× magnification, NA0.3 immersion-free objective was used with 0.645 × 0.645 μm pixel size. The detailed images were recorded with a 20×/NA0.8 immersion-free objective lens at 0.3225 × 0.3225 μm pixel size. The Z-stack images were acquired at 2 μm Z-slice distance. The various fluorescence channels were recorded with the following filter settings: excitation centered at 353 nm, 488 nm, 548 nm and 653 nm; emission centered at 465 nm, 509 nm, 561 nm, 572 nm and 668 nm, respectively. The images were recorded with an AxioCam MRm camera with 6.45 μm pixel size at 1388 × 1040 pixels (total area 8952.6 × 6708 μm). The camera exposure time was set at 20, 50, 100, 200, 1000 or 2000 ms, according to the intensity of the labeling.

1.12. Image analysis and quantification
Images were provided for analysis as Z stacks in the Zeiss native image format ‘CZI’. This data contained the Apotome images that were already processed by the ZEN software (Carl Zeiss Ag, Oberkochen, Germany) to create optical sections. During preprocessing, the Z-stack images were first deconvolved using the Huygens Professional software (SVI, Hilversum, Holland), where the spinning disk deconvolution module was utilized by setting the pinhole spacing at 4 micrometers, according to the corresponding SVI guidelines for handling Apotome image data. The deconvolved image stacks were segmented in Imaris 9.2.1 (Bitplane, Zürich, Switzerland) using either the ‘Cells’ module (for per-cell analysis of the endo- and epithelial cells) or the ‘Surfaces’ and ‘Spots’ modules (for per-image analysis and to characterize individual macrophages). Here the blue (DAPI) channel was selected to segment the cell nuclei, whereas either the orange or the green channel was chosen to identify either the E-(VE-) cadherin or the ZO-1 labeling, respectively. The segmented nuclei (from the blue channel) served as seeds to assist the search for the cell membranes (from the orange or the green channel). After the cell membranes were successfully segmented, the area between the nuclei and the cell membrane were identified as the cytoplasm. For the endothelial side of the samples the Imaris ‘Cells’ module was restricted to segment only the nuclei (DAPI labeling in blue) and the cell membranes (VE-cadherin in orange). The quantification of the segmented images was carried out in Imaris 9.2.1. Here we measured the mean value and the standard deviation of the fluorescence intensity for all channels; the volume and the sphericity of the cells; the number of cells per Z stack. Similarly, the S. aureus content of the entire fields of view of the images was determined from the corresponding fluorescence channel, using the ‘Spots’ module of Imaris that allowed the determination of the number of S. aureus, in addition to their individual and summed-up fluorescence intensity.

1.13. Statistical analysis
Statistical analysis was performed with GraphPad Prism software version 7 and 8 (GraphPad Software, La Jolla, CA, USA) or Microsoft Excel (Microsoft Office, 2010). Comparison between more than two groups was performed using a one-way or two-way ANOVA test. Where indicated, comparison between two groups was performed using a paired, two tailed Student’s t-test. P ≤ 0.05 was considered significant.

1.14. Informed consent
The study was approved by the ethics committee of the Jena University Hospital, Germany (3939-12/13). Informed consent was obtained from all individuals participating in the study who provided blood in accordance with the Declaration of Helsinki.

2. Results

2.1. Flow conditions and macrophage coculture increase barrier integrity
To establish the alveolus-on-a-chip model, we used the microfluidic MOTIF biochip with a suspended porous membrane serving as a perusable cell substrate [17] (figure 1). At the membrane facing the lower chamber, HUVECs were cultured. On the top of the membrane NCI-H441 cells and monocyte-derived macrophages were cocultured. To verify the key-findings of the NCI-H441 cultured model, we used also human primary epithelial cells (NHBE). The design of the chip resulted in a stable ALI 14 d after cultivation (supplementary figure 1). Pores in the
membrane allow molecules, cells and pathogens to translocate between the chambers and allow for an exchange of nutrients and oxygen during perfusion of the endothelial cell layer. In this context, the establishment of a selective biological barrier formed by confluent cell layers expressing proteins of the apical junctional complex is a central morphological feature of the lung. Immuno-fluorescence staining for epithelial and endothelial cell layers of the alveolus-on-a-chip model demonstrated the expression of zona occludens-1 (figure 2) and the alveolar epithelial type I specific marker AQP-5 (supplementary figure 2) as well as the detection of SP-A, an alveolar epithelial type II marker (figure 3, supplementary figure 2).

We are able to show, that SP-A is well distributed over a wide area. Although we are able to stain AQP-5, its expression pattern in NCI-H441 cells does not fully reflect the situation found on primary cells. As published before [18] NCI-H441 cells show excellent properties regarding their barrier function as well its surfactant production. Although the expression of the water channel AQP-5 on the apical surface of AT1 cells is not present, this fact does not affect the barrier function determined by TEER-measurement or protein quantification based on immuno-fluorescence staining.

Furthermore, E-cadherin signal (figure 3) for the epithelium and expression of CD68 by macrophages (figure 2) were detected. In addition, the mRNA expression of SP-A by NCI-H441 cells was significantly higher compared to A549 epithelial cells (supplementary figure 2). A549 cells are frequently used in infection research but do not have specific advantages such as the production of surfactant, demonstrating the higher comparability of NCI-H441 cells and primary human alveolar epithelial cells.

The endothelium was stained with VE-cadherin and VWF (figure 4). Furthermore, NCI-H441 cells were shown to form microvilli under perfused culture.
conditions (figure 5). Our results show that both perfusion and coculture with monocyte-derived macrophages improved barrier formation, which was confirmed by the reduced permeability of the cell layers for FITC-labeled dextran (figure 6(A)).

Interestingly, the perfusion further increased the tightness of the lung tissue in the presence of macrophages (supplementary figure 3). Thus, subsequent infection studies were performed only in presence of macrophages. In contrast, stimulation with LPS caused a significant reduction in barrier function as a result of a provoked proinflammatory immune response (figure 6(B)) and led to significant reduction of VE-cadherin expression (supplementary figures 4, 5).

2.2. Bacterial infections, in particular, co-infections, induce a high inflammatory response and spread to the endothelium

Based on the fact that macrophages lead to higher barrier integrity we next addressed their function in the context of infection. We established protocols to stimulate the human alveolus-on-a-chip model comprising primary macrophages with LPS as well as infection protocols with living pathogens *S. aureus* and IV. To determine the response of our model we analyzed cytokine secretion in the upper and lower chambers of the lung model after exposure to LPS or infection with virus and/or bacteria. After 14 d of culture, LPS was exposed to the epithelial cell layer for 24 h, and media from both chambers were collected. A considerable release of pro-inflammatory interleukin (IL)-1β, IL-6, TNF-α and anti-inflammatory IL-10 was observed (figures 7(A)–(H)) in the presence of macrophages.

Furthermore, the presence of bacteria attached to the microvilli was confirmed by SEM (figure 8). It is important to note that IV is able to infect but not to perform a complete replication cycle within macrophages [19]. Thus, infection experiments in monocultures of macrophages were not performed.

Next, we established a protocol to infect the human alveolus-on-a-chip model with integrated macrophages and live pathogens *S. aureus* and IV. For infection, we used the strain *S. aureus* (MOI 5) and IV (MOI 1), which were applied over the epithelial cell layer. We were able to show an infection by *S. aureus* through the determination of CFUs (figures 9(A), (B)) after an additional antibiotic washing step removing remaining live bacteria in the extracellular environment. In addition, the presence of intracellular *S. aureus* was validated and quantified by immunofluorescence staining (figure 9(C)). We have shown that the number of *S. aureus* particles during co-
infection was significantly higher compared to the single infection without IV.

The human alveolus-on-a-chip model was tested for its ability of productive viral replication. Based on the detection of viral RNA we were able to confirm infection of the assembled human alveolus-on-a-chip model 3 h and 6.5 h after incubation with a MOI 1 (supplementary figure 6). To investigate the individual ability of the virus to replicate within the epithelial and endothelial cells used in our model we validated the replication of a single cell culture based on the immunofluorescence staining of the viral nucleoprotein (supplementary figure 7).

The inflammatory response of the epithelial and endothelial cell layer differs significantly between single and co-infection (figures 10(A)–(H)). To determine the pro- versus anti-inflammatory responses, we tested for various cytokines and chemokines. Interestingly, co-infection results in higher amounts of IL-1β, IL-6, MCP-1 (CCL-1, monocyte chemotactic protein 1) and interferon (IFN)-γ than single bacterial and viral infections. In general, viral infection resulted in a lower inflammatory response, whereas bacterial infection and particularly co-infection induced higher cytokine expression. To determine the effect of intra-cellular pathogens, we measured the production of IFN-γ within the endothelial and epithelial supernatants. Here, the co-infection of the epithelial layer shows significantly higher IFN-γ release compared to the single virus infection.

2.3. Co-infection leads to disruption of the endothelial barrier but does not damage the integrity of the epithelium

The infection of the epithelial layer in the single or co-infection scenarios does not result in significant differences concerning the integrity of the epithelial layer (figures 11(A), 12(A)). This result was also proven for human primary epithelial cells (NHBE) (supplementary figure 8). Further, also a quantitative analysis of adherence and tight-junction proteins E-cadherin (figure 11(B)) and ZO-1 (figure 12(B), supplementary figure 8) showed no significant alteration of protein expression upon infection.

In contrast, significant alterations in the expression of adherence junctional complex proteins within the endothelial layer were observed. We demonstrated that the expression and localization of VE-cadherin was not affected by a single viral or bacterial infection. However, co-infection with both agents results in a significant reduction of VE expression levels of HUVEC cells cultured within the alveolus-on-a-chip-model (figures 13(A)–(C)). Likewise, SEM shown the damage of the endothelium caused by the co-infection (supplementary figure 9).
2.4. Break down of the barrier function during co-infection

To analyze the integrity of the whole biochip system, we performed permeability assays using FITC-dextran to verify the integrity of the endothelial and epithelial cell layers by detecting fluorescence. We were able to show that co-infection significantly reduced the barrier function compared to the mock-treated chamber (figure 14). Furthermore, bacteria were found to translocate from infected epithelial cells into the...
endothelial cell layer (figures 9(A), (B)). An additional washing step with lysostaphin after bacterial infection indicated that intracellular bacteria leave their host cells during the infection process. The number of S. aureus particles within the co-infection scenario could be shown as higher than for the single infection (figure 9(C)). Extracellular bacteria were detected 6.5 h p.i within the endothelial chamber after infection of the epithelial cell layer (figures 9(A), (B)). In addition, the loss of integrity of the endothelium is determined by immunofluorescence staining of VE-cadherin (supplementary clip 1). These findings could be confirmed with human primary epithelial cells (NHBE) on the top of the chip-model (supplementary figure 10).

3. Discussion

In this study, we established a human alveolus-on-a-chip model to study cellular alterations associated with bacterial and viral infections of the lung [20]. Epithelial cells are able to produce cytokines and chemokines that influence the endothelial barrier.

The expression and localization of functional cell type- and barrier-specific markers of our model is similar to human lung tissue [21]. The alveolar surface is composed of type I and type II alveolar epithelial cells. Around 95% of the surface is covered with type I cells. Type II alveolar cells are characterized by the production of pulmonary surfactant and apical microvilli [22]. Our results clearly demonstrate that
NCI-H441 cells are able to produce the alveolar epithelial type I specific marker AQP-5 [23] as well as SP-A, an alveolar epithelial type II marker. NCI-H441 cells used in our model are immune responsive and secrete a variety of cytokines and chemokines involved in the activation of immune cells [24]. Furthermore, type II alveolar cells contribute to lung defense by producing surfactant proteins. Our findings show that the human alveolus-on-a-chip is able to secrete pulmonary surfactant that is necessary for the maintenance of the barrier function, as well as of the host defense against microbial infections [25].

To verify the major findings of our model with human primary epithelial cells, we perform co-infection with NHBE cells at the top of the membrane. Our results clearly demonstrate that primary epithelial cells form a stable barrier equally to NCI-H441 cells (supplementary figure 8). Furthermore, we are able to detect a loss of barrier integrity caused by viral-bacterial co-infection (supplementary figure 10). This finding leads us to conclude that NCI-H441 cells represent a low cost and easy to use cell line and in addition a suitable tool to gain detailed insight into the molecular mechanisms of viral and bacterial infection.

Macrophages play a crucial role in host defense against infection in the lung [26]. The presence of macrophages in an in vitro infection model of the lung therefore represents an important feature. We thus aimed to further characterize their contribution to the cytokine production during lung inflammation [27]. We emulated the function of alveolar macrophages by tissue integration of monocyte-derived macrophages. Alveolar macrophages originate from fetal monocytes during embryogenesis and thus differ from macrophages replenished by monocytes upon lung injury in their epigenetic landscape [28, 29]. However, several studies show that the lung microenvironment supplies various cues supporting a functional phenotype in monocyte-derived macrophages that is comparable to alveolar macrophages [30]. Furthermore, recently published data indicate that the self-renewal of macrophages is supplemented by a contribution from blood monocytes [31]. Thus, the monocyte-derived macrophages used in this study represent suitable surrogates to model alveolar macrophage function in the alveolus infection model [32]. Our results clearly indicate that macrophages are essential for the maintenance of barrier integrity in our model. This first relevant aspect of our study demonstrates that dynamic culture conditions improve the barrier function compared to static conditions. In this context, an improved expression of junction complex proteins upon exposure to shear forces.

Figure 8. Visualization of S. aureus on the epithelial cell side by scanning electron microscopy (SEM). Visualization of the infection of the epithelial cell layer of the human alveolus-on-a-chip model caused by S. aureus via the epithelial side. For the infection with S. aureus USA300 (MOI 5) the epithelial side of the model was incubated for 90 min and washed afterwards by using lysostaphin to kill extracellular bacteria. Scanning electron microscopy was performed with 2,000 x and 10,000 x magnification. Scale represents 5 μm (2,000 x magnification) and 1 μm (10,000 x magnification).
stress has been described [33]. Our findings further demonstrate the key role of macrophages in maintaining the barrier integrity of the alveolus model. The presence of several cell types within the human alveolus results in a solid barrier function [8]. In comparison to this, we are able to show that the TEER-measurement as well as the permeability assays of our in vitro model demonstrates a high barrier formation of the human-alveolus-on-a-chip after 14 d of cultivation suggesting a comparable barrier function to the human alveolus.

However, there is still a notable controversy in the literature regarding the role of alveolar macrophages during microbial infections. On the one hand, it is well known that alveolar macrophages comprise the largest population of resident cells in the respiratory tract [34] and that impairment of their function is one of the main risk factors for invasive mycoses [35]. On the other hand, based on experiments with mice, Mircescu et al claimed that neutrophils rather than alveolar macrophages play the essential role in the immune response against fungal pathogens [36]. A game-theoretical model revealed that these seemingly contradictory viewpoints can be reconciled by accounting for the impact of the infection-dose on the time course of the infection-inflammation scenario [37]. However, due to the lack of live cell imaging data for infection scenarios in the lung, it remains unclear what distinguishes between low and high infection doses. The human alveolus-on-a-chip model established in our work represents the most realistic infection model today that allows for tracking cell migration and cell-cell interactions in space and time by live cell imaging [38–40]. Thus, going beyond current state-of-the-art analysis that is often based on endpoint experiments on host-pathogen confrontation assays in a petri dish [41–44], the alveolus model will have an essential bridging function in systems biology approaches by providing quantitative information on as of yet unknown parameter values for virtual models of lung infection [45–47].

The responsiveness of the immunocompetent alveolus model to pathogen-associated molecular patterns was confirmed in a model of endotoxemia by exposure of epithelial cells to LPS. In the model, LPS triggered the release of cytokines that was associated with a reduction in the barrier integrity. Importantly, this effect was observed only in the presence of functional macrophages. The inflammatory response of the immunocompetent alveolus consists of a variety of mechanisms leading to the production of diverse cytokines and chemokines. By using the human alveolus-on-a-chip-model, we were able to analyze the expression of these molecules separately within the upper and lower chambers, indicating air- and blood-side. We have shown that the release of cytokines is mainly

![Figure 9. Bacterial spread of the human alveolus-on-a-chip model.](image)
dependent on macrophages. Consequently, integrated immune cells induce a significant stimulation of resident cells. Obviously, epithelial and endothelial cells are able to generate pro- and anti-inflammatory responses.

The human alveolus-on-a-chip model was validated for its ability to enable bacterial and viral replication. Based on the detection of viral RNA we were able to confirm viral infection. However, we were interested in the immune response during early infection phase up to 6.5 h p.i, where an increase of infectious virus particles is not detectable because the complete IV replication and production of maturated and fully infectious virus particles requires 6–8 h [48].

Endothelial dysfunction results in vascular leakage, which is considered the main driver of acute lung injury [49]. Our data suggest that a single infection of the epithelium does not affect the integrity of the endothelium within one replication cycle of IV. However, a viral/bacterial co-infection resulted in significantly impaired endothelial barrier integrity, suggesting that infection with IV most likely predisposes the lung endothelium to

Figure 10. (A)–(H). Cytokine secretion on the epithelial and endothelial side after infection with influenza virus and S. aureus or with both pathogens (coinfection). Production of cytokines and chemokines (IL-1β, IL-6, MCP-1, and IFN-γ) in the epithelial (A)–(D) and endothelial (E)–(H) cell layers of the human alveolus-on-a-chip model in pg/ml. Infection was performed from the epithelial side for mock infection (blue bar), with IV (red bar), S. aureus (green bar) and co-infection (purple bar). For single infection an incubation with IV (MOI 1) or S. aureus (MOI 5) was performed. Co-infection experiments were carried out with IV with MOI 1 which was incubated for 30 min on the epithelial side followed by a S. aureus (MOI 5) infection for 90 min. After 3 h supernatants were collected separately from upper and lower chamber and measured for cytokine production. N = 6, mean value shown as line, ordinary one-way ANOVA with Tukey’s multiple comparisons test, *p < 0.05, **p < 0.01.
leak after superinfection with *S. aureus*. Diffuse alveolar damage and massive infiltration of macrophages lead to inflammation and subsequent damage of the alveoli, which is largely dependent on the cell interaction and cannot be reproduced in single cell cultures. Our findings indicate that analyzing infection processes within the lung requires the interaction of different cell types. Epithelial cells within the alveolus are important for the first step of infection. In a second step, endothelial cells become also infected via epithelial cells. The

**Figure 11.** Analysis of adherence junction protein E-cadherin of the epithelial layer after infection. (A) Immunofluorescence staining of the epithelial layer shows E-cadherin (orange) for mock, *S. aureus* and influenza virus infection and coinfection. Infection was performed from the epithelial side for mock infection (blue bar), with IV (red bar), *S. aureus* (green bar) and coinfection (purple bar). For single infection an incubation with IV (MOI 1) or *S. aureus* (MOI 5) was performed. Co-infection experiments were carried out with IV (MOI 1) which was incubated for 30 min on the epithelial side followed by a *S. aureus* (MOI 5) infection for 90 min. Scale bar represents 50 μm. (B) Quantification of mean fluorescence intensity (MFI) for E-cadherin per cell. *N* = 4 (for each experiment at least 5 regions of interest were randomly selected), Whisker Plot with min. and max. value, two-way ANOVA, Tukey’s multiple comparison test, not significant.

**Figure 12.** Analysis of the barrier function of the epithelial layer. Analysis of tight junction protein ZO-1 of the epithelial layer after infection. (A) Immunofluorescence staining of the epithelial layer shows ZO-1 (green) for mock, *S. aureus* and influenza virus infection and co-infection. Infection was performed from the epithelial side for mock infection (blue bar), with IV (red bar), *S. aureus* (green bar) and coinfection (purple bar). For single infection an incubation with IV (MOI 1) or *S. aureus* (MOI 5) was performed. Co-infection experiments were carried out with IV (MOI 1) which was incubated for 30 min on the epithelial side followed by a *S. aureus* (MOI 5) infection for 90 min. Scale bar represents 50 μm. (B) Quantification of the mean fluorescence intensity (MFI) of ZO-1 per cell. *N* = 4 (for each experiment at least 5 regions of interest were randomly selected), Whisker Plot with min. and max. value, two-way ANOVA, not significant.
combined cell activation results in a robust inflammatory response of immune cells, which also contributes to lung damage. A bacterial co-infection with bacteria and viruses induced the highest immune response regarding cytokine expression and barrier function loss compared to the single infections. This can explain the severe clinical outcome observed in patients with superinfection and secondary pneumonia.
By using the human-specific alveolus-on-a-chip system, the investigation of bacterial toxins, e.g. staphylococcal pore-forming toxins, is feasible. For this, the analysis of Panton-Valentine leukocidin (PVL) [50] as a human-specific toxin is possible, and our model is an excellent complement to the next generation of humanized mouse models [51]. For viral-bacterial co-infections neutrophil granulocytes play a crucial role, mainly due to the fact that bacterial toxins like PVL target these immune cells and thereby lead to severe necrotizing pneumonia plays a key part for the infection. PVL acts in a highly human specific manner in targeting human neutrophil granulocytes [52]. These results should be addressed in follow up by integration of circulating neutrophil granulocytes into the alveolus model.

Our findings indicate that complex cell systems are suitable for the human-specific effects of pathogens. Future work will assess the potential of the human alveolus-on-a-chip model with respect to the investigation of pathogenesis and testing of various therapeutic compounds to treat lung infections.

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Conflict of interest

KR is CEO of Dynamic42 GmbH. KR and ASM are both shareholders of Dynamic42. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Contribution of authors

SDE, KR, and CE conceived and designed the experiments. Preparation of HUVECs was performed by the RH and ASM groups. SEM was performed by SN, SC, ZC, and MTF evaluated the light microscopy data and performed image analysis. MS maintained cell cultures. SDE, KR, ES, MW, KFH, and FS performed the infection experiments. SDE and KR and CE analyzed the data. SDE and BL wrote the manuscript. ASM provided tools for the establishment of the alveolus-on-a-chip model. All authors critically read and commented the manuscript.

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References

[1] Azoulay E et al 2019 Acute respiratory failure in immunocompromised adults Lancet Respiratory Med. 7 173–86
[2] Deinhardt-Emmer S et al 2018 Virulence patterns of Staphylococcus aureus strains from nasopharyngeal colonization J. Hosp. Infect. 100 309–15
[3] Lloyd C M and Marsland B J 2017 Lung homeostasis: influence of age, microbes, and the immune system Immunity 46 549–61
[4] Masopust D, Sivula C P and Jameson S C 2017 Of mice, dirty mice, and men: using mice to understand human immunology J. Immunol. 199 383–g
[5] Mestas J and Hughes C C W 2004 Of mice and not men: differences between mouse and human immunology J. Immunol. 172 2731–8
[6] Henneke P and Golenbock D T 2004 Phagocytosis, innate immunity, and host-pathogen specificity J. Exp. Med. 199 1–4
[7] Oliveira D, Borges A and Simões M 2018 Staphylococcus aureus toxins and their molecular activity in infectious diseases Toxins 10 252
[8] Wang L et al 2013 Human alveolar epithelial cells attenuate pulmonary microvascular endothelial cell permeability under septic conditions PLoS One 8 e55311
[9] Dohle E et al 2018 Human Co- and triple-culture model of the alveolar-capillary barrier on a basement membrane mimic Tissue Eng. C 24 495–503
[10] Schildberger A et al 2013 Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide Mediators Inflamm. 2013 697972
[11] Raasch M et al 2015 Microfluidically supported biopipet design for culture of endothelial cell layers with improved perfusion conditions Biofabrication 7 015013
[12] Rennert K et al 2016 Recruitment of CD16 (+) monocytes to endothelial cells in response to LPS-treatment and concomitant TNF release is regulated by CX3CR1 and interfered by soluble fractalkine Cytokine 83 41–52
[13] Warnking K et al 2015 Super-infection with Staphylococcus aureus inhibits influenza virus–induced type I IFN signalling through impaired STAT1–STAT2 dimerization Cell Microbiol. 17 305–17
[14] Mosit S et al 2009 Different functions of monocyte subsets in familial hypercholesterolemia: potential function of CD14 + CD16 + monocytes in detoxification of oxidized LDL FASEB J. 23 866–74
[15] Au-Lehmann J S et al 2017 Multiplex cytokine profiling of stimulated mouse splenocytes using a cytometric bead–based immunoassay platform JoVE 129 e56440
[16] Hubatsch I, Ragnarsson E G E and Artursson P 2007 Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers Nat. Protocols 2 2111–9
[17] Raasch M et al 2016 An integrative microfluidically supported in vitro model of an endothelial barrier combined with cortical spheroids simulates effects of neuroinflammation in neocortex development Biomicrofluidics 10 044102
[18] Ren H, Birch N P and Suresh V 2016 An optimised human cell culture model for alveolar epithelial transport PLoS One 11 e015225
[19] Cline T D, Beck D and Bianchini E 2017 Influenza virus replication in macrophages: balancing protection and pathogenesis J. Gen. Virol. 98 2401–12
[20] Huh D et al 2010 Reconstituting organ–level lung functions on a chip Science 328 1662–8
[21] Herzog E L et al 2008 Knowns and unknowns of the alveolus Proc. Am. Thorac. Soc. 5 778–82
[22] Wang J et al 2007 Differeniated human alveolar epithelial cells and reversibility of their phenotype in vitro Am. J. Respir. Cell Mol. Biol. 36 661–8
[23] Flodby P et al 2017 Cell-specific expression of aquaporin-5 (Aqp5) in alveolar epithelium is directed by GTA6/Spl via histone acetylation Sci. Rep. 7 3473
[24] Chuaquiúmia O D et al 2013 Alveolar epithelial cells are critical in protection of the respiratory tract by secretion of factors able to modulate the activity of pulmonary macrophages and directly control bacterial growth Infect. Immun. 81 381–9
[25] Glasser J R and Mallampalli R K 2012 Surfactant and its role in the pathobiology of pulmonary infection Microbes Infection 14 17–25
[26] Cheung D O, Halsey K and Speert D P 2000 Role of pulmonary alveolar macrophages in defense of the lung against Pseudomonas aeruginosa Infection Immun. 68 4585–92
[27] Moldoveanu B et al 2009 Immunological mechanisms in the lung J. Inflammation Res. 2 1–11
[28] Lavin Y et al 2014 Tissue–resident macrophage enhancer landscapes are shaped by the local microenvironment Cell 159 1312–26
[29] Gautier E L et al 2012 Gene–expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages Nat. Immunol. 13 1118–24
[30] Morales–Nebreda L et al 2015 The heterogeneity of lung macrophages in the susceptibility to disease Eur. Respiratory Rev. 24 505–9
[31] Roszer T 2018 Understanding the biology of self-renewing macrophages Cells 7 103
[32] Allard B, Panariti A and Martin J G 2018 Alveolar macrophages in the resolution of inflammation, tissue repair, and tolerance to infection Frontiers Immunol. 9 1777
[33] Tarbell J M 2010 Shear stress and the endothelial transport barrier Cardiovasc. Res. 87 320–30
[34] Philippe B et al 2003 Killing of aspergillus fumigatus by alveolar macrophages is mediated by reactive oxidant intermediates Infection Immunity 71 3034–42
[35] Rollides E, Katila H and Walsh T J 1998 Pulmonary host defences against aspergillus fumigatus Res. Immunol. 149 154–65
[36] Mircescu M M et al 2009 Essential role for neutrophils but not alveolar macrophages at early time points following Aspergillus fumigatus infection J. Infect. Dis. 200 647–56
[37] Pollmacher I et al 2016 Deciphering the counterplay of aspergillus fumigatus infection and host inflammation by evolutionary games on graphs Sci. Rep. 6 27807
[38] Brandes S et al 2017 Migration and interaction tracking for quantitative analysis of phagocyte–pathogen confrontation assays Med. Image Anal. 36 172–83
[39] Al-Zaben N et al 2019 Automated tracking of label–free cells with enhanced recognition of whole tracks Sci. Rep. 9 3317
[40] Brandes S et al 2015 Automated segmentation and tracking of non–rigid objects in time–lapse microscopy videos of polymorphonuclear neutrophils Med. Image Anal. 20 34–51
[41] Cseresnyes Z, Kraiboek K and Figge M T 2018 Hessian–based quantitative image analysis of host–pathogen confrontation assays Cytometry A 93 346–56
[42] Kraiboek K et al 2015 Automated quantification of the phagocytosis of aspergillus fumigatus conidia by a novel image analysis algorithm Frontiers Microbiol. 6 549
[43] Kraiboek K et al 2014 Virulent strain of Lichtheimia corymbifera shows increased phagocytosis by macrophages as revealed by automated microscopy image analysis Mycoses 57 56–66
[44] Mech F et al 2011 Automated image analysis of the host–pathogen interaction between phagocytes and Aspergillus fumigatus PLoS One 6 e19591
[45] Blickensdorf M, Timme S and Figge M T 2019 Comparative assessment of aspergillus by virtual infection modeling in murine and human lung Frontiers Immunol. 10 142
[46] Pollmacher J and Figge M T 2015 Deciphering chemokine properties by a hybrid agent–based model of Aspergillus fumigatus infection in human alveoli Frontiers Microbiol. 6 503
[47] Pollmacher J and Figge M T 2014 Agent–based model of human alveoli predicts chemotactic signaling by epithelial cells during early Aspergillus fumigatus infection PLoS One 9 e111630
[48] Dou D et al 2018 Influenza a virus cell entry, replication, virion assembly and movement Frontiers Immunol. 9 1581
[49] Huertas A et al 2018 Pulmonary vascular endothelium: the orchestra conductor in respiratory diseases: highlights from basic research to therapy Eur. Respir. J. 51 1700745
[50] Tromp A T et al 2018 Human CD45 is an F–component–specific receptor for the staphylococcal toxin Panton–Valentine leukocidin Nat. Microbiol. 3 708–17
[51] Parker D 2017 Humanized mouse models of staphylococcus aureus infection Frontiers Immunol. 8 812
[52] Loffler B et al 2010 Staphylococcus aureus panto–valentine leukocidin is a very potent cytotoxic factor for human neutrophils PLoS Pathog. 6 e1000715