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REVIEW

Human lung *ex vivo* infection models

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Abstract Pneumonia is counted among the leading causes of death worldwide. Viruses, bacteria and pathogen-related molecules interact with cells present in the human alveolus by numerous, yet poorly understood ways. Traditional cell culture models little reflect the cellular composition, matrix complexity and three-dimensional architecture of the human lung. Integrative animal models suffer from species differences, which are of particular importance for the investigation of zoonotic lung diseases. The use of cultured *ex vivo* infected human lung tissue may overcome some of these limitations and complement traditional models. The present review gives an overview of common bacterial lung infections, such as pneumococcal infection and of widely neglected pathogens modeled in *ex vivo* infected lung tissue. The role of *ex vivo* infected lung tissue for the investigation of emerging viral zoonosis including influenza A virus and Middle East respiratory syndrome coronavirus is discussed. Finally, further directions for the elaboration of such models are revealed. Overall, the introduced models represent meaningful and robust methods to investigate principles of pathogen-host interaction in original human lung tissue.

Keywords Pneumonia · Human lung · Bacteria · Virus · Immunity

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Introduction

Pneumonia is counted among the group of widespread diseases and lower respiratory tract infections belong to the five most common causes of death worldwide (Global Burden of Disease Study 2015). The high burden of disease consequently entails a great economic burden to the general public (Welte et al. 2012). Pneumonia is a severe inflammatory condition of the lung affecting primarily the peripheral alveolar compartment. Clinically, patients suffer from a productive or dry cough, chest pain, fever and compromised respiration. A simple pneumonia may progress to a life-threating condition with subsequent respiratory failure and systemic inflammation (Bauer et al. 2006). Usually, infection with viruses or bacteria and less commonly with other microorganisms causes pneumonia. Despite applicable vaccination strategies against pathogens commonly causing pneumonia, such as *Streptococcus pneumoniae* (Mehr and Wood 2012; Scott et al. 2012) and influenza A virus (IAV; Ortiz et al. 2016), being available, these pathogens still cause tremendous morbidity and mortality worldwide. Furthermore, antibiotic resistance is an emerging problem in infectious diseases *per se* (Brown and Wright 2016). Risk factors for pneumonia comprise further lung diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) and asthma and include other problematic conditions such as diabetes, heart failure, a history of smoking and alcohol abuse (Torres et al. 2015). In addition, children younger than 5 years of age (Walker et al. 2013) and people older than 65 years (Torres et al. 2013) are at a higher risk for developing pneumonia.

Why is the investigation of host-pathogen interaction, especially in human lung tissue, the key for developing new intervention strategies in pneumonia? Classical cell culture models are useful for the mechanistic and functional analysis of the one-to-one interaction between cell and microbe. In an

expanded version, a second implemented cell type could, for example, reflect the simple influence of additional host factors. However, these basic in vitro systems neither reflect tissue diversity at a cellular level, nor recapitulate the typical alveolar cell-cell interaction in the natural matrix (e.g., alveolar epithelial cell [AEC]-I interaction with AEC-II and the capillary endothelium) of the three-dimensional architecture. Therefore, if researchers would like to investigate the cellular tropism of a virus causing pneumonia in a realistic model, this is only possible if all cell types forming the potential habitat are present in the dish at once. Frequently passaged primary immortalized cells (such as bronchial epithelial BEAS-2B; Ke et al. 1988) or tumor-derived cells (such as alveolar A549 cells; Smith 1977), by using proteases such as trypsin for cell splitting, may lack central biological characteristics important for host-pathogen interactions. These include, for example, the capability to form tight junctions (Rothen-Rutishauser et al. 2008) or to express receptors involved in pathogen-binding or recognition. In addition, the use of wild-type encapsulated bacteria is limited in most cell cultures because of the cell toxic effects of the capsule (N'Guessan et al. 2005; Schmeck et al. 2004) motivating researchers to use unencapsulated laboratory-derived strains. Since, for the pathogenicity of, for example, the over 90 serotypes of *S. pneumoniae* (the major cause of pneumonia; Bauer et al. 2006; Bogaert et al. 2004; Drijkoningen and Rohde 2014; Jefferson et al. 2006), capsule-related effects are of enormous significance for virulence and tissue invasion (Geno et al. 2015), the use of original encapsulated patient-derived strains is important for the realistic modeling of pathogen-host interaction.

Animals such as mice represent integrative models and modern genetic animal manipulation allows the sophisticated analysis of host-pathogen interaction, including that of the lung (Baron et al. 2012; Hraiech et al. 2015; Thangavel and Bouvier 2014). Nevertheless, significant differences in anatomy, innate and adaptive immunity have long been known to exist between humans and, in particular, small rodents (Mestas and Hughes 2004). For many results obtained in such models, their translation to humans remains unclear (Mak et al. 2014). With respect to the investigation of infectious diseases in rodents, including pneumonia, a major problem is the host specificity of most pathogens (Bean et al. 2013; Bouvier 2015; Gretebeck and Subbarao 2015; Sutton and Subbarao 2015; Ware 2008; Wolfe et al. 2007). For example, the human nasopharynx seems to represent the natural reservoir for *S. pneumoniae* (Bogaert et al. 2004; Kadioglu et al. 2008) and only rare observations of this pathogen in wildlife species without contact to humans are documented (Chi et al. 2007). Some animal models, such as ferrets and guinea pigs, are naturally susceptible to infection by human influenza A strains (IAV); others, such as mice, require adaptation of the virus (Bouvier 2015). In particular, the majority of IAV research in mice employs either BALB/C or C57BL/6 strains in conjunction with the laboratory adapted A/Puerto Rico/8/1934 (H1N1) (PR8) or A/WSN/1933 (H1N1) (WSN). Such adaptation of pathogens to animal model species involves serial passaging to increase virulence, a procedure that inevitably alters pathogen behavior. Emerging viral lung diseases, such as the severe acute respiratory syndrome coronavirus (SARS-CoV; Peiris et al. 2004; Poon et al. 2004), Middle East respiratory syndrome coronavirus (MERS-CoV; Fehr et al. 2016; Mohd et al. 2016; Zumla et al. 2015) and new IAV strains infecting humans (e.g., H5N1, H1N1, H7N9; Lai et al. 2016; Novel Swine-Origin Influenza et al. 2009; Peiris et al. 2009; Zhu et al. 2016) represent classical zoonotic diseases, indicating that the species used for research is of great importance. Noteworthy, although, for example, IAV infects a broad variety of species, it does not infect rodents such as mice in nature. For some diseases, such as MERS-CoV, the available animal models do not represent the human clinical disease (Gretebeck and Subbarao 2015; Sutton and Subbarao 2015) and the lack of receptors for viral binding (Raj et al. 2013) has forced researchers to create complex transgenic humanized models (Gretebeck and Subbarao 2015; Sutton and Subbarao 2015).

Overall, in order to investigate the biology and to estimate the virulence potential of such pathogens, we need to complement in vitro cell culture studies and animal studies with suitable human-derived models. In the following, the use of *ex vivo* cultured and infected human lungs for the investigation of bacterial and viral infections will be discussed and further directions for model development will be revealed.

Ex vivo bacterial infection of human lung tissue

Streptococcus pneumoniae

Pneumococci frequently colonize the human nasopharynx (Bogaert et al. 2004; Scott et al. 2012), which seems to be indeed the primary natural habitat of this important human pathogen. *S. pneumoniae* infections of animals such as race horses, rhesus monkeys, or chimpanzees occur mostly in animals held in human captivity and are suspected to be attributable to human-animal transmission (Chi et al. 2007). In all studies searching for the causative agent of pneumonia, pneumococci are the most frequently isolated pathogen, in both out-patient and in-inpatient settings (including severe pneumonia treated at intensive care units; Drijkoningen and Rohde 2014). *S. pneumoniae* strains differ considerably in their capacity to cause a disease in humans in general and some strains may primarily cause invasive disease, whereas others predominately induce otitis media (Jefferson et al. 2006; Mehr and Wood 2012). Since pneumococci are a true human-specific pathogen of high clinical importance with a

high diversity of diseases caused by the various clinical isolates, we urgently need to study the biology of this pathogen in human tissue.

The *ex vivo* infection of human lungs with pneumococci induces the expression of immunomodulatory molecules such as tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), granulocyte-macrophage colony-stimulating factor, platelet-derived growth factor (PDGF), IL-6, IL-8, IL-10, IL-15, IL-17, or prostaglandin-E_2 (PGE_2; Fatykhova et al. 2015; Szymanski et al. 2012; Xu et al. 2008). Experiments performed with the clodronate-induced depletion of alveolar lung macrophages (AM) indicated that these cells might, in particular, contribute to the induction of IL-6 and TNFα (Xu et al. 2008). Fatykhova et al. (2015) involved the use of various clinical isolates to investigate their capacity to induce the potent pro-inflammatory cytokine IL-1β. These clinical strains differed in the expression of the cholesterol-dependent cytolysin pneumolysin (PLY), a major virulence factor of pneumococci (Kadioglu et al. 2008): Whereas serotypes 2, 3, 6B and 9N pneumococci express fully hemolytic PLY, serotype 1 and 8 strains express non-hemolytic PLY (Fatykhova et al. 2015). The authors demonstrated that only strains expressing lytic PLY induce IL-1β in a NLRP3-inflammasome-dependent manner. Experiments with purified allele 1 PLY (capable of causing pores) and allele 5 PLY (not causing pores) verified that pore formation is a pre-requisite for PLY-related IL1β induction in human lung tissue. In addition to the mechanistic information about IL-1β regulation in human lungs, this study highlighted the suitability of the model to analyze the biology of patient-derived bacterial isolates and showed the meaningfulness of the results obtained.

Cyclooxygenases (COX) produce fatty acid mediators, including prostaglandins such as PGE_2, which play an important role in the regulation of lung immunity (Claar et al. 2015; Zhou et al. 2016). In human lungs, pneumococci induce the strong up-regulation of the inducible form of COX, namely COX-2, in particular, in AM, AEC-II (but not AEC-I) and the vascular endothelium. Notably, same results have been seen in the tissue of patients suffering from pneumonia (Szymanski et al. 2012). Inhibition of p38 MAPK (mitogen-activated protein kinase) or ERK1/2 (extracellular signal-regulated kinase 1/2) blocked both the induction of COX-2 and the release of PGE_2. In addition to PGE_2, the authors showed the release of 6-keto $PGF_{1\alpha}$ and thromboxane B_2 into the infected tissue. Tissue expresses predominately the E prostanoid receptor 4 (EP4) and EP4 stimulation results in an increased cAMP production in lung tissue. Such PGE_2 production by lung cells may contribute to the control of inflammatory mediator production, such as that of IL-1β (Mortimer et al. 2016) in pneumococcal pneumonia.

Furthermore, Xu et al. (2008) noted the increased expression of Toll-like receptor 2 (TLR2) and TLR4 mRNA in pneumococci-infected tissue; however, neither the way that this translates into protein expression nor the effected cells are known. In accordance, the means by which the observed activation of signaling pathways such as MAPKs (Szymanski et al. 2012; Xu et al. 2008) is made cell-specific is unknown.

In addition to PLY, pneumococci liberate significant amounts of hydrogen peroxide and thus we can reasonably suggest that this causes oxidative stress to the lungs (Kadioglu et al. 2008). However, although pneumococci cause oxidative stress in human lungs, as evidenced by a decreased ratio of glutathione to glutathione disulfide in infected tissue (Zahlten et al. 2015b), this seems not to depend on oxygen radicals. Unexpectedly, a pneumococcal autolysin A (LytA)-dependent process turns out to induce oxidant stress. LytA is the major autolysin of pneumococci (Lopez and Garcia 2004) and causes the release of intrabacterial components such as PLY and bacterial DNA. Since the pneumococci-related expression of the immunomodulatory transcription factor Krueppel-like factor (KLF) 4 (McConnell and Yang 2010) in human lungs seems also to depend on LytA-related pneumococcal autolysis (Zahlten et al. 2015b) and as KLFs have an impact on lung cell activation in pneumococcal infection (Zahlten et al. 2010, 2013, 2015a, b), further investigation of LytA-related activation of lung tissue is highly recommended.

The above-mentioned studies indicate strong pro-inflammatory mediator release in pneumococci-infected lung tissue (Fatykhova et al. 2015; Szymanski et al. 2012; Xu et al. 2008) and massive inflammation, in particular during severe pneumonia, is suggested to foster the progression of the disease to acute respiratory failure, sepsis and multiorgan dysfunction (Bauer et al. 2006). Quinolones, such as moxifloxacin, have been hypothesized to exert anti-inflammatory (beneficial) effects, in addition to their well-established antimicrobial properties (Dalhoff and Shalit 2003). However, when investigating the effect of moxifloxacin on pneumococci- or TNF-α-stimulated IL-6 and IL-8 release in human lung tissue, Müller-Redetzky et al. (2015) found that only TNF-α-related IL-6 release was reduced by moxifloxacin. Accompanying investigations in a pneumococcal mouse pneumonia model showed similar results and, thus, did not support the hypothesis that moxifloxacin exhibits potent anti-inflammatory potency in pneumococcal pneumonia.

Bacillus anthracis

Although *Bacillus anthracis* does not cause pneumonia, the lung is the entry site for *B. anthracis* in inhalation anthrax, which is the most deadly form of the disease. It seems that inhaled spores escape from the alveolus into regional lymph nodes. Therein, spores may germinate and induce disease after having reached the circulatory system (Moayeri et al. 2015). Important virulence factors of *B. anthracis* include two toxins: both lethal toxin (LT) and edema toxin (ET) share the

protective antigen (PA) as a common receptor-binding component. PA allows the transport of the catalytic components LF and EF into the cytosol of target cells (Friebe et al. 2016; Moayeri et al. 2015). Chakrabarty et al. (2007) tested *B. anthracis* spore-related inflammatory tissue activation by utilizing spores prepared from the *B. anthracis* Sterne strain 7702(pX01$^+$, pX02$^-$). A significant increase in IL-6, TNFα, IL-8, monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 1 (MIP)-1 α/β was noted in the lung tissue supernatants; however, although a tremendous upregulation of IL-1β mRNA was documented, the authors noted no increase in IL-1β protein. Nevertheless, supernatants of spore-exposed lung tissue stimulated neutrophil and monocyte chemotaxis. *B. anthracis* spores caused strong activation of the MAPKs ERK, JNK and p38 and chemical inhibition of the kinases reduced chemo- and cytokine liberation. Finally, immunohistochemistry revealed the presence of IL-6 and IL-8 in epithelial cells and AM. Overall, the study (Chakrabarty et al. 2007) revealed that *B. anthracis* spores initiated a prominent pro-inflammatory response.

An important unresolved question is: how do *B. anthracis* spores escape from the alveolar airspace into the systemic circulation (Friebe et al. 2016; Moayeri et al. 2015)? In principle, transport might occur via host cells used as Trojan horses (AM, dendritic cells [DC], or a hitherto unidentified "carrier" cell) or spores may cross the alveolar epithelial wall without the help of migratory cells. Finally, a sequence of spore clustering, germination and production of *B. anthracis* virulence toxins has been proposed to cause epithelial damage that allows free spore passage (Friebe et al. 2016; Goossens and Tournier 2015; Moayeri et al. 2015), the so-called "jailbreak" model (Weiner and Glomski 2012). By using the above-mentioned model (Chakrabarty et al. 2007), the same group (Booth et al. 2016) aimed to identify the role of carrier cells and of *B. anthracis* toxins in this process. Around 5 % of spores were internalized in APC and around 13 % in AEC, whereas around 80 % of the spores were free after 2 h post-infection and these numbers did not change over time. Importantly, the clustering of spores occurred only in infected cells. The clear identification of the AEC cells that take up spores (AEC I, AEC II), by means of appropriate imaging techniques, would be of interest. Interestingly, the addition of PA or LT neither significantly influences spore uptake nor causes any cytotoxicity (as spore treatment alone also does not). Overall, the data suggest that *B. anthracis* spores migrate through the lung soon after exposure. The primary initial phase of spore movement from the alveolar space across the alveolar epithelial barrier may not essentially require a cellular carrier. However, the above study showed no translocation of spores into lung blood vessels (e.g., alveolar capillaries) and further investigations are required to demonstrate that the presence of spores in alveolar cells finally results in spore movement into the circulation.

Mycobacterium tuberculosis

Mycobacterial lung infections are still a major cause of morbidity and mortality worldwide. In 2012, around 9.0 million people developed tuberculosis (TB) and 1.5 million people died of this chronic infectious lung disease (Zumla et al. 2013). Although the innate immune system may clear early infection of *M. tuberculosis* in a significant number of cases (Khan et al. 2016; Morrison et al. 2008), little is known about the initial interaction of this important pathogen with human lung tissue. Ganbat et al. (2016) have now started to explore such interactions by establishing an *ex vivo* human lung infection model with various mycobacterial strains (*M. tuberculosis*, *M. abscessus*, *M. avium*). The authors note that AM, monocytes, neutrophils and AEC-II cells are infected by the mycobacterial strains. Interestingly, AEC-II seem to be infected in a significantly higher frequency by *M. tuberculosis* than by *M. abscessus* or *M. avium*. The presented results indicate the occurrence of cell death in all infected cell types but this also differs among strains. Although the study is limited by the relatively short time period post-infection (16 h post-infection), this model in principle will allow the detailed analysis of the initial mycobacterium-alveolar interaction, including the activation of inflammation-regulating mediators by, for example, clinical isolates. Thus, the model adds an important step forward for the investigation of these important events in early mycobacterial infection.

Nontypeable *Haemophilus influenzae*

Nontypeable *Haemophilus influenza* (NTHI) infections, in particular newly acquired strains, may trigger infection-related exacerbation in COPD and recurrent infections may induce overall disease progression (Duell et al. 2016). On using the NTHI strain Rd KW20 isolated from a COPD patient suffering from invasive pneumonia, Wagner et al. (2015) noticed strong IL-8 induction in *ex vivo* infected human lungs; this was reduced in tissue pre-treated with the anti-inflammatory drug budenoside. Interestingly, the steroid reduced not only the induction of IL-8; it furthermore reduced the presence of intracellular bacteria in the tissue by a hitherto unexplored mechanism. How the C-type lectin receptor Dectin-1, which has recently been found to be expressed apically on human bronchial and alveolar epithelium (Heyl et al. 2014), participates in the pro-inflammatory activation of human lung tissue, in addition to the "classic" innate immune receptors such as TLRs, needs to be determined. In a subsequent study, the group of Drömann (Dromann et al. 2010) used two clinical isolates: one isolated from a COPD patient with invasive disease and one from a patient without COPD and invasion. Both strains infected AM and lung epithelial cells; however, AEC subtypes were not identified in this

study. Notably, the authors observed a moderate induction of TGF-β but a strong upregulation of the TGF-β-pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) on both the alveolar wall epithelium and AM. This was accompanied by p38 MAPK-triggered induction of the pro-inflammatory mediators IL-8 and TNF-α. TGF-β is an important mediator of inflammatory and remodeling events in the lung and elevated levels of BAMBI protein in plasma were observed in COPD patients (Zhang et al. 2016). In these patients, increased BAMBI expression on human $CD4^+$ T cell membranes was noted and the enhanced plasma BAMBI levels in COPD positively correlated with increased plasma TGF-β1 levels and the Th17/Treg ratio indicating that an impaired TGF-β/BAMBI pathway can promote inflammation in COPD (Zhang et al. 2016). Furthermore, studies in the lungs of idiopathic pulmonary fibrosis patients and the mouse model of bleomycin-related lung fibrosis (Murphy et al. 2016) have demonstrated increased levels of BAMBI (and related BMP accessory proteins noggin and FSTL1) in the lungs suggesting a role of these molecules in inflammatory and fibrotic lung injury. Since other studies have suggested a role of TGF-β and related molecules in acute lung infection, such as IAV-related pneumonia (Furuya et al. 2015; Woods et al. 2015) and IAV-bacterial coinfection (Li et al. 2015), a more detailed analysis of the function of these molecules in human lungs is highly desirable.

Legionella pneumophila

Legionella pneumophila (*L. pneumophila*) is the causative agent of Legionnaires' disease. The inhalation of contaminated water droplets causes disease outbreaks of public health significance (Phin et al. 2014). The bacterium interacts with the human host by the release of extracellular proinflammatory outer membrane vesicles (OMV) and uses the Dot/Icm type IVB translocation system to inject over 300 effector proteins into the infected cell (Ensminger 2016). It creates a replicative niche by avoiding fusion of phagosomes with the lysosome, interacts with endoplasmic reticulum–Golgi traffic (Prashar and Terebiznik 2015) and induces the massive pro-inflammatory activation of human lung epithelium in vitro (Schmeck et al. 2007, 2008). Jäger et al. (2014) started their investigation of *Legionella* infection in human lung tissue by using wild-type strain Corby and a DotA-negative mutant (defect for the Dot/Icm type IVB translocation system). Furthermore, they explored the role of OMV, suspected of inducing massive lung cell activation (Galka et al. 2008). They showed that extracellular adhesion to the alveolar epithelial barrier took place before *Legionella* entered macrophages. Whereas wild-type bacteria multiplied more than 10-fold within 48 h, DotA-negative bacteria could not replicate within the tissue. Of note, infection caused damage to both the infected AM and the alveolar wall. Bacteria deficient for DotA induced less damage than wild-type bacteria highlighting the importance of this virulence system. The AM surface and cytoplasm were decorated by OMV suggesting that these cells are particularly targeted by pro-inflammatory *Legionella* vesicles. OMV induced damage to the lung comparable with that caused by wild-type bacteria, shedding light on the possible important role of OMV in *Legionella* pathogenesis. By using transcriptome analysis, the authors showed the differentiated expression of more than 2400 genes in *Legionella*-infected human lung tissue, including genes related to extracellular proteins, components of the immune response and lipoprotein transport proteins. The pathophysiological role of, for example, the observed downregulation of the protein content of immunoregulatory uteroglobin (a member of the secretoglobin superfamily; Mukherjee et al. 2007) and the downregulation of MARCO, a class A scavenger receptor, which seems to be involved in, for example, the pathogenesis of the pneumococcus (Dorrington et al. 2013), remains unknown. In addition, the further characterization of the way that vacuole formation (Naujoks et al. 2016) and the activation of innate immune receptors (Cunha and Zamboni 2014) take place in original human lung tissue would be of great interest.

Coxiella burnetii

The obligate intracellular pathogen *Coxiella burnetii* causes Q fever, a disease starting with flu-like symptoms, which, in cases of prolonged infections, may proceed to severe endocarditis. Similar to Legionnaires' disease, the public health system noticed Q fever mostly in the form of localized outbreaks resulting from the inhalation of contaminated aerosols from farm and domestic animals (in particular sheep and goats; Cilloniz et al. 2016). *Coxiella* replicates within an acidic lysosome-like parasitophorous vacuole (PV), mostly in macrophages and uses a Dot/Icm type IV secretion-system-based molecule delivery into host cells to hijack host cell signaling cascades, thereby creating its replicate niche (Moffatt et al. 2015). Graham et al. (2016) compared the behavior of avirulent *C. burnetii* NMII (RSA439, clone 4, a frequently used laboratory strain) with virulent *C. burnetii* in human precision-cut lung slices. Although single bacteria were detected in the alveolar epithelium, bacteria replicated sufficiently only in AM. Of note, only live avirulent bacteria induced the liberation of IL-1β and IL-18, whereas virulent bacteria did not. Subsequently, on using human AM, the authors found that a human-specific noncanonical inflammasome dependent on caspase-4/-5 might induce IL-1β release without the induction of pyroptosis. Since caspase-4 and caspase-5 are human-specific proteins, caspase-5 is undetectable in the THP-1 human macrophage-like cell line model and mouse macrophages do not respond with IL-1β liberation to *C. burnettii* infection (Cunha et al. 2015); this discovery was essentially based on

the use of original human tissue. Notably, since numerous studies involving the use of avirulent and virulent *C. burnettii* indicated widely identical intracellular behavior of the bacteria (Moffatt et al. 2015), further investigations in primary human tissue (and cells) are needed in order to discover the way that virulent *C. burnetii* suppress IL-1β production, thereby inhibiting one of the most powerful pro-inflammatory mediators in the host.

Ex vivo viral infection of human lung tissue

Influenza virus

Because of the enormous clinical importance and continuous emergence of new influenza A strains (IAV; Lai et al. 2016; Trombetta et al. 2015), various workers have investigated IAV infection of human lung tissue. Research has focused, in most reports, on viral replication, tissue tropism and tissue activation in the sense of cytokine and chemokine liberation. Knowledge of the level of efficiency of an emerging IAV replicate in human lung tissue is of enormous importance to estimate its potential to spread in the community and to gauge the potential of the virus to cause harm to the lungs. For example, the avian IAV H5N1 has caused typically severe human infections with a high fatality rate over many years (Lai et al. 2016). By using human lung tissue *ex vivo* infected with IAV H5N1, several groups have demonstrated strong viral replication (R.W. Chan et al. 2009; Hocke et al. 2013a; Nicholls et al. 2007; Weinheimer et al. 2012) comparable with or even higher than that observed with classic pandemic strains such as IAV H3N2 (Weinheimer et al. 2012). In 2009, the first pandemic of the 21st century started with a novel swine-originated IAV H1N1 virus (Novel Swine-Origin Influenza et al. 2009). In most cases, this virus caused a disease of moderate severity as observed in most seasonal influenza (Peiris et al. 2009). In accordance, compared with highly virulent IAV strains such as H5N1 or classic pandemic strains such as IAV H3N2, this virus showed intermediate replication in *ex vivo* infected human lung tissue (M.C. Chan et al. 2010; Weinheimer et al. 2012; Wu et al. 2010b, 2012; Zhang et al. 2010) thus reflecting the moderate disease caused in humans. A new IAV H7N9 virus resulting from sequential reassortments in ducks and chickens has been shown to infect human beings since 2013. Originated from eastern China, the virus has caused repeated waves of outbreaks and has thus raised concerns of a pandemic threat (Zhou et al. 2016). Two reports have revealed that IAV H7N9 efficiently replicates in human lung tissue (M.C. Chan et al. 2013; Knepper et al. 2013). Noticeably, whereas virus isolated from a fatal human infection (A/Anhui/1/2013 (H7N9)) replicated comparably with seasonal IAV, classic avian H7 subtype viruses propagated poorly indicating that this new H7N9 virus is well adapted to replicate in the human host (Knepper et al. 2013). In agreement, several studies have demonstrated that IAV strains not adapted to humans (such as classic swine or avian strains) do not propagate efficiently in human material (M.C. Chan et al. 2010, 2013; Knepper et al. 2013; Weinheimer et al. 2012; Wu et al. 2010b; Zhang et al. 2010). Overall, the *ex vivo* models used in these studies seem to robustly reflect the capacity to infect and propagate in humans.

Infection of a host cell with IAV results in numerous alterations in cell function, ranging from the release of immune-regulating mediators to changes in sodium pump activity and finally the killing of IAV-infected cells (Herold et al. 2012; Short et al. 2014). Thus, the identification of the primary target cells of IAV in the human lung is of primary importance if we are to estimate the possible effects of cell damage for organ function and to investigate possible cell-based interventions. Irrespective of the virus strain used in the *ex vivo* infection models, most reports identify AEC II cells as the primary replicative niche in the peripheral human lung (Fig. 1; M.C. Chan et al. 2010, 2013; Hocke et al. 2013b; Knepper et al. 2013; Weinheimer et al. 2012; Zhang et al. 2010). Patients with lung fibrosis typically show AEC II hyperplasia

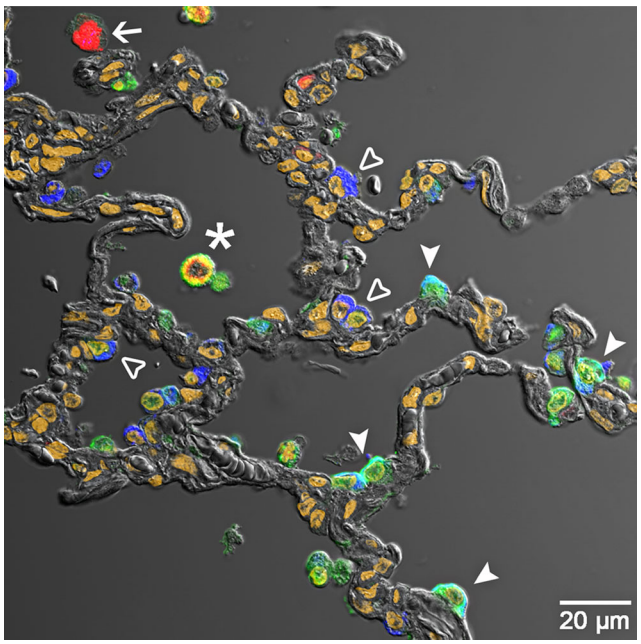

Fig. 1 Influenza A virus (IAV) targets AEC-II in *ex vivo* infected human lung tissue. Cross-sections from infected lung explants were stained for IAV antigen (*green*), for prosurfactant protein C (*blue*) to detect alveolar epithelial cell II (AEC-II, T II; *white arrowheads* infected cells [*cyan*], *open arrowheads* uninfected cells [*blue*]) and for CD68 (*red*) to detect alveolar lung macrophages (*asterisk* infected cell [*yellow*]). Nuclear staining with 4,6-diamidino-2-phenylindole is shown in *dark orange* and lung structure is visualized with differential interference contrast. The stains were visualized by confocal microscopy and tissue autofluorescence was separated from specific fluorescence by spectral unmixing. From Weinheimer et al. (2012). Reproduction by permission of Oxford University Press

(Fernandez and Eickelberg 2012). Hocke et al. (2013b) demonstrated massive infection of AEC II cells of fibrotic lungs and Fujino et al. (2013) noticed increased IAV replication in AEC II cells derived from patients with pulmonary fibrosis thus indicating that the AEC II cell is the primary target cell of IAV in the human lung. In contrast, the presence of viral antigen is detected much less frequently in human alveolar macrophages (M.C. Chan et al. 2010, 2013; Weinheimer et al. 2012) but nevertheless these cells are of importance for the immune response to IAV (Halstead and Chroneos 2015). Although (1) IAV H1N1 particles have been identified in endothelial cells of fatal cases (Ru et al. 2011), (2) in vitro experiments indicate IAV propagation in endothelial cells (Wang et al. 2015) and (3) the endothelium might significantly contribute to the course of the disease (Teijaro et al. 2011), IAV antigen has not been detected in the *ex vivo* models discussed here.

The tissue responded to IAV infection with the release of multiple immunomodulatory mediators including IL-1β, IL-6, IL-8, MCP-1, MIP-1α/β, interferon-gamma inducible protein 10 kDa (IP-10) and interferon-beta (IFN-β) in a somewhat strain-specific manner (Knepper et al. 2013; Weinheimer et al. 2012; Wu et al. 2010b, 2012). However, a subsequent strain-related analysis of mediator expression is still lacking and limited information is available about the mediator origin (Wu et al. 2010b). Experiments with chemical inhibitors have suggested an important role of MAPKs for the regulation of inflammatory mediator expression (Wu et al. 2010b) and IAV seem to interfere with the expression of the pattern-recognition receptor retinoic acid-inducible gene I (RIG-I; Wu et al. 2012). Patients with COPD are prone to IAV infections, and IAV vaccination is highly warranted in those suffering from COPD (Sehatzadeh 2012). By using human lung explants exposed to cigarette smoke extract before IAV infection Wu et al. (2011) demonstrated altered IFN and IP-10 expression and IAV-mediated RIG-I upregulation, suggesting that smoking impairs the host response to IAV.

That these models might be useful for the testing of new therapeutic approaches is indicated by a report of Chan et al. (R.W. 2009). The binding to sialic acids (alpha2-6-linked and alpha2-3-linked depending on the viral strain) on the host cell membrane is an integral step of the IAV infection process. R.W. Chan et al. (2009) demonstrated that DAS181 induces de-sialylation of both sialic acids in *ex vivo* human lung tissue and that two doses of DAS181 treatment given 12 h post-infection are sufficient to block H5N1 infection in *ex vivo* lung tissue culture.

Adenovirus 7

The human adenovirus 7 belongs to the Adenoviridae subgroup B (HAdV-B7), which causes pneumonia and systemic disease in both immuno-compromised and non-immuno-compromised hosts, mainly in regional outbreaks (Ng et al. 2015; Scott et al. 2016). In addition to being human pathogens, recombinant adenoviruses attracted the interest of researchers, several years ago, as promising tools for gene therapy but the lack of suitable animal (Ginsberg et al. 1990) and cell culture (Jogler et al. 2006) models supporting human HAdV replication hampered research progress. Booth et al. (2004) thus used a human *ex vivo* infection model with human HAdV-B7 and showed efficient viral replication. HAdV-B7 provoked activation of ERK kinases and ERK inhibition blocked the release of IL-8. In a subsequent study (Wu et al. 2010a), the same group showed the liberation of IL-6, IL-8, IP-10, MIP-1α/β and MCP-1 in infected tissue. Multicolor immunostaining documented the infection of AEC-I and AEC-II cells. The authors identified lung AEC as the primary source for IL-8, whereas IP-10 was found in AM and epithelial cells. Overall, this model now gives a solid basis for further assessment of HAdV viral replication and pathology.

Coronaviruses

Infections with coronaviruses (CoVs) in humans primarily target the upper respiratory tract and, in most cases, induce a rather mild, self-limiting disease, such as the common cold (Su et al. 2016). However, SARS-CoV and MERS-CoV differ from the other CoV. In 2002/2003, SARS-CoV caused a global outbreak of a severe respiratory disease killing over 700 people and illustrating the potential worldwide impact of a new interspecies transmission of a highly pathogenic zoonotic virus (Peiris et al. 2004). An important initial step for the understanding of such emerging viral infections of the lung is the identification of host cell receptors and primary target cells. By using human SARS-CoV-infected lung tissue slices, the function of angiotensin-converting enzyme 2 as a human receptor for SARS-CoV could be substantiated (V.S. Chan et al. 2006). Furthermore, results indicate that a subpopulation of lung cells expressing stem/progenitor cell markers CD34 and Oct-4 (while being negative for cytokeratin or surfactant) may be important target cells of SARS-CoV in human lungs (Chen et al. 2007). However, the way in which SARS-CoV interacts with the human lung alveolus is far away from being understood and research efforts to elucidate its pathobiology have been paralyzed with the temporal distance of the outbreak.

In 2012, MERS-CoV (originally named human coronavirus-EMC) emerged as a new CoV causing an acute respiratory syndrome in humans (Fehr et al. 2016; Mohd et al. 2016; Zumla et al. 2015). Probably originating in bats, the MERS-CoV infection is endemic in dromedary camel populations of East Africa and the Middle East (Mohd et al. 2016). Most human cases are based on dromedary camel to human transmission, although, under some circumstances, significant

human-to-human transmission also occur (Cho et al. 2016; Drosten et al. 2014, 2015). MERS-CoV uses, as a receptor, the human Dipeptidyl peptidase 4 (DPP4; Raj et al. 2013), which is not present in the most frequently used animal models (Gretebeck and Subbarao 2015; Sutton and Subbarao 2015), thus impeding its analysis in complex lung tissue.

Hocke et al. (2013a) and R.W. Chan et al. (2014) used *ex vivo* infection of human lungs to assess viral replication and cellular tropism of MERS-CoV. Both groups infected the tissue with the original human MERS-CoV Erasmus Medical Center strain and the group of R.W. Chan (2014) expanded the observations to strains isolated from dromedaries. Both groups showed strong viral replication in lung tissue. Bronchial epithelial cells, AEC-I, AEC-II cells and endothelial cells were shown to be infected (R.W. Chan et al. 2014; Hocke et al. 2013a). Indeed, electron microscopy showed the presence of MERS-CoV in the AEC-I and AEC-II cells (Hocke et al. 2013a). The presence of virus particles in the basal lamina below intact AEC suggested the basolateral release of MERS-CoV (Hocke et al. 2013a). Notably, both reports showed no evidence for the infection of AM. DPP4 was present in all cell types infected (Hocke et al. 2013a) and this broad receptor expression might be one of the major factors for the observed widespread cellular tropism of MERS-CoV. To gain insight into the mechanistic cause of lung failure in MERS-CoV, Hocke et al. (2013a) assessed the cell death of epithelial cells and the integrity of the alveolar tight junction protein occludin. Detachment of apoptotic MERS-CoV–infected AEC-II from the alveolar base membrane with disruption of alveolar tight junctions (indicated by the disintegration of the occludin protein band) indicated structural lung damage caused by MERS-CoV (Fig. 2). Notably, the first (and only) published autopsy performed on a fatal case of MERS-CoV essentially confirmed the results of the *ex vivo* models with respect to viral tissue tropism and damage (Ng et al. 2016). Because of the persistent reintroduction of the virus into the human population and the lack of specific therapies, an ongoing need exists to investigate MERS-CoV interaction with the human host.

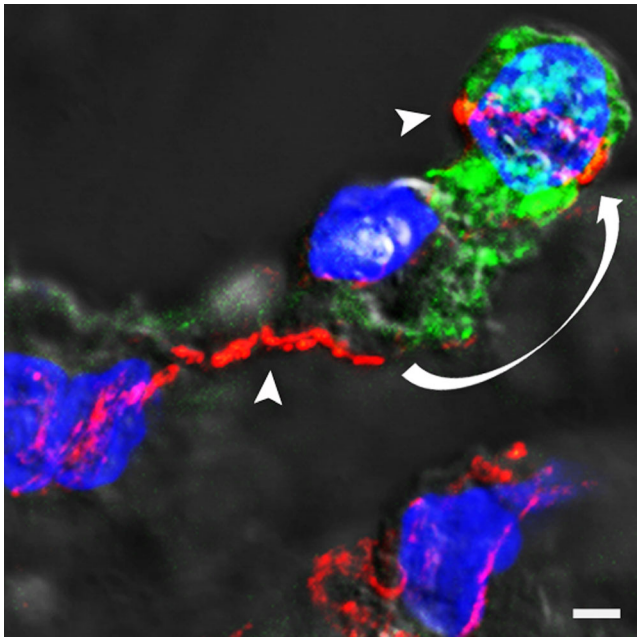

Fig. 2 MERS coronavirus (MERS-CoV) causes structural damage in *ex vivo* infected human lungs. Detachment of MERS-CoV-infected cell (*green*) from the alveolar epithelial layer disrupts epithelial continuity. An annular formation of tight junction protein occludin (*red*, *white arrowheads*) still surrounds the detached cell and is dissolved from the alveolar junctional band (*white arrow*). Stain visualization by confocal microscopy and separation of tissue autofluorescence from specific fluorescence by spectral unmixing. From Hocke et al. (2013a). Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society

Summary and outlook

The presented studies indicate that *ex vivo* infected human lung tissue is useful for the investigation of basic principles of pathogen-host interaction in the lung. A great advantage of this model is the possibility of using wild-type patient isolates of bacteria and viruses for the investigation of general pathogenicity and risk assessment of emergent pathogens (e.g., new zoonotic viruses) in original three-dimensional tissue. Many studies focus on pathogen replication, cellular tropism and tissue activation in the sense of the release of immunomodulatory factors. Despite this obvious value, many areas of human tissue culture *per se* can be improved and, in particular, many further directions can be taken in the investigation of infectious lung diseases.

One important issue is that we need to know, in more detail and on several levels, "what is in the box". Most studies involve the use of peripheral (alveolar) lung material obtained from surgery because of lung cancer; some groups have investigated lung tissue not used for transplantation. Although tumor-free tissue is used, we cannot rule out that the very presence of the tumor in the lung has altered tissue responses or that, for example, mechanical ventilation before explantation has affected sample responses. Donors of lung tissue might have a long history of smoking and might suffer from additional diseases. However, if sample numbers in the laboratories increase, a detailed stratification of patient history and an analysis of tissue responses may help us to understand the way that smoking, COPD, asthma, or diabetes mellitus and chronic heart disease lead to increased susceptibility to pneumonia (Torres et al. 2015). Lung tissue contains a huge variety of cellular components and even nowadays, new cell types are being identified (Franks et al. 2008). We need to use robust technologies such as advanced cell sorting (Fujino et al. 2012; Gross et al. 2015) and microscopy to identify cells present in

our samples and to investigate any changes in these populations over culture time and during lung-pathogen interaction. This step is a prerequisite for the in-depth analysis of the role of those hitherto neglected cells for the responses to lung infection. For example, Piet et al. (2011) used human lung tissue-derived T cells to investigate the specificity and function of $CD103^{+}CD8^{+}$ T cells. Their study indicated that the human lung might harbor local virus-specific epithelial $CD8^{+}$ T cells that might protect the lung against recurring IAV infection (Piet et al. 2011). Techniques such as laser-assisted cell picking for the extraction of single cells out of infected lung tissue (Fink et al. 1998) combined with recently developed assays for single-cell transcriptome analysis (Bell and Eberwine 2015; Grun et al. 2015; Hodne and Weltzien 2015; Macaulay et al. 2016) might help to expand our knowledge of cell-specific responses in the human lung. Indeed, the combination of such cutting-edge technologies with complex human samples is a challenge and needs strong interdisciplinary collaboration. Microscopy-based documentation of live-dead staining of cells and subsequent three-dimensional reconstruction will help us to gain insight into tissue viability over time and into the specific effects of pathogens and their virulence factors (e.g., PLY release of pneumocooci).

The preservation of the complex three-dimensional structure of the lung is one of the great advantages of these models and high-end microscopy is a key to obtaining information about pathogen-host interaction in this complex organ architecture. Unfortunately, human lung tissue contains very strong autofluorescent structures (e.g., collagen, elastin) causing significant overlap with fluorophore emission spectra. Spectral confocal microscopy can be used to achieve higher signal-to-noise ratios as demonstrated in some studies (Hocke et al. 2013a, b; Szymanski et al. 2012; Weinheimer et al. 2012). On the other hand, autofluorescence itself may be useful for the visualization of tissue morphology and cellular dynamics in human lung tissue by performing autofluorescence multiphoton microscopy (Kretschmer et al. 2016). No technical reason exists as to why the real-time imaging of pulmonary reactions combined with micropuncture techniques, which have previously been successfully used in animal experiments (Islam et al. 2012; Kreisel et al. 2010; Looney and Bhattacharya 2014; Westphalen et al. 2014) should not also be used for the dissection of intra-alveolar host-pathogen interactions in *ex vivo* human lung models.

A next step is to advance from phenomenological studies to mechanistic investigations in order to improve the analytical potency of the model. Beyond the use of chemical inhibitors or the testing of innovative anti-infective drugs, viral transformation (McBride et al. 2000) allowing cell-specific functional analysis is a possible strategy. This can be combined with gene editing approaches such as the CRISPR-Cas9 system (Chen and Goncalves 2016; Wang and Qi 2016), thereby permitting mechanistic studies.

In general, we need to be able to establish longer durations of tissue cultivation. For example, viral transformation and subsequent gene editing, together with studies addressing tissue injury and repair, would profit from expanded observation times. The rapid emergence of sophisticated microfluidic systems (Esch et al. 2015) better mimicking organ supply and possibly including physical forces might lead to longer periods of lung tissue cultivation. Finally, studies are hampered by limited tissue availability, a limitation that could, at least in part, be overcome by the rigorous improvement of human lung-specific cryopreservation methods (Baatz et al. 2014; Bai et al. 2016; Rosner et al. 2014).

Beyond the analytical power of these models, we wish to stress that the use of human lung tissue reduces the burden of animal experiments by contributing to the 3-R principle to replace, reduce, or refine animal experiments (Russell 1995).

Overall, *ex vivo* infection models of human lung tissue are today of great value for the investigation of pneumonia-related host-pathogen interaction. The combination of these models with the now available cutting-edge technologies will booster the mechanistic understanding of pneumonia in humans.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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