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Endothelial–platelet interactions in influenza-induced pneumonia: A potential therapeutic target

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Abstract

Every year, influenza viruses spread around the world, infecting the respiratory systems of countless humans and animals, causing illness and even death. Severe influenza infection is associated with pulmonary epithelial damage and endothelial dysfunction leading to acute lung injury (ALI). There is evidence that an aggressive cytokine storm and cell damage in lung capillaries as well as endothelial/platelet interactions contribute to vascular leakage, pro-thrombotic milieu and infiltration of immune effector cells. To date, treatments for ALI caused by influenza are limited to antiviral drugs, active ventilation or further symptomatic treatments. In this review, we summarize the mechanisms of influenza-mediated pathogenesis, permissive animal models and histopathological changes of lung tissue in both mice and men and compare it with histological and electron microscopic data from our own group. We highlight the molecular and cellular interactions between pulmonary endothelium and platelets in homeostasis and influenza-induced pathogenesis. Finally, we discuss novel therapeutic targets on platelets/endothelial interaction to reduce or resolve ALI.

KEYWORDS

endothelial cell, influenza, interaction, laboratory animals, lung injury, platelet, pneumonia, therapy

1 | THE IMPACT OF INFLUENZA ON HUMAN HEALTH

Influenza, commonly known as 'the flu', is a viral infection in humans and animals caused by the influenza virus. It occurs in seasonal outbreaks (epidemic influenza) caused largely by influenza A and B viruses, in sporadic pandemics caused mainly by influenza A viruses, or by zoonotic swine or avian influenza virus subtypes crossing the species barrier to humans. In Europe, every year around 4–50 million symptomatic seasonal influenza virus cases are reported, resulting in 15,000–70,000 deaths (European Centre for Disease Prevention & Control, 2019). The natural reservoirs for most influenza A viruses are waterfowl and shore birds, where infection is generally asymptomatic. Nevertheless, influenza A virus can spread from these reservoirs to susceptible birds and mammals, including humans, where it continuously evolves through antigenic drift (selective mutations in the viral genome as a result of pressure from the host immune system) and antigenic shift (re-assortment of genome segments between two different influenza subtypes co-infecting one host cell), resulting in the emergence of a novel influenza strain. Consequently, it is challenging to predict the dominant influenza strains that will circulate in any given year.

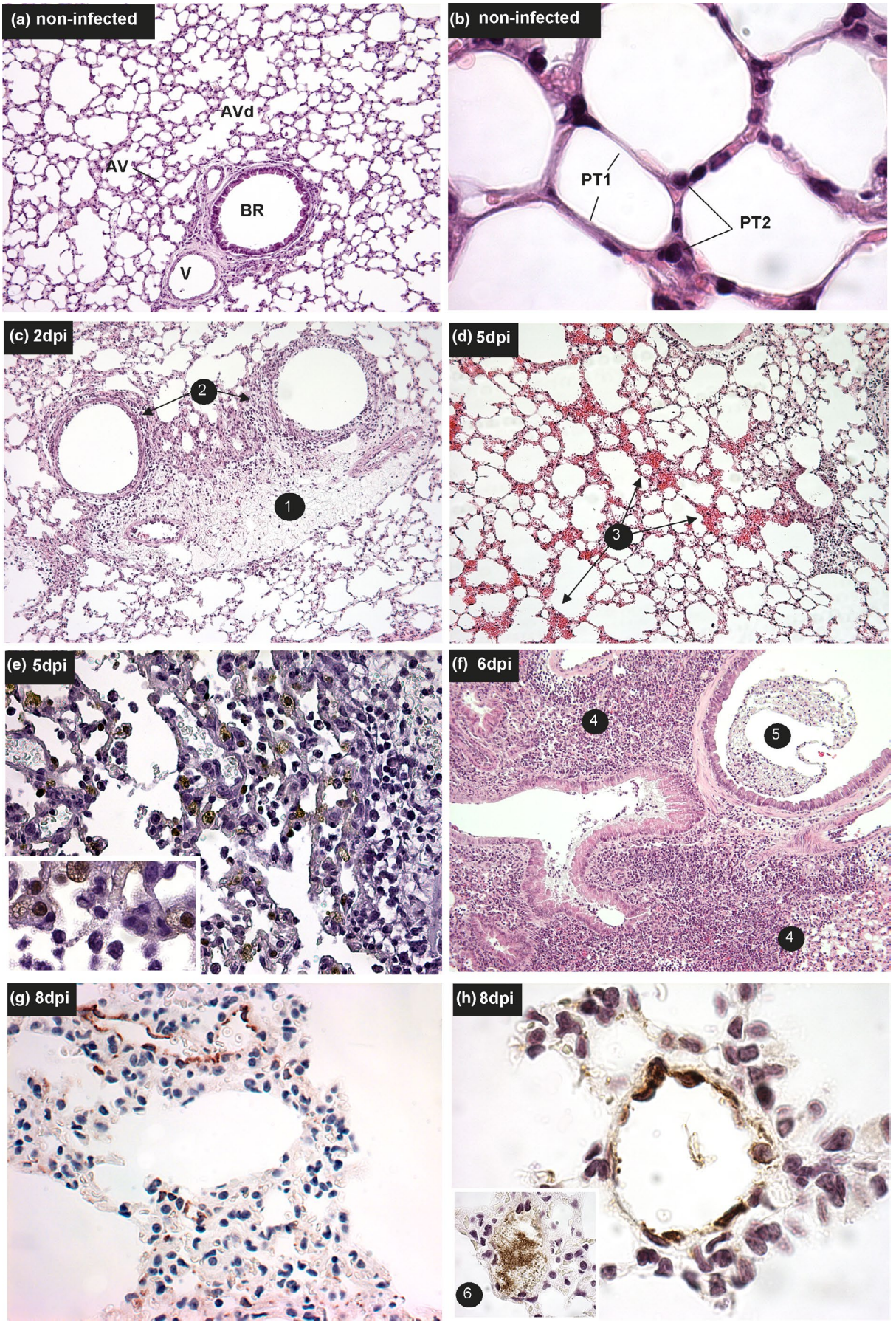

(a) non-infected
AVd
AV
BR
V
(b) non-infected
PT1
PT2
(c) 2dpi
2
1
(d) 5dpi
3
(e) 5dpi
(f) 6dpi
4
5
4
(g) 8dpi
(h) 8dpi
6

FIGURE 1 Histopathology of lung alveolar tissue from influenza A PR8 H1N1-infected mice at different days post infection (dpi). C57Bl6 mice were infected with IAV PR8 H1N1 (10,000 tissue culture infectious dose 50) intranasally. Formalin-fixed paraffin-embedded sections of infected and non-infected lung tissue. (a) Overview of the lung architecture with bronchiole (BR), adjacent pulmonary vessel (V), alveolar ducts (AVd) and alveoli (AV) (hematoxylin/eosin (HE) staining, ×100 magnification). (b) Thin lining of pneumocyte type I (PT1) and interspaced pneumocyte type II (PT2) surround the alveoli; the presence of erythrocytes indicates the capillaries. (HE staining ×1,000). (c) Lung alveolar tissue presenting (1) perivascular and alveolar oedema and (2) peri-bronchial infiltration of leucocytes (HE staining, ×100). (d) Several bleedings (3) into the alveolar lumen (HE staining, ×100) (e) Immunohistochemistry (IHC) detects influenza A-positive PT2 (brown colored deposits). 1° antibody goat polyclonal anti-influenza A H1N1 1:50 (OBT1551, AbDSerotec/Bio-Rad) and 2° antibody donkey polyclonal anti-goat IgG H&L horseradish peroxidase (HRP) 1:1,000 (Abcam Cambridge GB), substrate 3,3′-diaminobenzidin (*DAB*) developed brown coloured deposits, the tissues were counterstained with haematoxylin (×400 and insert ×1,000). (f) Massive infiltration (4) of leukocytes into the alveolar lumen. The typical alveolar structure is nearly invisible. (5) Exudate of dead cells in the bronchioles (HE staining, ×200). (g and h) IHC staining against von Willebrand factor (VWF) which detects the lining of intact pulmonary endothelium in capillaries. 1° rabbit polyclonal anti-vWF 1:250, (Agilent Dako Waldbronn DE) and 2° antibody donkey polyclonal anti-rabbit IgG HRP 1:1,000 (GE Healthcare Amersham GB), DAB as substrate, the tissues were counterstained with hematoxylin (G ×400 and insert H ×1,000). (6) Small brown DAB deposits in small pulmonary vessels indicate platelet accumulation (×400). Pictures were taken with Axiophot/Axiocam (Zeiss Jena DE) and DM RBE (Leica Wetzlar DE) with Infinity2 camera (Teledyne Lumenera Ottawa CA)

2 | INFLUENZA VIRUS STRUCTURE

Influenza viruses are enveloped negative-stranded RNA viruses with a segmented genome consisting of seven to eight RNA segments. The six genera of the family *Orthomyxoviridae* include *Influenzavirus A* (IAV), *Influenzavirus B* (IBV), *Influenzavirus C* (ICV), *Influenzavirus D* (IDV), *Thogotovirus* and *Isavirus*. Although the four influenza virus genera share similar structures and particle composition, their host ranges and pathogenicity differ substantially. While IAV infects many species, including humans, swine, birds and horses, the host ranges of IBV and ICV are more restricted and they have only been isolated from human, dog and swine (Wright, Neumann, & Kawaoka, 2007). Recently, new IDVs have been isolated from clinically ill pigs and cattle (Su, Fu, Li, Kerlin, & Veit, 2017).

Influenzavirus A's occur either as spherical particles or as filaments that are enveloped by a lipid membrane derived from the infected host cell (Badham & Rossman, 2016). The eight RNA genome segments of negative polarity encode at least twelve proteins, with some produced from alternate open reading frames or via mRNA splicing. The envelope contains the two surface glycoproteins, the haemagglutinin (HA), which carries receptor-binding and membrane fusion function, and the neuraminidase (NA), which facilitates viral release, as well as the ion channel protein matrix protein 2 (M2), which is responsible for uncoating and viral genome release into the host cell cytoplasm. The matrix 1 (M1) protein is associated with the inner side of the lipid bilayer, and it interacts with the surface glycoproteins as well as the viral ribonucleoproteins (vRNPs).

In electron microscopic images of IAV, one can recognize the HA and NA proteins as spikes embedded in the spherical virion (Figure 2c,d, inserts). IAVs are classified into different subtypes based on antigenic characterization of the HA and NA proteins. To date, 16 HA (H1–H16) and nine NA (N1–N9) variants have been characterized (Taubenberger & Kash, 2010). Virus structure and replication has been reviewed in depth by experts in the field (Dou, Revol, Östbye, Wang, & Daniels, 2018; Krammer et al., 2018) and is not further discussed here.

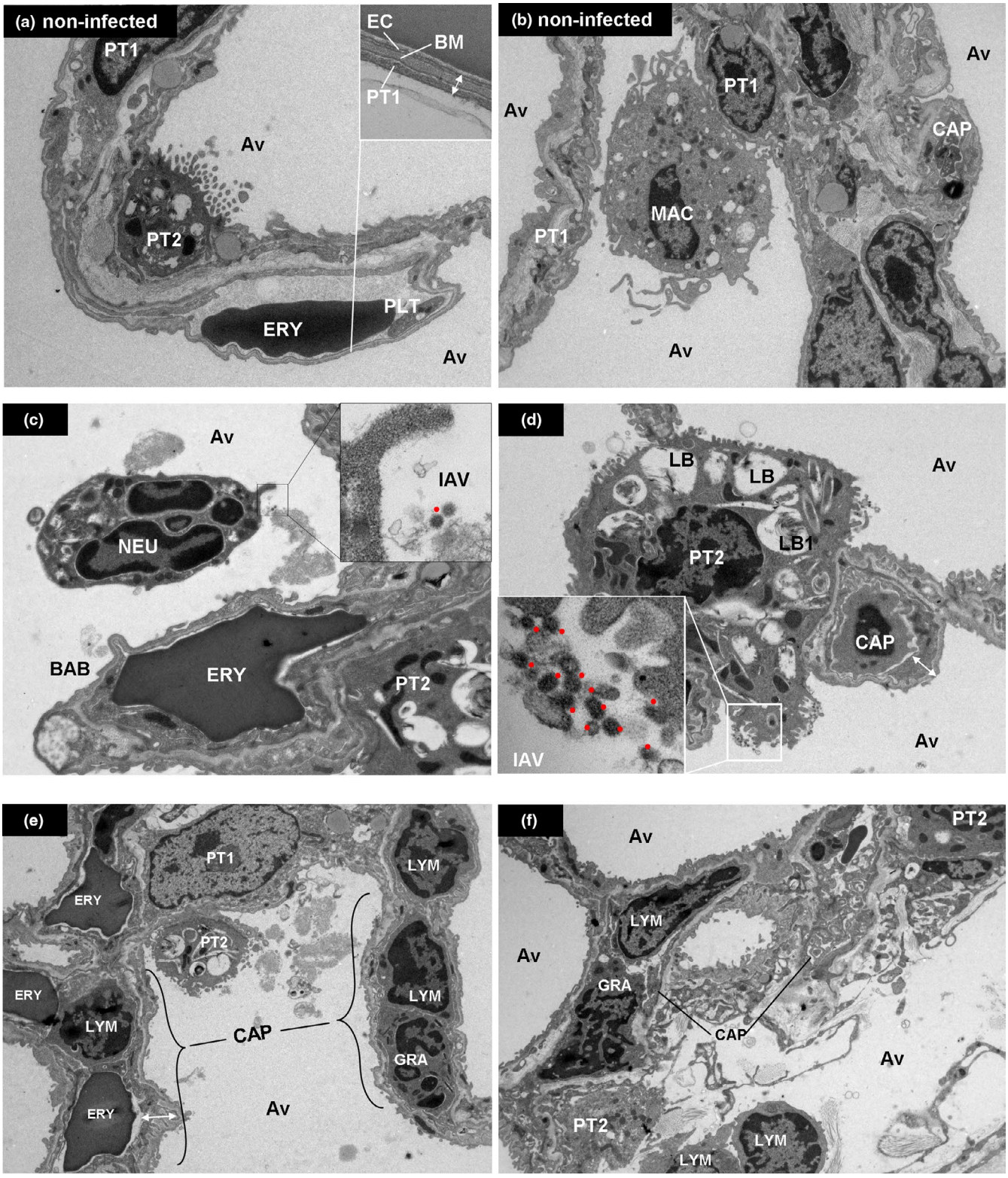
3 | CLINICAL SYMPTOMS IN HUMAN PATIENTS

Humans infected with influenza can remain asymptomatic or exhibit a wide range of symptoms, with potentially lethal outcomes. After infection with seasonal influenza virus, the first symptoms develop after an incubation period of 18–72 hr. A sudden onset of symptoms is typical for influenza infections, as well as a continuous fever between 38 and 41°C, rhinitis, paranasal sinusitis, myalgia, headache, an unproductive cough and a sore throat. Arthralgia and conjunctivitis are less common. Most patients exhibit symptoms for 2–5 days. The viral inflammation of the lung, pneumonia, is considered a complication. Patients with viral pneumonia, due to their influenza infection, usually have dyspnoea, hypoxia and a mostly unproductive cough. Diffuse crackling, a sound typically found in lung oedema, can be heard in the auscultation of some patients. The lung damage from infection with IAV often leads to a bacterial superinfection of the airways. These bacterial superinfections seem to be the main cause of lethality in seasonal and pandemic outbreaks (Mulder & Hers, 1972).

Macroscopic pathology is mainly reported from pandemics and may therefore be biased because of the lethality required for a pathological examination (Taubenberger & Morens, 2008). Autopsy reports from the 1918 IAV H1N1 outbreak describe reddened and swollen mucosal surfaces in the lung, which are sometimes overlaid with mucopurulent material (Lucke, 1919). These findings are usually described as haemorrhagic tracheitis and bronchitis.

4 | HISTOPATHOLOGY OF INFLUENZA INFECTION

In the lung, the body's gas exchange to the blood takes place. Air enters the lung via the upper airways (pharynx, larynx), the trachea and the tracheal branches (bronchi and broncheoli) into the alveolar ducts and alveoli (Figure 1a). The epithelial cells lining the alveolar ducts and alveoli are termed pneumocytes, which can be further divided into type I and type II pneumocytes (Figure 1b). Type I pneumocytes



line most of the alveolar surface and minimize the diffusion barrier for gases through their flat shape, while type II pneumocytes are cuboidal cells, typically located at the transition of the alveolar duct to the alveolus (Figures 1b and 2a). Another important cell type in the lung are alveolar macrophages (AM). These cells are resident next to the pneumocytes (Figure 2b) and have important roles during infection such as the clearing of debris and antigen presentation to leucocytes. Tight junctions between pneumocytes and epithelial cells in general separate the air containing parts from the blood vessels and their surrounding structures. Type I pneumocytes actively pump (Na/K-ATPase) ions from the air compartment, thereby preventing vascular leakage and pulmonary oedema (Berthiaume & Matthay, 2007; Matthay, 2014). This barrier is apparently more important to prevent pulmonary oedema than the endothelial barrier

FIGURE 2 Ultrastructure of lung alveolar tissue from non-infected, healthy and influenza A PR8 H1N1-infected mice 3 days post infection by transmission electron microscopy. (a) Non-infected healthy murine alveolar lung tissue with alveoli (Av). The Av duct is lined by pulmonary epithelial cells so-called pneumocyte type I (PT1) and pneumocytes type II (PT2). PT1 are thin and flat and form the structure of the Av. Insert: The epithelial basal membrane of PT1 is fused with the basal membrane (BM) of the endothelial cells (EC) forming the blood-air barrier (BAB, around 200–600 nm) to minimize the diffusion barrier for the gas exchange. The blood capillary is lined by non-fenestrated EC. PT2 are located at the transition of the alveolar duct to alveolus and contain secretory granules so-called lamellar bodies. ERY = erythrocyte, PLT = platelet (×2,000 magnification and insert BAB ×15,000). (b) Non-infected healthy murine alveoli with a resident alveolar macrophage (MAC) forming pseudopodia and containing several granules, CAP = capillary (×2,000). (c) C57Bl6 mice infected with IAV PR8 H1N1 (10,000 tissue culture infectious dose 50) intranasally. Lung tissue 3 days post-infection (dpi). Extravasated neutrophil (NEU) in the Av forming a pseudopodium in the direction to spherical IAV particles (red dot in insert). IAV is characterized by an electron dense core (dark) and a spiked surface membrane with HA and NA (×4,000 and IAV ×20,000). (d) Lung tissue 3 dpi. PT2 infected with IAV which bud from PT2 into the Av. Insert: IAVs are spherical shaped with 100 nm in diameter (red dots in insert). The lamellar bodies (LB) contain less precursor of pulmonary surfactant which forms thin electron dense lamellae (LB1). CAP with thickened BAB (white arrow, around 800 nm) filled with a leucocyte (×8,000 and insert IAV ×25,000). (e) Lung tissue 3 dpi. CAP filled with lymphocytes (LYM) or granulocytes (GRA) (more compared to 2a). Partial thickened BAB (white arrow, 2 μm) through swelling of the endothelium (×2,000). (f) Lung tissue 3 dpi. Extravasated LYM at the bottom of the picture. Loss of EC integrity in alveolar CAP. Diffuse alveolar damage and shedding thin cellular compartments from CAP and PT1 (×2,000). Tissues were fixed in 2.5% glutaraldehyde (Sigma Aldrich Taufkirchen DE) in PBS pH 7.1 for 24 hr/4°C and embedded in epoxy resin (Epon812, Sigma Aldrich Taufkirchen DE). Electron microscopic pictures were taken with JEM-1400 Flash (Joel Peabody US) and Xarosa Camera (Emsis Münster DE)

(Gorin & Stewart, 1979). In the upper airways, the epithelial cells lie on a basal membrane. This epithelial basal membrane is fused to the basal membrane of the endothelial cells (ECs) lining the capillaries which surround the alveoli (Figure 2a, insert).

Influenzavirus A infection occurs via the airways and attaches with its HA protein to α2,3 or α2,6 sialic acids (SA). Because most seasonal human IAVs attach to α2,6 SA presented on the epithelial cells of the upper airways (Nicholls, Bourne, Chen, Guan, & Peiris, 2007; van Riel et al., 2007), this is also the primary location for IAV replication and, as a consequence, most cell death is found in this cell population (Kuiken & Taubenberger, 2008; Mulder & Hers, 1972). A flattened lining of bronchial epithelial cells usually remains, while many cells desquamate into the lumen or show signs of apoptosis, such as cell shrinkage and vacuolization. In cases where IAV travels further into the lung, replication can destroy the alveolar epithelium causing denudation of the alveolar septa, designated diffuse alveolar damage (Wolbach, 1919). In the human lung, the primary targets of human H1N1 are type I pneumocytes and, to a lesser extent, type II pneumocytes or alveolar macrophages (van Riel et al., 2007). The alveolar septum is widened and infiltrated by neutrophils. Due to impaired pump function in damaged pneumocytes, barrier integrity is hampered. The alveolar lumen fills with fluid (edema), fibrin, erythrocytes, macrophages, neutrophils and desquamated pneumocytes. The latter cells show signs of apoptosis, including nuclear pyknosis, karyohexis and cytoplasmatic vacuolization; some cells are hypereosinophilic. Lipids and cytoplasmic remains of necrotic cells and fibrin-rich fluid form hyaline membranes and line some of the alveoli and bronchioles (Kuiken & Taubenberger, 2008). The gas exchange is hampered resulting in severe to fatal respiratory dysfunction and can manifest as acute respiratory distress syndrome (ARDS).

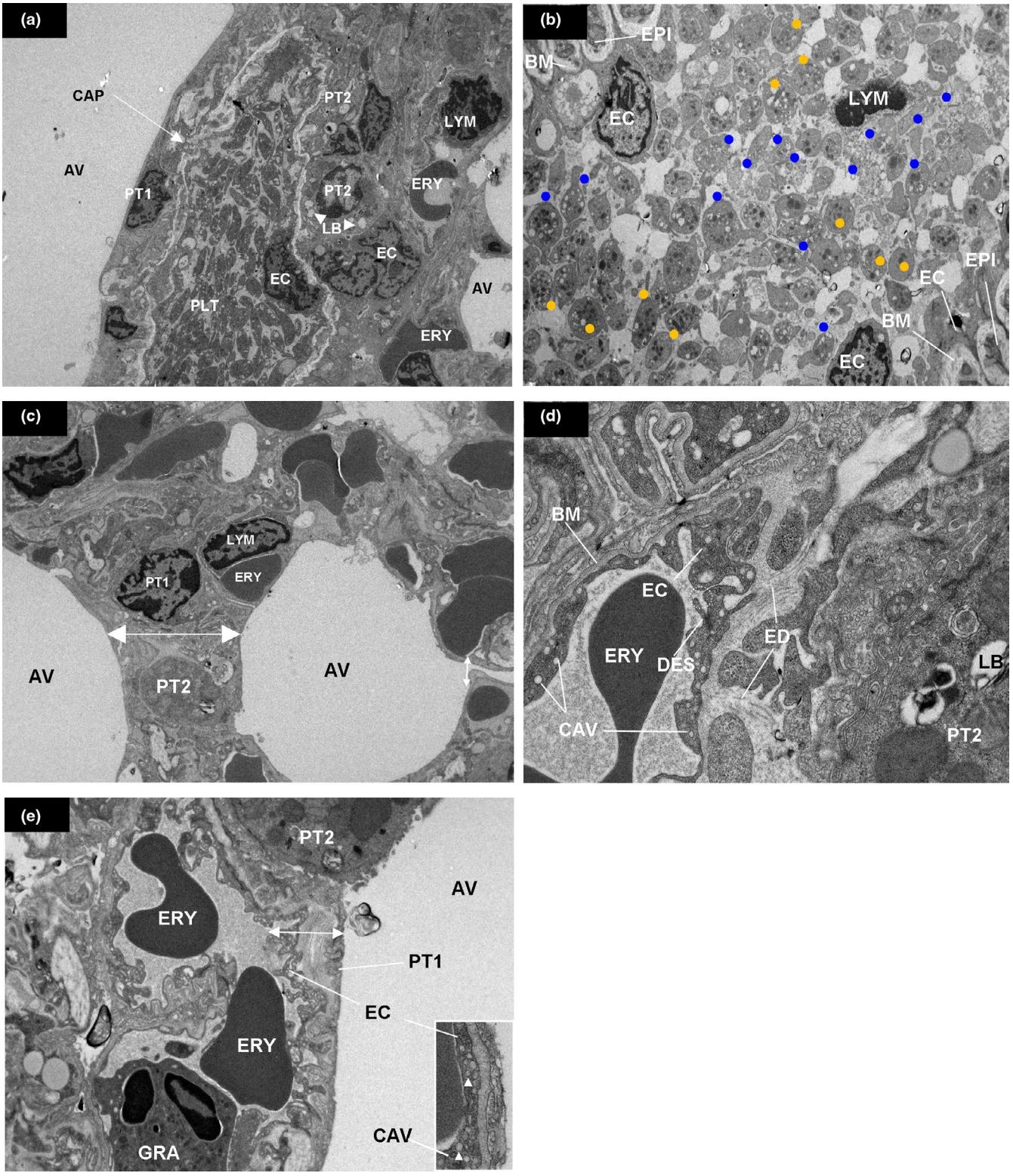
5 | ANIMAL MODELS FOR INFLUENZA RESEARCH

To study influenza virus-induced pathogenesis and develop antiviral therapies or vaccines, preclinical animal models are required. However, the choice of animal model in influenza research must also take into account the susceptibility of the respective animal model to influenza infection and the virus-induced pathology (Bouvier, 2015; Bouvier & Lowen, 2010; Radigan, Misharin, Chi, & Budinger, 2015).

Mouse models are the most frequently used in biomedical research due to short generation times, small size and ease of handling and housing. The mouse is, although well established and accepted, not the best suited host for human influenza infection studies. IAV strains are used that are adapted to mice, such as A/Puerto Rico/8/1934 (H1N1) [PR8] or A/WSN/1933 (H1N1) [WSN] influenza viruses. During adaptation, the virus is passaged through mouse lungs in vivo resulting in amino acid changes in viral proteins. With these mutations, the influenza virus can bind more efficiently to the mouse receptor, enabling better entry and replication. However, this alteration also makes the virus very different to its ancestor virus. Clinical symptoms observed in human patients (coughing, fever, nasal discharge) are not observed in infected mice (these animals develop hypothermia instead). Mice present with lethargy, anorexia (loss of bodyweight), huddled posture, ruffled fur and eventually death (euthanasia at humane endpoints).

Furthermore, most inbred laboratory mouse strains (including BALB/c, C57BL/6 and DBA/2) carry defective *Mx1* alleles (Staeheli, Grob, Meier, Sutcliffe, & Haller, 1988). *Mx1* codes for a very important antiviral protein that is induced by interferon α/β and inhibits transcription of viral RNAs (Staeheli, Haller, Boll, Lindenmann, & Weissmann, 1986). Although all three mouse lines have *Mx1* deletion, BALB/c and C57BL/6 mice are much more resistant to infection than DBA/2 mice.

When analysed by histopathology, infected mice show pulmonary oedema and inflammatory cell infiltrations (Figure 1, see also ref. Dietert et al. (2017)). In PR8 H1N1-infected mice, we could demonstrate many of the pathological findings also found in humans: oedema, bleeding and leucocyte infiltration in to the alveolar lumen, as well as exudates with dead cells in the bronchioli (Figure 1c, d and



f). In the alveoli, we identified type II pneumocytes to be IAV antigen positive, indicating that these cells are the primary target of PR8 H1N1 infection in mice (Figure 1e). Type II pneumocytes shed IAV virus particles (Figure 2d); therefore, IAV replicates in these cells. The extravasated neutrophils are one cell population which takes up IAV particles (Figure 2c). ECs are not affected, and continuous lining of cells can be detected via electron microscopy (Figure 3d,e) and immunohistochemical staining for von Willebrand factor (VWF) (Figure 1g,h). However, through the massive recruitment of immune effector cells in early days post-infection (dpi), including granulocytes and lymphocytes, the alveolar septum is thickened (Figure 3c,e) and the alveolar lumen is completely filled causing diffuse alveolar

FIGURE 3 Ultrastructure of lung alveolar tissue from influenza A PR8 H1N1-infected mice 8 days post-infection by transmission electron microscopy. C57Bl6 mice infected with IAV PR8 H1N1 (10,000 tissue culture infectious dose 50) intranasally. (a) IAV infected murine alveolar lung tissue representing many accumulated platelets (PLT) in a capillary (CAP). Furthermore there are no LBs in the PT2 (×1,000 magnification). EC = endothelial cell, ERY = erythrocyte, LYM = lymphocyte, (b) Overrepresented non-activated PLT (yellow dots; discoid shape and presences of different granules) and activated PLT (blue dots; shape change, protrusions formation and loss of granular content) next to the bronchial epithelium (EPI) LYM is enclosed into activated PLT. PLT show contact with EC. BM = basal membrane (×4,000). (c) Reduction of the alveolar lumen by increased alveolar interstitial width (white arrow), by swelling of the PT1 cytoplasm. PT2 are inbound and contain less LBs compared to the PT2 in healthy lung tissue (×2,000). (d) BM of the CAP is widened through oedema (ED) formation. EC lining is continuous. The cell–cell junction integrity is not altered, indicated by the presence of desmosomes (DES). The dotted white circle could represent a damaged tight junction. EC membrane with caveolae (CAV) which regulate the transport of macromolecules from blood to tissue space (×10,000). (e) EC lining is continuous with a thickened and ruffled blood air barrier (BAB, around 1 μm). Increased presence of CAV (above the white arrowheads in insert) in the EC membrane. GRA = granulocytes (×2,000 and insert EC ×15,000). Tissues were fixed in 2.5% glutaraldehyde (Sigma Aldrich Taufkirchen DE) in PBS pH 7.1 for 24 hr/4°C and embedded in epoxy resin (Epon812, Sigma Aldrich Taufkirchen DE). Electron microscopic pictures were taken with JEM-1400 Flash (Joel Peabody US) and Xarosa Camera (Emsis Münster DE)

damage at later dpi (Figures 1f and 2f). In contrast to humans and due to the difference in distribution of α2,6-SA, which are the attachment sites for human IAV, the prevalent site of disease manifestation in mice is the lower respiratory tract. With increasing virus doses, mice develop more severe pneumonia which can be fatal. Highly pathogenic strains such as the pandemic H1N1 virus from 1918 or 2009 or the human highly pathogenic avian IAV H5N1 strain can infect mice directly. H5 and H7 subtypes can also spread to other tissues (brain, spleen, thymus, kidney, liver and heart) in mice.

Ferrets are naturally susceptible to a wide range of human influenza viruses (Enkirch & Messling, 2015). Inoculation can be intranasally or intratracheally. The symptoms in ferrets are very similar to symptoms in human patients: fever, nasal discharge, lethargy and signs of upper and sometimes lower respiratory infection. Changes in the application route or the viral strain can alter symptoms and grade of severity in infected ferrets; in some cases, pathology can even involve non-respiratory organs, such as the gut or neuronal tissue (Bodewes et al., 2011; Meunier et al., 2012). In addition, old ferrets develop more severe symptoms than young, newly weaned animals (Huang et al., 2012). In histopathological analysis, bronchiolitis, interstitial pneumonia and haemorrhages can be diagnosed; after infection with highly pathogenic strains, these defects become more extensive including also necrosis of the bronchial epithelium or purulent exudates in the bronchiolar lumen.

The breeding of ferrets for influenza research is, however, more laborious, as these animals need to be seronegative against influenza before they are used in experiments. In addition, reagents directed against ferret antigens are limited in availability.

Guinea pigs are naturally susceptible to human influenza virus infection, including H3N1 and H1N1, but do not develop severe symptoms. Viral replication takes place in the upper airways with titres of ~10^7 plaque forming unit (PFU) two days after intranasal infection with 10^4 PFU (Cal/04/09 (H1N1) virus) (Steel et al., 2010). Viral clearance was observed after 8 days. Guinea pigs develop signs of mild listlessness but no coughing/sneezing, no fever or weight loss and only mild mucosal discharge. The virus also does not spread systemically throughout the body. Histopathologically, a mild-to-severe interstitial bronchopneumonia and rhinitis can be detected. Most influenza virus strains can spread between guinea pigs; however, due to the low visibility of clinical symptoms, the guinea pig model is not often used for vaccine or antiviral drug studies (Lowen, Mubareka, Tumpey, García-Sastre, & Palese, 2006).

Pigs are natural hosts for the same influenza subtypes as humans. Similar to humans, pigs express α2,6 SA in the tracheal epithelial cells (Ito et al., 1998). Pigs can transmit the virus to other pigs, but also humans, and pigs are often intermediate hosts between birds and humans. Therefore, the pig is the so-called mixing vessel.

Non-human primates are the most closely related animal model to humans. Macaques are most frequently used including rhesus macaques (Macaca mulatta), pig-tailed macaques (Macaca nemestrina) and cynomolgus macaques (Macaca fascicularis). Macaques are susceptible to non-adapted IAV subtypes such as H1N1, H5N1 and H3N2. Infection with these viruses can induce fever, rush, cough and ARDS. Histopathological analysis of cynomolgus macaques after lethal H1N1 infection showed alveolitis, oedema and haemorrhages (Cillóniz et al., 2009). In contrast, infection of macaques with the mouse adapted PR8 virus did not induce visible signs of disease except for leukopenia (Saslaw, Wilson, Doan, Woolpert, & Schwab, 1946).

6 | THE ROLE OF THE ENDOTHELIUM IN IAV-INDUCED PNEUMONIA

Pulmonary ECs are directly and indirectly activated by IAV infection triggering them to express and secrete inflammatory cytokines, procoagulation factors, change their cell–cell junctions, and recruit leucocytes and platelets (Liu, Zhou, & Yang, 2016). Besides pulmonary epithelial cells being the primary source, cytokines are also secreted from ECs in the lung and contribute majorly to pneumonia pathology (Teijaro et al., 2011). Excessive cytokine production is part of the severe pathology in influenza infection and often referred to as a 'cytokine storm'.

In pulmonary epithelial cells, pathogen-associated molecular patterns (PAMPs) of IAV are sensed by pattern recognition receptors (PRRs). PRRs include retinoic acid-inducible gene I (RIG-I), which senses viral RNA (Liu & Zhou, 2019), and toll-like receptors (TLRs) which detect viral proteins on the cell surface or on endosomes (Pothlichet et al., 2013). IAV can also induce endoplasmatic reticulum

stress through the HA glycoprotein (Frabutt, Wang, Riaz, Schwartz, & Zheng, 2018). PRRs then initiate a rapid antiviral signalling cascade. Epithelial cells produce primary cytokines: type I and type II interferons, IL-1β, IL-6 and TNF-α. The highly pathogenic avian H5N1 induces strong IL-6 secretion to the basolateral face in primary pulmonary epithelial cells in culture, which can directly stimulate subjacent pulmonary ECs (Chan et al., 2009). The combination of TNF-α, IL-1β and IL-6 promotes the upregulation of trypsin in ECs, loss of tight junction protein ZO-1 and hyper permeability through PAR-2 signalling in mice (Wang et al., 2010). Higher TNF-α levels induce changes in the microtubule network in ECs. This increase of intercellular gaps and cell permeability can lead to endothelial barrier dysfunction (Petrache, Birukova, Ramirez, Garcia, & Verin, 2003).

It has been demonstrated that lung ECs expressing the sphingosine-1-phosphate receptor 1 (S1P-R1) mediate the cytokine storm (Teijaro et al., 2011). Application of a S1P1 receptor agonist (small molecule drug, CYM-5442) during severe IAV infection reduced the global pro-inflammatory cytokine response. The S1P1 receptor agonist thereby diminished the expression of type I interferons, especially IFN-α. Pulmonary ECs are not the primary target for influenza infection. Some subtypes of IAV (H3N2 or H5N1) are capable of infecting primary lung ECs in vitro, thereby altering tight junction integrity by degradation of claudin-5 without changes to adherent junctions (Armstrong et al., 2012). To date, only the avian H5N1 strain was able to infect murine pulmonary ECs in vivo (Ogiwara et al., 2014); however, in several animal studies with human IAV, infection of the pulmonary endothelium is rarely observed (Kuiken, Brand, Riel, Pantin-Jackwood, & Swayne, 2010).

Activated or even damaged pulmonary endothelium presents a pro-thrombotic-milieu to the local blood system. The pro-inflammatory cytokines TNF-α, IL-1β and IL-6, or viral RNA sensed by endothelial pattern recognition receptors give rise to the production of tissue factor (TF) in ECs (Liu, Pelekanakis, & Woolkalis, 2004). TF is one of the most potent activators of the coagulation cascade when presented to the blood, mediating the generation of thrombin, that activates platelets via the protease-activated receptor (PAR)-1, PAR-3 and PAR-4 (Antoniak & Mackman, 2014). PARs are expressed on the surface of ECs, platelets, macrophages and bronchial epithelial cells. The role of the thrombin/PAR-1 signal transduction pathway during IAV infection is poorly defined. Investigations of Mackmann and colleagues showed that PAR-1-KO mice have reduced innate immune responses to IAV H1N1 PR8 and were therefore more susceptible to infection. However, successful resolution of infection indicates that the adaptive immune responses in these mice are normal (Antoniak et al., 2013). In the study by Khoufache and colleagues, activation of PAR-1 increased inflammation, virus replication, weight loss and mortality of mice infected with IAV PR8 H1N1. In agreement with this observation, administration of PAR-1 antagonists in mice, or infection of PAR1 knockout mice, reduced inflammation and prolonged survival (Khoufache et al., 2013). In vitro infection of epithelial cells with IAV PR8 H1N1 increased PAR-2 expression. Subsequent PAR-2 activation decreased viral replication and increased IFN-γ release (Khoufache et al., 2009). Furthermore, infiltration of innate immune cells regulates the expression of cell adhesion molecules (VCAM-1, ICAM-1, E- and P-selectin) on pulmonary ECs.

The endothelium may contribute to lung repair after IAV infection by supporting nearby epithelial cells through secretion of growth factors or barrier protecting proteins. How endothelial damage in the lung after IAV infection is repaired is under debate. One possible explanation is repair by so-called endothelial progenitor cells (EPC) which reside in the bone marrow and can be released in the case of need. EPC circulate then to the side of endothelial damage and give rise to new ECs or release factors to support the local repair (Asahara et al., 1997; Yoder, 2012).

7 | PLATELETS IN INFLUENZA-INDUCED PNEUMONIA

The endothelial barrier integrity is regulated by platelets under physiological conditions; however, there is contradictory evidence of the role of platelets in either controlling or contributing to vascular leakage during inflammation in the lung (Ho-Tin-Noé, Demers, & Wagner, 2011). Early experiments in sheep report that low platelet numbers elevate the permeability of pulmonary vessels to proteins, based on the lymph-to-plasma ratio. The application of bovine platelet-rich plasma reversed the observed leakage while thrombocytopenic plasma had no effect (Lo, Burhop, Kaplan, & Malik, 1988; Pearse, Brower, Adkinson, & Sylvester, 1989). In isolated rabbit lungs, after oxidant-induced edema formation, alveolar protein concentrations were reduced by infusion of human platelets (Heffner, Cook, & Halushka, 1989). In this model, platelet antioxidant enzymes were key players in reconstituting the endothelial barrier integrity.

7.1 | Platelet activation

After influenza infection, platelets are sequestered in the lung, resulting in thrombocytopenia in the peripheral blood (Chen et al., 2013; Lê et al., 2015). Platelets are activated by cell surface molecules on inflamed ECs, or by the exposed basement membrane in damaged vessels. Pre-activated platelets can be detected in the peripheral blood of mice infected with IAV PR8 (Lê et al., 2015). Activated platelets were also isolated from human patients with ARDS after IAV infection (2009/H1N1) (Rondina et al., 2012). In agreement with these studies, we observed activated platelets in pulmonary vessels and capillaries by electron microscopy (Figure 3b, indicated by blue circles). Platelet activation may be beneficial: during the 2009 H1N1 influenza pandemic, patients who did not recover platelet counts had a poor prognosis, when compared to survivors who had significantly higher platelet counts (Ríos et al., 2011). In contrast, PAR-4 activation on platelets in mice aggravated ARDS with massive platelet aggregation in the lung capillaries causing high lethality (Lê

et al., 2015). In mice, PAR-4 is expressed on platelets and required for thrombin induced activation, while on human platelets, PAR-1 and PAR-4 are present. Increased expression of TF and VWF in ECs, the release of collagen by EC damage or binding of platelets to ECs trigger the extrinsic coagulation cascade. Consequently, platelet aggregation takes place and micro-thrombi form in the pulmonary capillaries, which passively reduces the number of platelets and leads to disseminated intravascular coagulation (DIC) (Armstrong, Darwish, & Lee, 2013). We were able to confirm the formation of microthrombi in the murine lung capillaries by VWF immunohistochemical staining (Figure 1h, insert) and the presence of accumulated platelets by electron microscopy (Figure 3a,b). Capillary thrombosis is described as one of the main features in the 1918 pandemic influenza (Taubenberger & Morens, 2008) and was also observed in the 2009 pandemic (Bunce et al., 2011). Recently, IAV particles were detected within platelets from patients infected with IAV H1N1, H3N2 and IBV, potentially taken up by phagocytosis (Koupenova et al., 2019). Similarly, IAV was detected in platelets of PR8 H1N1-infected mice (Lê et al., 2015).

7.2 | Secretion from activated platelets

Activation of platelets triggers secretion from their granules which is accompanied by a change in platelet morphology. This has been reported to be both beneficial and harmful to the outcome of influenza infection. In vitro studies with endothelial cells co-cultured with platelets or with conditioned media from platelet cultures identified that bioactive lipids (sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA)), pro-angiogenic cytokines and growth factors (vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), angiopoietin-1) released by platelets are key molecules for the maintenance of endothelial barrier integrity (Ho-Tin-Noé et al., 2011; Nachman & Rafii, 2008; Singleton, Dudek, Chiang, & Garcia, 2005; Su et al., 1999). In addition to the leakage of protein, water and solutes, severe thrombocytopenia leads to extravascular escape of erythrocytes (Nachman & Rafii, 2008). The presence of erythrocytes in the alveolar lumen was observed in our IAV infection studies at later time-points (Figure 1d). Bioactive lipids, including S1P, are secreted from platelets and bind to their receptor to induce the rearrangement of the actin cytoskeleton and formation of VE-cadherin positive adherent junctions in ECs. It has been shown that S1P reduced alveolar edema in models of acute lung injury (ALI) and lung inflammation.

In contrast, some studies indicate activated platelets to be involved in destabilization of alveolar-capillary integrity and therefore increase pulmonary oedema in viral and bacterial infection. We detected activated, de-granulated platelets with protrusions during IAV PR8 infection via electron microscopy (Figure 3a,b). Platelets release endothelial integrity destabilizing cytokines, such as histamine, IL-1β, CD40L, CCL5, CXCL4, CXCL7, TGF-β or thromboxane A2 (Rossaint, Margraf, & Zarbock, 2018). Furthermore, human and murine platelets are capable of synthesizing IL-1β from stored mRNA pools (Brown & McIntyre, 2011; Lindemann et al., 2001), which is a central mediator of enhanced vascular permeability in the context of inflammation and infection (Pober & Sessa, 2014; Zhu et al., 2012). Platelet derived IL-1β stimulates expression of the intracellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1) (Hawrylowicz, Howells, & Feldmann, 1991). In addition, neutrophils adhered better to cultured ECs in the presence of IL-1β from activated platelets. Other investigations have shown that CD40L induced expression of E-selectin, ICAM-1 and VCAM-1 on human ECs in vitro (Danese et al., 2004; Henn et al., 1998).

7.3 | Interaction of platelets with other blood cells

Activated platelets interact with various blood cells: neutrophils, eosinophils, basophils, lymphocytes, dendritic cells, monocytes and macrophages at extravascular sites in the lung and other organs (Middleton, Weyrich, & Zimmerman, 2016). Platelet–neutrophil interactions in the lung, under physiological conditions and in the context of viral or bacterial infections, are the focus of intensive study. Neutrophils scan for activated platelets and engage them in the circulation to sites where neutrophils attach to activated endothelium (Sreeramkumar et al., 2014). This interaction is mediated by P-selectin on activated platelet membranes and PSGL-1 on polarized neutrophils. This platelet–neutrophil interaction forces the formation of neutrophil extracellular traps (NETs), which seem to be cytotoxic to ECs. The mechanism of cytotoxicity is suggested to be caused by the extracellular presence of histones and DNA fibres in the alveoli and terminal bronchioles, and the generation of reactive oxygen species (Narasaraju et al., 2011; Saffarzadeh et al., 2012). In the blood of critically ill patients from the 2009 H1N1 pandemic, more platelet/monocyte aggregations could be demonstrated (Rondina et al., 2012). Platelets in the blood of these patients also bind more antibody specific to the activated form of integrin $\alpha_{IIb}\beta_3$, indicating that activated platelets circulate in the blood.

8 | POTENTIAL THERAPEUTIC TARGETS OR TREATMENTS IN ACUTE RESPIRATORY LUNG INJURY

To date, only two specific treatments against influenza are on the market: live virus or inactivated viral vaccines, and the application of viral entry inhibiting (NA-inhibitors; oseltamivir or zanamivir) or replication inhibiting (M2 ion channel blockers; amantadine or rimantadine) antiviral drugs. These treatments are mainly prophylactic strategies, and there is no specific pharmacological treatment of ARDS/ALI in severe flu patients. Instead, the current therapy revolves around the optimization of the supply and transfer of oxygen by ventilation. There are no additional pharmacological

therapies that have demonstrable clinical benefits, except for the inhalation of surfactant in children with ARDS. Activated and damaged ECs, activated platelets and platelet–cell interactions promote IAV replication and infectivity as well as influenza-induced ARDS. Understanding the endothelium/platelet interaction in influenza infection will identify potential targets for the development of novel therapeutics.

Administration of pharmaceutical agonists that target endothelial cell activation by the S1P-S1P-R signalling pathway successfully reduces the cytokine storm and innate immune responses in PR8 H1N1-infected mice (Marsolais et al., 2009; Teijaro et al., 2011). This treatment did not alter the viral titre in the lung, but limited the damage by the overshooting immune system, as demonstrated in histological analysis. Furthermore, the treatment with SP1PR agonists protected mice and ferrets from mortality by human IAV H1N1 infection more successfully than the application of oseltamivir (Teijaro et al., 2014; Walsh et al., 2011), and combination of both drugs resulted in a 96% survival rate in mice. The exogenous addition of S1P or S1P-mimetics to human, bovine or canine lungs also enhanced endothelial barrier integrity by actin filament re-arrangement to stabilize adherens junctions (Camp et al., 2009; Garcia et al., 2001; McVerry et al., 2004). However, in bleomycin-induced acute lung injury in mice, repeated or prolonged administration of S1P-agonists increased vascular leakage (Shea et al., 2010). The barrier protective activity of S1P and S1P-analoga is highly concentration dependent in murine lungs indicating a narrow therapeutic window.

Activation of the coagulation cascade, and therefore formation of micro-thrombi in the lung, is observed in lethal influenza infection. Activation of PAR-1 by administrating recombinant activated protein C (APC) during IAV PR8 infection resulted in lower virus titres and loss of coagulopathy in mice (Schouten et al., 2011). However, treatment did not alter the histopathology and infiltration of neutrophils in the lung or lethal outcomes. Preventing the activation of PAR-1 by PAR-1 antagonists should inhibit platelet activation. PAR-4 antagonism by treatment with pepducin p4pal-10 protected mice from death in H1N1 and H3N2 infection (Lê et al., 2015). A recent study observed more lung injury and lethality by a more pronounced cytokine immune response in the lung of PAR-4-deficient mice infected with IAV H1N1 (Tatsumi et al., 2019). An additional approach to stop coagulation in IAV infection is the direct inhibition of thrombin by anti-thrombin III (AT, serine protease inhibitor). AT-thrombin binding suppressed the activation of ECs and platelets. Moreover, several serine proteases are upregulated during IAV H1N1 infection (Bahgat, Błazejewska, & Schughart, 2011). In agreement with that observation, AT strongly inhibits IAV infection in cultured MDCK cells dependent on the haemagglutinin subtype. Intranasal application of AT in mice reduced lethal outcomes only in the high dosage groups (Smee, Hurst, Day, & Geiben-Lynn, 2014).

Overshooting platelet activation by influenza infection causes thrombosis in the lung and is associated with the severity of ARDS. As a consequence, the administration of anti-platelet drugs (platelet aggregation inhibitor) should diminish ARDS. Eptifibatide, an RGD mimetic (reversible inhibition of platelet aggregation via competitive binding of the GPIIb/IIIa receptor (CD41/CD61) with fibrinogen), protects mice from lethal influenza infection (Lê et al., 2015). Consequently, GPIIIa-deficient mice survive infection better compared to wild-type mice. Lê and colleagues investigated other anti-platelet drugs, irreversibly inhibiting the ADP receptor P2Y1/2 receptor by clopidogrel and MRS 2179, and observed protection from ARDS and lethality from infection with different influenza strains (Pulavendran et al., 2019). To inhibit platelet aggregation, aspirin (acetylsalicylic acid: ASA) is widely used. The primary mode of function in IAV infections is based on reduction of pro-inflammatory NF-kB signal transduction (Mazur et al., 2007). However, retrospective evidence suggests the high-fatality rates observed with pandemic 1918 influenza H1N1 were inflated by ASA treatment (Starko, 2009). Moreover, the prophylactic usage of ASA to prevent ARDS in risk-patients was tested in a randomized clinical study and was associated with a negative outcome (Kor et al., 2016).

In the last two decades, cell therapies have become exciting approaches to treat ARDS/ALI. In this respect, the application of stem and progenitor cells is currently the subject of intensive ongoing research with the aim to restore the lung tissue after injury. In particular, the application of mesenchymal stem/progenitor cells (MSCs) shows an anti-inflammatory function and reduces the severity of histopathology after lung injury (see also ref. (Lee, Fang, Krasnodembskaya, Howard, & Matthay, 2011). Moreover, there is evidence that MSCs improve oxygenation and resolve pulmonary oedema (Asmussen et al., 2014). The main therapeutic effect involves the release of soluble factors such as cytokines or mRNA/miRNA in micro-vesicles, which alter gene expression in epithelial and endothelial cells. Several studies where EPCs were administered intravenously in rats or mice have been reported. EPCs migrated to the damage endothelium in the lung, reduced the inflammatory milieu and increased the alveolar barrier (Rafat, Tönshoff, Bierhaus, & Beck, 2013). However, the all aforementioned studies were performed in models of lung injury due to bacterial infection. Beneficial effects of MSC transplantation/infusion after viral infection should be further investigated.

9 | CONCLUSION

IAV PR8 H1N1 infection in mice induces pulmonary oedema, infiltration of immune effector cells and bleeding into the alveolar lumen, leading to ARDS/ALI with very similar histopathological findings to those reported in human patients. However, pathology is dependent on the specific influenza subtype for any animal model or human infection. The primary target of seasonal IAV is the pulmonary epithelium and only high pathogenic IAV infect pulmonary ECs. Pulmonary EC activation is responsible for the massive cytokine storm which can be diminished by agonists of the S1P/S1P-R signalling pathway. Endothelial–platelet interactions initiate a pro-thrombotic milieu in the lung capillaries, and platelet aggregation can be inhibited by the administration of approved

anti-platelet drugs, anti-coagulants or serine protease inhibitors to intervene or reduce ARDS/ALI caused by IAV infection. An understanding of the consequences of platelet–endothelial interactions in IAV infected lungs will identify further therapeutic targets for pharmacological intervention.

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