Endothelial activation and dysfunction in the pathogenesis of influenza A virus infection

Susan M Armstrong,[1,†] Ilyse Darwish,[2,†] and Warren L Lee[3,4,*]

[1]Institute of Medical Science; Faculty of Medicine; University of Toronto; Toronto, ON Canada; [2]Sandra A. Rotman Laboratories; Sandra Rotman Centre for Global Health; University Health Network-Toronto General Hospital; Toronto, ON Canada; [3]Division of Respirology and Interdepartmental Division of Critical Care Medicine; Faculty of Medicine; University of Toronto; Toronto, ON Canada; [4]Keenan Research Centre of the Li Ka Shing Knowledge Institute; St. Michael's Hospital; Toronto, ON Canada

[†]These authors contributed equally to this work.

The development of severe influenza has been attributed, in part, to a heightened innate immune response. Recent evidence suggests that endothelial activation, loss of barrier function, and consequent microvascular leak may also serve important mechanistic roles in the pathogenesis of severe influenza. The aim of this review is to summarize the current evidence in support of endothelial activation and dysfunction as a central feature preceding the development of severe influenza. We also discuss the effect of influenza on platelet–endothelial interactions.

Introduction

Influenza A viruses commonly infect the upper respiratory tract resulting in a self-limited infection with mild respiratory symptoms. In severe cases, which typically occur in elderly or immunocompromised patients, influenza may spread to the lower respiratory tract, causing viral pneumonia. This may progress to acute lung injury, a syndrome of increased pulmonary microvascular leakage leading to pulmonary edema, hypoxemia and respiratory failure.[1,2] In this setting, the use of an antiviral drug for the specific treatment of influenza is only partially effective at reducing mortality,[3] underlining the urgent need for additional therapies.

The inadequacy of current therapeutic approaches is reinforced by the rapid development of viral resistance to anti-viral drugs and their limited efficacy when administered late in the course of disease. Instead, the lung microvascular leak that occurs in severe influenza virus infections may represent an attractive adjunctive therapeutic target. Major advances have emerged in our understanding of the role of the microvascular endothelium during infections, particularly in the field of sepsis.[4] Despite this, the contributions of the lung microvascular endothelium to the pathogenesis of severe influenza have been largely uninvestigated. The aim of this review is to summarize the literature on endothelial activation and dysfunction in severe influenza. Specifically,

we will highlight the role of inflammation, permeability, and thrombosis as it pertains to the pulmonary microvascular endothelium during influenza virus infection. We conclude by discussing areas for future research in the field.

Endothelial Permeability and Activation during Influenza

Influenza A virus primarily binds the respiratory epithelium. Subsequent infection of epithelial cells can lead to pulmonary edema by interfering with epithelial sodium channel (ENaC) function, an important mediator of alveolar fluid clearance.[5-7] However, endothelial dysfunction is thought to be the main factor involved in the development of pulmonary edema.[8] Thus, understanding how the influenza virus causes disruption of the endothelial barrier is critical.

Endothelial barrier dysfunction, a phenomenon that is particularly problematic in the lung due to the resultant alveolar flooding, may be a deleterious consequence of excessive cytokine production. Indeed, many investigators have implicated a heterogenous subset of elevated cytokines and chemokines known as a "cytokine storm" in the pathogenesis of severe influenza.[9-15] Tumor necrosis factor (TNF) is one of multiple cytokines implicated in the pathogenesis of influenza[16,17] that has been shown to mediate endothelial barrier dysfunction. Elevated TNF levels have been shown to increase Rho kinase-induced stress fiber formation leading to induction of endothelial apoptosis.[18-20] Increased TNF, IL-6, and IL-1β following influenza virus infection has also been shown to upregulate trypsin resulting in the loss of the endothelial tight junction protein, zonula occludens-1 (ZO-1), and subsequent vascular hyperpermeability.[21] Elevated chemokine expression during influenza virus infection may also play a role in endothelial barrier dysfunction. For instance, upon avian influenza H5N1 virus infection, Chan and colleagues[22] observed chemokine expression from epithelial cells at the basolateral surface, which would about the microvascular endothelium in the alveolo-capillary membrane. Intriguingly, this effect was not observed with a low pathogenic H1N1 influenza virus, suggesting that chemokine-induced endothelial activation may contribute to the development of severe disease during H5N1 influenza infection.

Recent evidence suggests that the lung endothelium itself plays a previously unsuspected role in mediating the cytokine response. In particular, the endothelial sphingosine-1-phosphate receptor 1 ($S1P_1$) was found to regulate the cytokine storm caused by severe influenza.[23] Influenza-infected mice that received an $S1P_1$ specific agonist (CYM-5442) had significantly decreased early cytokine and chemokine levels as well as reduced inflammatory cell infiltrates in the lung. This resulted in a 60% improvement in survival. Teijaro and colleagues[23] demonstrated that $S1P_1$ located on the pulmonary endothelium was responsible for mediating this effect. While CYM-5442 attenuated leukocyte recruitment, this did not explain the reduction in cytokines. Instead, SIP_1 agonism was shown to dampen global proinflammatory cytokine responses by blunting IFN-α production, an upstream regulator of early cytokine production. Unfortunately, the effects of SIP_1 agonism on microvascular leak per se were not reported in this study. However, S1P is known to promote endothelial barrier integrity by inducing endothelial Rac activity, thereby increasing cortical actin formation and stabilizing the adherens junctions.[24,25] Thus, the protective effect of the $S1P_1$ agonist may have been due to both global cytokine repression and improved lung endothelial stability.

Cytokine production also leads to upregulation of adhesion molecules that facilitate leukocyte recruitment. Indeed, an influx of leukocytes into the lungs is critical to mounting a healthy innate immune response to infection. However, recent evidence in humans, murine models, and cell lines suggests an important contribution of neutrophil infiltration to the development of acute lung injury and the acute respiratory distress syndrome (ARDS) in influenza pneumonia.[26,27] Activated neutrophils release neutrophil extracellular traps (NETs), macromolecular structures formed of extruded nuclear chromatin and bactericidal proteins.[28] While postulated to play a role in host defense against infection, NETs have been shown to exert cytotoxic effects upon endothelial cells[29] and to contribute to lung damage in influenza-infected mice;[26] A/PR/8 (H1N1)-infected mice developed lung damage with extensive focal NET formation within pulmonary lesions. In this study, NETs were found entangled with alveolar epithelium and small blood vessels in areas with hemorrhagic lesions, suggestive of NET-induced alveolo-capillary damage.[26] In addition, small airway occlusions were attributed in part to NET-endothelial or -epithelial cell interaction/attachment. Redox enzyme-dependent NET-enhanced endothelial cell damage was confirmed in vitro.

In addition to cytokine-mediated endothelial activation and endothelial damage from infiltrating leukocytes, direct invasion of the endothelium by influenza virus may activate the endothelium resulting in functional changes in endothelial protein expression, enhanced endothelial permeability, and significant vascular destruction. Viral replication within the alveolar epithelium leads to apoptosis, exposing the underlying basolateral surface of the pulmonary endothelium which expresses α2,6-linked sialic acid residues, the receptors for the human influenza virus.[30] In vitro, HPAIV H5N1 and H3N2 influenza subtypes have been shown to replicate within human lung microvascular endothelial cells.[22,30-32] However, in vivo, direct infection of the lung microvascular endothelium has only been demonstrated for avian influenza H5N1 viruses.[33] Influenza virus infection of the endothelium can lead to activation of the transcription factor NFκB resulting in upregulated cytokine and chemokine production giving rise to vascular leak.[21,31] In the presence of the NFκB dominant negative mutant IKK2, Schmolke et al.[31] reported a 46% reduction in H5N1-induced mRNA expression in endothelial cells, including IFN-β. NFκB-dependent activation of influenza-inducible genes may be more pronounced during H5N1 infection of the endothelium compared with infection by other influenza subtypes.[34] While NFκB signaling in the endothelium may be important for influenza-induced cytokine production, NFκB signaling within the endothelium may play a more direct role in regulating vascular permeability in vivo.[35] For instance, in a murine model of *E. coli* sepsis, mice that overexpressed an endothelial-specific NFκB inhibitor had decreased endothelial permeability, decreased expression of markers of organ injury, and improved survival. Importantly, systemic and tissue inflammation were similar in the transgenic mice compared with wild-type mice.[35]

In contrast, a recent report demonstrated a negligible role for NFκB in mediating endothelial stability.[36] Instead, Zhu and colleagues[36] reported an IL-1β-induced MYD88-ARNO-ARF6 signaling pathway that regulates vascular stability independent of NFκB function. This cytokine-mediated pathway may also be of importance for influenza-induced vascular leak.[36,37]

Ultimately, an increase in endothelial permeability almost always reflects endothelial apoptosis or remodeling of endothelial cell–cell junctions (adherens junctions and tight junctions) (**Fig. 1A**). Adherens junction proteins, notably VE-cadherin, possess an extracellular domain that connects endothelial cells, and an intracellular domain that connects to the actin cytoskeleton via catenin proteins.[38] Recently, London et al.[39] identified Slit and its cognate receptor, Robo-4, as important mediators of VE-cadherin retention at the plasmalemma. Increased cell surface VE-cadherin was mediated by enhanced association of p120-catenin to VE-cadherin, which prevented VE-cadherin internalization. Slit2N-mediated effects were abrogated in the presence of VE-cadherin antibody. Of key interest, in vivo administration of Slit2N, the biologically active component of Slit, significantly improved lung injury, lung endothelial integrity, and survival in H5N1-infected mice. Remarkably, these effects were observed in the absence of an effect on pulmonary inflammation, cytokine levels, and viral load, suggesting that strengthening the microvascular endothelial barrier may be sufficient to improve clinical outcome.

Alterations in tight junction proteins[38] may also mediate endothelial leak during influenza infection.[21] Tight junction-mediated vascular leak may play a role in influenza pathogenesis, independent of adherens junction protein modification. Replication-deficient influenza A/X-31 virus induced degradation of the tight junction protein claudin-5 in vitro, thereby augmenting endothelial permeability in the absence of changes in adherens junctions.[32] This finding was demonstrated to be independent of influenza-induced endothelial apoptosis, indicating a specific involvement of claudin-5 tight junction proteins in influenza
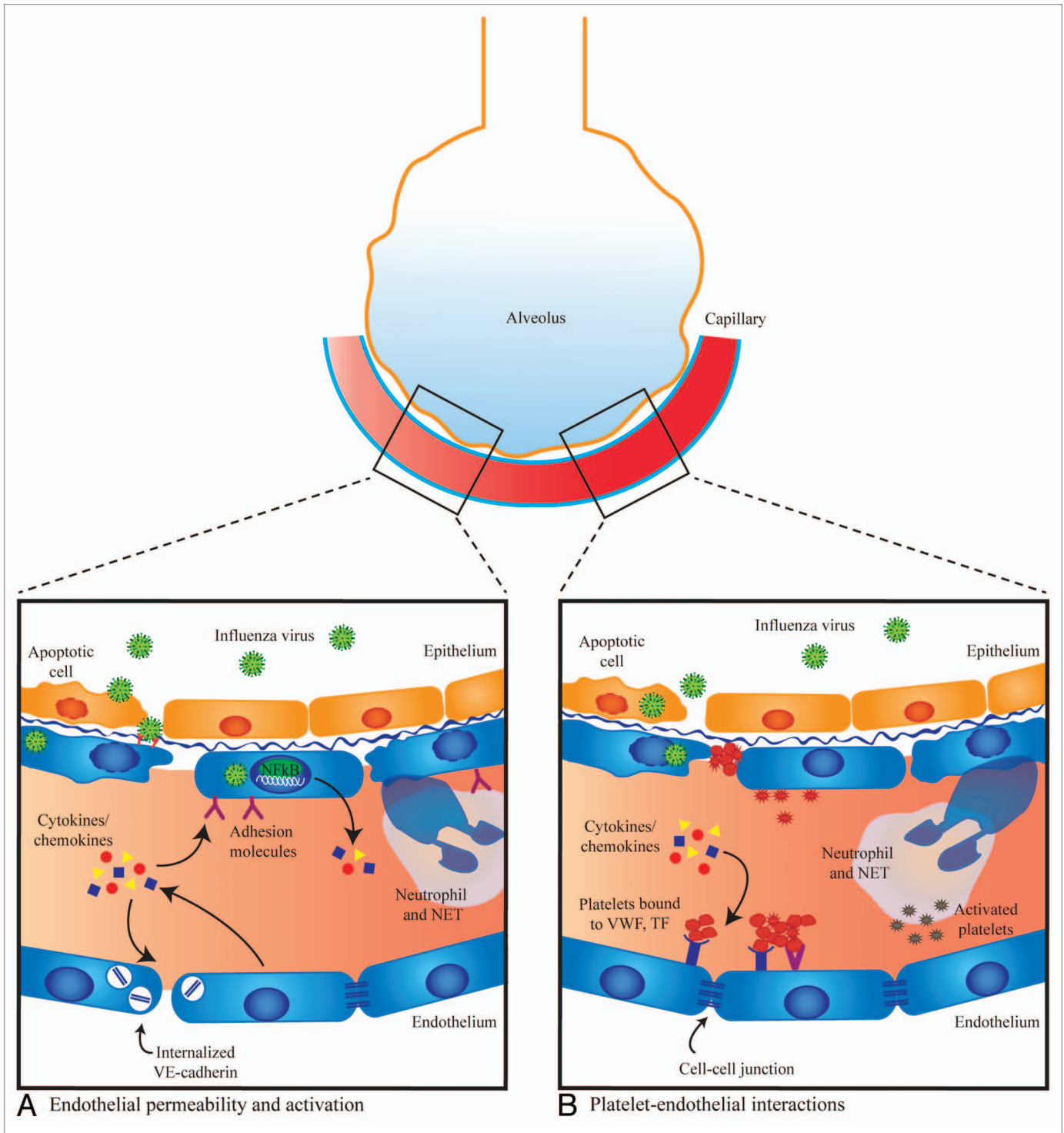
Figure 1. Mechanisms of endothelial dysfunction in influenza virus infection. (**A**) Endothelial permeability and activation. Elevated levels of pro-inflammatory cytokines/chemokines can directly induce endothelial leak through disruption of cell–cell junctions and may also cause endothelial cells to express elevated levels of adhesion molecules that promote leukocyte recruitment. Neutrophils release neutrophil extracellular traps (NETs), which can damage endothelial cells. There is in vitro evidence that influenza can directly infect lung endothelial cells and cause activation of NFκB, endothelial apoptosis, and loss of junctional proteins. In vivo, only avian H5N1 influenza has been shown to directly infect endothelial cells. (**B**) Platelet–endothelial interactions. Circulating cytokines/chemokines cause increased expression of platelet-binding receptors. Influenza virus can directly infect lung endothelium and induce endothelial apoptosis exposing the extracellular matrix, which has a high affinity for platelets. Influenza may directly induce platelet activation and activated platelets bind to endothelium. Activated platelets may interact with neutrophils triggering the production of NETs.

pathogenesis. Furthermore, formoterol, a β2-agonist that induces claudin-5 expression, attenuated influenza-induced pulmonary edema in a murine model of influenza.[32] As mentioned earlier, other investigators have proposed that influenza-induced cytokine production upregulates the human trypsin/hPRSS gene, which in turn mediates the loss of endothelial tight junction protein ZO-1, thereby increasing endothelial permeability.[21]

Influenza and Platelet–Endothelial Interactions

In addition to leak, there is evidence to suggest that endothelial dysfunction following influenza virus infections may manifest as altered thrombogenicity. Healthy endothelial monolayers are anti-thrombogenic; during the H1N1 pandemic in 2009 (H1N1pdm09), there were multiple reports of flu-associated thrombosis. A retrospective review of 119 hospitalized patients found that 7 patients (5.9%) had thrombotic vascular events that were diagnosed or occurred during hospitalization.[40] Three of these patients had arterial thrombosis and four had venous thrombosis. A study in Michigan looked at 10 patients with H1N1pdm09 influenza who were admitted to the intensive care unit with ARDS.[41] Five had pulmonary emboli, while two others showed evidence of hypercoagulation. Whether these complications are directly attributable to the virus or simply reflect the overall severity of illness remains unclear.

Similarly, epidemiological evidence supporting a link between influenza and cardiovascular disease has been reported for decades.[42-44] A temporal relationship between influenza infections and the incidence of cardiovascular disease has been reported by a number of groups[42-47] and several studies have found the influenza vaccine to be associated with a reduction in stroke, transient ischemic attack, and hospitalization due to cardiac disease.[48-50]

The link between influenza infection and cardiovascular disease has also been reported in animals. In one study, apoE$^{-/-}$ mice (an accepted murine model of atherosclerosis) were infected with influenza A/Hong Kong/68 (H3N2) and vascular histology of the aorta was compared with apoE$^{-/-}$ uninfected mice as well as infected wild-type mice. Infected apoE$^{-/-}$ mice showed increased subendothelial cellular infiltration in atherosclerotic plaques compared with uninfected apoE$^{-/-}$ mice. Infected wild-type mice showed no evidence of cellular infiltration of the vascular intima.[51] This group found clustered platelets on the plaques of the majority of infected animals, but none in uninfected animals. Thus, influenza may worsen existing vascular disease resulting in increased endothelial damage and platelet adhesion.

While a causal link between influenza and thrombotic disease has not yet been definitely established,[52] a variety of plausible mechanisms have been proposed that highlight the relationship between the influenza virus, platelet activation, and endothelial dysfunction. Elevated levels of circulating cytokines associated with influenza infection[53] can induce endothelial activation leading to upregulation of cell surface adhesion molecules that favor platelet adhesion. In HUVECs, TNF and IL-1β have been shown to induce the expression of type 1 plasminogen activator inhibitor (PAI-1), which promotes platelet binding, while inhibiting the expression of tissue-type plasminogen activator (tPa) and

thrombomodulin, which are anticoagulant.[54] Similarly, infusion of IL-1 into rabbits caused a time-dependent increase in tissue factor (TF) expression on aortic endothelial cells;[55] TF is known to be a key player in the coagulation process.[56] Endothelial cells also increase production of platelet activating factor (PAF) upon stimulation with TNF and IL-1α.[57] Cytokines can also induce endothelial cell retraction, exposing the pro-atherogenic extracellular matrix.

Influenza-induced lung injury itself may be an important factor promoting thrombosis. Patients with severe influenza require supplemental oxygen because of profound hypoxemia.[1,2] Hypoxia has been shown to induce a pro-inflammatory state in the endothelium causing the increased release of IL-1, IL-6, PAF, ICAM-1, p-selectin, and VWF,[58] all of which are associated with platelet activation and adhesion.

There is also evidence that the influenza virus per se may directly affect the endothelium resulting in platelet adhesion. Influenza H3N2 virus has been shown to infect endothelial cells in vitro and to trigger endothelial cell apoptosis,[32] which is known to enhance platelet adhesion.[59] Endothelial cell death would cause exposure of the extracellular matrix to circulating blood, favoring platelet binding.[60] Cultured HUVEC monolayers infected with influenza have been shown to reduce clotting times by 55% after 3 h of infection and by 66% after 24 h of infection, compared with uninfected monolayers.[61] This was attributed to an increase in TF expression, but at least some of the effect may have been mediated through cytokines and/or the induction of apoptosis as neither was measured in this study.

In addition to affecting the endothelium, the influenza virus may have a direct effect on platelets. An H3N2 virus added directly to platelets was found to induce clumping of both human and rabbit platelets.[62] In this study, both live and dead virus were adsorbed by platelets and the adsorption period was linked to clumping. Infusion of influenza into rabbits induced rapid thrombocytopenia. Platelet activation by influenza has also been documented in humans. In one prospective study comparing patients with severe influenza (H1N1) and patients with severe bacterial pneumonia (all being treated for ARDS) to healthy controls, patients with influenza showed the greatest degree of baseline platelet activation as evidenced by increased formation of platelet-monocyte aggregates and increased binding of the PAC-1 antibody, which binds to the active conformation of $\alpha_{IIb}\beta_3$ integrin on platelets.[63] Platelets could also promote endothelial damage during influenza infection through their interaction with neutrophils. As mentioned earlier, excessive neutrophils have been associated with worse lung pathology in mice infected with H1N1 influenza, attributed to the formation of neutrophil extracellular traps.[26] Intriguingly, it has been reported that addition of activated platelets to neutrophils in vitro is sufficient to induce NET formation.[64] In a mouse model of transfusion-related lung injury, inhibition of platelets either with aspirin (acetylsalicylic acid) or a glycoprotein IIb/IIIa inhibitor reduced both NET formation and lung injury.[64] In a study of acid-induced lung injury in mice, platelet depletion reduced lung neutrophil infiltration, improved histological changes, and increased survival.[65] Depletion of platelets was also found to reduce neutrophil influx and pathological

changes associated with LPS-induced lung injury.[66] Remarkably, however, little is known about platelet–endothelial interactions and their contribution to acute lung injury during severe influenza. This is the subject of ongoing work in our laboratory.

In summary, influenza virus, platelets, and endothelial cells may interact in a variety of ways to induce lung endothelial dysfunction. These include induction of pro-coagulant pathways, activation of platelets and the endothelium, and enhanced interaction between platelets and neutrophils leading to the breakdown of the lung endothelial barrier (**Fig. 1B**).

Future Directions

Microvascular endothelial barrier activation/dysfunction due to influenza virus infection may contribute to the development of severe lung injury that occurs in a subset of patients with influenza. As mortality in this group remains high, novel therapeutic strategies that target the lung endothelium represent a promising therapeutic approach and may elicit a synergistic effect when combined with antiviral and supportive treatments.

A number of agents which enhance the endothelial barrier have been described (e.g., S1P and Slit2N) and may prove effective in combating influenza-induced lung injury.[39,67-69] As platelets may play a detrimental role in the pathogenesis of influenza, treatments that blunt platelet activation or prevent their interaction with endothelial cells also warrant investigation.

In conclusion, influenza virus may interact with the lung microvascular endothelium both directly and indirectly causing damage to the alveolo-capillary membrane resulting in severe disease. Targeting of the microvascular endothelium in conjunction with antiviral administration is thus an attractive therapeutic strategy to improve patient outcomes.

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