Influenza-Induced Priming and Leak of Human Lung Microvascular Endothelium upon Exposure to *Staphylococcus aureus*

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**Abstract**

A major cause of death after influenza virus infection is lung injury due to a bacterial superinfection, yet the mechanism is unknown. Death has been attributed to virus-induced immunosuppression and bacterial overgrowth, but this hypothesis is based on data from the preantibiotic era and animal models that omit antimicrobial therapy. Because of diagnostic uncertainty, most patients with influenza receive antibiotics, making bacterial overgrowth unlikely. Respiratory failure after superinfection presents as acute respiratory distress syndrome, a disorder characterized by lung microvascular leak and edema. The objective of this study was to determine whether the influenza virus sensitizes the lung endothelium to leak upon exposure to circulating bacterial–derived molecular patterns from *Staphylococcus aureus*. In *vitro* as well as in *vivo* models of influenza followed by *S. aureus* superinfection were used. Molecular mechanisms were explored using molecular biology, knockout mice, and human autopsy specimens. Influenza virus infection sensitized human lung endothelium to leak when challenged with *S. aureus*, even at low doses of influenza and even when the pathogens were given days apart. Influenza virus increased endothelial expression of TNFR1 both *in vitro* and in intact lungs, a finding corroborated by human autopsy specimens of patients with influenza. Leak was recapitulated with protein A, a TNFR1 ligand, and sequential infection caused protein A–dependent loss of IkB, cleavage of caspases 8 and 3, and lung endothelial apoptosis. Mice infected sequentially with influenza virus and *S. aureus* developed significantly increased lung edema that was protein A and TNFR1 dependent. Influenza virus primes the lung endothelium to leak, predisposing patients to acute respiratory distress syndrome upon exposure to *S. aureus*.

**Keywords:** influenza; pneumonia; superinfection; pulmonary edema; tumor necrosis factor receptor type I

**Clinical Relevance**

A common cause of death after influenza virus infection is bacterial superinfection, typically with gram-positive organisms such as *Staphylococcus aureus*, but the mechanism is unclear. Our study demonstrates that infection with even low titres of influenza virus primes the lung endothelium to become leaky upon exposure to *S. aureus*, even when the two pathogens are separated by days; the interaction between bacterial protein A and endothelial TNFR1 is the key determinant of this response. Thus, infection with influenza virus predisposes patients toward the development of acute respiratory distress syndrome upon subsequent exposure to *S. aureus*.
Influenza is a major cause of deaths annually, yet many deaths occur due to bacterial superinfection rather than primary viral pneumonia (1, 2). One of the commonest bacterial pathogens to cause this phenomenon is Staphylococcus aureus (3–7), but the mechanism is unclear. The commonly held hypothesis, that the virus causes immunosuppression leading to diminished bacterial clearance (8, 9), is based on autopsy data from the 1918 flu pandemic (2), before the discovery of antibiotics, and from animal models in which antibiotics are not typically used. In the 1957 flu pandemic, when antibiotics were readily available, many autopsy lung cultures were negative (3, 10). Thus, the almost universal administration of empiric broad-spectrum antibiotics (11, 12) to patients with severe influenza virus infection (due to diagnostic uncertainty) makes bacterial overgrowth per se unlikely as the cause of death. Instead, we hypothesized that superinfection-related disease might be related to an aberrant host response initiated by the viral infection and then potentiated by circulating bacterial-derived molecular patterns.

The predominant cause of death in patients with bacterial superinfection after influenza is acute respiratory distress syndrome (ARDS) (10, 13, 14), a potentially fatal illness featuring pulmonary edema and hypoxemia caused by increased permeability of the alveolar–capillary membrane (15). This structure comprises a single layer of alveolar epithelium abutting a single layer of lung microvascular endothelium, separated by scant interstitium, and is as thin as 100 nm in some regions (16); because the lung microvasculature is lined by a layer of continuous endothelium, loss of endothelial barrier integrity is necessary for the development of ARDS (17).

We hypothesized that infection with influenza virus could predispose the lung endothelium to leak after exposure to S. aureus. Viral replication in the alveolar epithelium, the primary target of the virus, leads to cell death (18), exposing the subjacent endothelium to low levels of virus (19). This exposure could trigger up-regulation of surface receptors that, when engaged by S. aureus–derived molecular patterns, could induce endothelial leak, culminating in ARDS.

Our results showed that low doses of influenza virus were sufficient to prime human lung endothelium to leak when subsequently challenged with S. aureus. This effect was due to the induction of TNFR1 surface expression after influenza infection, which was then engaged by protein A, a surface protein of S. aureus and known ligand of TNFR1. Mice infected with influenza virus followed by S. aureus developed marked lung edema that was protein A and TNFR1 dependent. Influenza infection induced up-regulation of TNFR1 on the lung endothelium of mice as well as humans. Thus, influenza virus alters or "primes" the lung endothelium to leak upon exposure to S. aureus, predisposing to ARDS and contributing to respiratory failure from bacterial superinfection. Some of the results of this study have been previously reported in the form of an abstract (20).

Materials and Methods

Full MATERIALS AND METHODS are available in the online supplement.

Priming Assay

Influenza A/HK-X31 (H3N2) virus was added to human microvascular endothelial cells derived from human lungs in serum-free media at a multiplicity of infection (MOI) of 0.1–1.0 for 1 hour, after which complete media were added for a final serum concentration of 2.5%. At 16–72 hours after influenza virus infection, the media were aspirated and replaced with complete media to which nonviable S. aureus (MOI of 100; ATCC 29213; ATCC, Manassas, VA) was added.

Statistical Analysis

Statistical analysis was performed by use of GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Data are presented as mean (±SEM) unless otherwise indicated. One-way ANOVAs were performed followed by post hoc between-group analysis by Bonferroni’s multiple comparison test. A P value less than 0.05 was considered significant.

Ethics

All procedures were performed in accordance with National Institutes of Health guidelines and were approved by the local animal care authorities. Written informed consent from the deceased next-of-kin was obtained before human autopsies and autopsies were performed within 72 hours of death.

Results

Exposure to Low-Dose Influenza Virus Primes Lung Endothelium to Leak after Exposure to S. aureus

Primary human lung microvascular endothelial cells were grown on transwells and infected with a low dose of influenza virus A virus (H3N2; MOI = 0.1); 16–72 hours later, the monolayers were exposed to nonviable S. aureus and the effect on endothelial permeability was determined. Dead instead of live bacteria were used for practical reasons, but also to mimic the clinical effect of antibiotics, sterilizing the bacteria while generating pathogen-associated molecular patterns (PAMPS).

At doses of influenza virus or S. aureus that alone caused minimal leak, sequential infection induced a synergistic increase in endothelial permeability, as measured by a marked fall in transendothelial electrical resistance; this priming effect was evident even when S. aureus was given up to 72 hours after viral infection (Figure 1A). The increased leakiness of the endothelium was to both ions and macromolecules, as we observed similar findings using a fluorescein tracer (Figure 1B). The identical phenomenon was observed using heat-killed or antibiotic-killed bacteria (Figure 1C). The effect was even greater at higher doses of influenza virus (MOI=1), despite a slight increase in permeability in response to the virus alone (Figure 1D).

As endothelial cells are polarized, we infected lung endothelial monolayers with influenza virus from the basal aspect, as might occur in vivo. Priming-induced leak still occurred, indicating that the effect is independent of cell polarity (Figure 1E).

The assay was order specific, as we did not observe synergistic leak in cells exposed first to S. aureus followed by influenza virus 24 hours later (Figure 1F). As bacterial superinfection is best described with gram-positive pathogens, like S. aureus, we tested whether priming would occur using the prototypical gram-negative organism, Escherichia coli. Gram-negative pathogens are a common cause of hospital-acquired pneumonia and, of these, E. coli is the main Enterobacteriaceae implicated (21). When lung endothelial cells infected with influenza virus were
Figure 1. Influenza virus primes endothelial cells to leak upon subsequent exposure to Staphylococcus aureus (SA). (A–G) Primary human lung microvascular endothelial cells were infected with influenza virus (flu multiplicity of infection [MOI] = 0.1) for 24 hours, followed by infection with SA. Transendothelial electrical resistance (TEER) was measured 24 hours later, and the fraction of baseline TEER was plotted relative to control cells. (A) Infection with SA was 16–72 hours after influenza virus infection. ANOVA, $P = 0.0011$ (16-h group), 0.0070 (24-h group), 0.0004 (48-h group), and 0.0017 (72-h group) ($n = 4$). Using Bonferroni’s multiple comparison test, $^*P < 0.05$ for the comparison of flu/SA versus control cells and versus flu-alone cells at 16 and 24 hours, and $P < 0.05$ for flu/SA versus all other groups at 48 and 72 hours. (B) Permeability of the endothelial monolayer to fluorescein-Na. Data were normalized to control. ANOVA, $P = 0.0009$ ($n = 4$). $^*P < 0.01$, $^**P < 0.001$. (C) As in A, but using cloxacillin-killed bacteria (CKSA). ANOVA, $P = 0.0034$ ($n = 4$). $^*P < 0.05$, $^**P < 0.01$. **References**
subsequently exposed to E. coli, increased leak did not occur (Figure 1G). Finally, we exposed endothelial cells to the synthetic RNA analog poly I:C, which is used to simulate viral infections (22). This exposure was sufficient to recapitulate the effect of the intact influenza virus (Figure 1H), suggesting that recognition of viral nucleic acid may underpin priming.

The simplest explanation for priming would be that infection of the endothelium causes retraction or sloughing of the cells, leading to increased binding of S. aureus to the underlying extracellular matrix. However, infection with influenza virus had no effect on attachment and internalization of S. aureus by endothelial monolayers (Figure 1I).

Influenza Virus Induces Up-Regulation of Endothelial Pattern-Recognition Receptors
Given that attachment and internalization of S. aureus were unchanged, we reasoned that priming might be attributable to increased detection of S. aureus by influenza virus–infected cells. Thus, we hypothesized that infection with even low doses of influenza virus could induce up-regulation of pattern-recognition receptors (PRRs) capable of engaging PAMPs derived from S. aureus. These include the peptidoglycan (muramyl dipeptide) receptor, nucleotide-binding oligomerization (NOD) 2, TLR1, which recognizes bacterial protein A, and Toll-like receptor (TLR) 2, which engages a heterogeneous group of PAMPs, including staphylococcal lipoteichoic acid (23).

Influenza virus induced a significant increase in NOD2 and TLR2 messenger RNA in lung microvascular endothelial cells (Figure 2A). As the increase in NOD2 was anticipated, given its involvement in the antiviral response (24), we also examined protein levels of RIP (receptor-interacting protein) 2, a downstream effector of NOD2 that is not required for viral immunity, but is important for endothelial activation. Influenza virus induced a modest increase in RIP2 protein (Figure 2B). However, we observed a significant and persistent increase in TNFR1 protein levels in endothelial cells after influenza virus infection, both on the cell surface (Figure 2C) and in whole-cell lysates (Figure 2D). Because poly I:C was able to prime endothelial cells to leak upon exposure to S. aureus (Figure 1H), we determined whether it could similarly increase PRR expression. As with the intact virus, exposure to poly I:C caused an increase in RIP2 and TNFR1 protein expression (Figure 2E).

If the interaction between S. aureus PAMPs and endothelial PRRs accounted for priming-induced leak, we hypothesized that the PAMPs alone should be able to recapitulate the leak induced by the intact bacterium. Purified peptidoglycan from S. aureus, a NOD2 ligand, could not recapitulate priming-induced leak in influenza virus–infected endothelium (Figure 2F). Similarly, purified lipoteichoic acid, a TLR2 ligand (25), did not cause priming-induced leak. However, exposure to purified protein A, a cell surface protein and TNFR1 ligand released from S. aureus (26, 27), caused increased endothelial leak in virally infected cells, albeit to a lesser degree than the intact bacterium (Figure 2G).

Priming-Induced Leak Is Dependent on TNFR1 and Protein A
To further delineate whether NOD2 might contribute to priming-induced leak upon exposure to the intact bacterium, we inhibited the NOD2/RIP2 signaling pathway using GSK2’14, a small-molecule inhibitor of RIP2 (28). This is preferable to inhibition or knockdown of NOD2, given its function in the viral innate immune response. In contrast, RIP2 is dispensable in that pathway, but essential for alternate downstream signaling pathways, such as NF-κB activation (29–31). Although GSK2’14 effectively blocked RIP2 activation in macrophages, as measured by IκB degradation (Figure 3A), it had no effect on priming-induced endothelial leak (Figure 3B), suggesting that NOD2/RIP2 is not essential for detection of S. aureus in this model.

To prove that protein A engagement of endothelial TNFR1 was involved, we obtained a strain of S. aureus–deficient in protein A (spa) (32). Sequential infection with spa almost completely attenuated priming when compared with wild-type S. aureus (Figure 3C). Reciprocally, knockdown of endothelial TNFR1 by small interfering RNA (Figure 3D) inhibited priming-induced leak (Figure 3E), although a comparison of the transendothelial electrical resistance of influenza/ S. aureus–treated cells that received scrambled-RNA versus TNFR1 small interfering RNA did not quite reach statistical significance (P = 0.07).

We considered the additional possibility that activation of the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome by α-hemolysin from S. aureus could contribute to priming-induced leak (33). To test this, we used the agr mutant of S. aureus that lacks hemolysins, but expresses protein A (34). Influenza virus–infected endothelium subsequently exposed to agr S. aureus continued to display a synergistic increase in leak (Figure 3F). Therefore, priming-induced leak does not require α-hemolysin, and is unlikely to involve the NLRP3 inflammasome.

TNFR1 Activation Leads to Endothelial Apoptosis and NFκB Activation
One of the most proximal events after TNFR1 engagement (35) is caspase 8 activation, triggering the extrinsic pathway of apoptosis, a known mechanism for endothelial leak (36–38). Accordingly, exposure to influenza virus followed by S. aureus led to significant cleavage of caspase 8; this was not apparent in cells exposed to spa, implying that it is protein A dependent (Figure 4A). Sequential infection with influenza virus and S. aureus caused a significant increase in endothelial apoptosis and cleavage of caspase 3, again
Figure 2. Influenza causes up-regulation of pattern recognition receptors on endothelial cells. (A) Cells were infected with influenza for 24 hours, and messenger RNA levels were measured by quantitative PCR. Data were normalized to 18S rRNA and are mean ± SE from three experiments. (B) Levels of RIP (receptor-interacting protein) 2 protein after 24 and 48 hours of influenza. *P < 0.05. (C) Measurement of surface TNFR1 expression by flow cytometry was performed in cells treated with influenza for 25 hours. Histogram is representative of five experiments. Control mean fluorescence intensity (MFI) = 11.50 ± 0.52; flu MFI = 16.47 ± 0.62. (D) TNFR1 protein levels in whole-cell lysates were measured after treatment for 24 and 48 hours with influenza. *P < 0.05, **P < 0.01 (n = 3). (E) Protein levels of RIP2 and TNFR1 in whole-cell lysates were measured after treatment for 24 hours with poly I:C (n = 3 for TNFR1; n = 4 for RIP2). *P < 0.05, **P < 0.01. (F–H) Cells were infected with influenza (MOI = 0.1) for 24 hours followed by treatment with SA-derived purified (F) peptidoglycan (PGN), (G) protein A (PA), or (H) lipoteichoic acid (LTA) given twice, 6 hours apart. The final TEER was measured 24 hours later. (F) ANOVA, P = 0.0048 (n = 5). **P < 0.01. (G) ANOVA, P = 0.001 (n = 3). ***P < 0.001, **P < 0.01, *P < 0.05. (H) n = 5. Ctrl, control; NOD, nucleotide-binding oligomerization; TLR, Toll-like receptor.
in a protein A–dependent manner (Figures 4B and 4C).

TNFR1 engagement also triggers activation of endothelial NF-κB, an established mechanism of increased vascular permeability (39) that is initiated by degradation of its inhibitor, IκB. Lung endothelium infected with influenza virus followed by S. aureus exhibited a significant loss of IκB that was protein A dependent (Figure 4D). As endothelial cytokine production could conceivably contribute to leak, we measured a panel of cytokines in the supernatant of sequentially infected cells. We detected only very modest levels of MCP-1 (monocyte chemoattractant protein-1), regulated upon activation, normal T cell expressed and secreted, and IL-10 from infected cells and a notable lack of other mediators including IL-1β and TNF (Figure 4E).

**Priming-Induced Lung Vascular Leak Occurs in a Mouse Model**

To validate our in vitro findings, we developed a mouse model in which mice were inoculated intranasally with influenza virus followed 24 hours later by intratracheal administration of nonviable S. aureus. Under these conditions, sequentially infected mice exhibited a marked increase in pulmonary edema and acute lung injury that was worse than singly infected animals (Figures 5A and 5B).

Interestingly, lung histological analysis of influenza virus–infected mice demonstrated colocalization of viral nucleoprotein with the endothelial marker, PECAM (platelet endothelial cell adhesion molecule)-1, indicating that endothelial exposure to the virus had occurred (see Figure E1 in the online supplement).

Because, under natural conditions PAMPs from S. aureus would be in the circulation (40), we varied the model to administer S. aureus by tail vein rather than intratracheally. This had the added benefit of minimizing confounding from the
Figure 4. TNFR1 activation leads to endothelial apoptosis and NF-κB activation. Cells were treated with influenza for 24 hours followed by infection with wild-type (SA) or Spa SA for 24 hours. (A) Western blot of whole-cell lysates for cleaved caspase 8. Lysates were harvested 6 hours after the addition of SA. ANOVA, $P < 0.0001$ ($n = 5$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. (B) Histogram for annexin V is representative of four independent experiments. Control MFI = 3.09 ± 0.44, Flu MFI = 4.78 ± 0.75, SA MFI = 5.27 ± 0.41, Flu + SA MFI = 7.48 ± 1.03. (C) Western blot of whole-cell lysates for cleaved caspase 3. ANOVA, $P < 0.0001$ ($n = 7$). ***$P < 0.001$. (D) Western blot of whole-cell lysates for IκB. ANOVA, $P = 0.0045$ ($n = 6$). **$P < 0.01$, *$P < 0.05$. (E) Cytokine levels were measured from cell supernatants of sequentially infected cells by Luminex (Luminex Corporation, Austin, TX) assays. Data are mean ± SD from 2 independent experiments. MCP, monocyte chemoattractant protein; RANTES, regulated on activation, normal T cell expressed and secreted.
respiratory epithelium. The interval between influenza virus and S. aureus exposure was 3 days to mimic the human scenario in which patients initially improve, followed 3–4 days later by respiratory deterioration (10). Under these conditions, sequentially infected mice developed significantly more lung edema and arterial hypoxemia than singly infected animals; importantly, these effects were protein A dependent, as they were absent after administration of the spa mutant (Figures 5C and 5D).

Given the up-regulation of TNFR1 in vitro, we considered the possibility that elevated circulating TNF could contribute to leak. We compared plasma TNF concentrations in mice sequentially infected with influenza virus followed by wild-type or spa S. aureus. Despite significant differences in lung edema, concentrations of TNF were similar in the two groups (Figure 5E).

Next, mice were injected with purified protein A alone or after infection with influenza virus. Similar to the in vitro data, mice exposed to influenza virus followed by circulating protein A developed the most lung edema (Figure 5F).

Influenza Virus Infection Is Associated with Increased Lung Endothelial TNFR1 Expression In Vivo; TNFR1 Is Required for Priming-Induced Leak

We then sought evidence of increased TNFR1 expression on the lung endothelium in vivo by immunohistochemistry (41). There was abundant endothelial TNFR1 expression in influenza virus–infected animals, which was absent in uninfected controls (Figure 6A). To confirm that TNFR1 was induced on the luminal aspect of the endothelium, where exposure to circulating PAMPs would occur, we injected lungs of control or infected mice ex vivo via the pulmonary artery with a fluorophore-tagged anti-TNFR1 antibody and washed away unbound fluorescence by subsequent buffer infusion. As predicted, the lung vasculature of influenza virus–infected mice exhibited a marked increase in luminal TNFR1 compared with uninfected controls (Figure 6B).

To validate our hypothesis in humans, we obtained lung tissue blocks from autopsies of intensive care unit patients who died after infection with influenza A virus or intensive care unit patients who died...
of another cause. We performed immunohistochemistry for TNFR1 and analyzed the slides in a blinded fashion. We observed marked up-regulation of TNFR1 on the lung endothelium of influenza virus–infected patients compared with uninfected controls (Figure 6C).

Finally, to prove the requirement for TNFR1 in priming-induced leak in vivo, we obtained TNFR1 knockout mice. As expected (42), these mice developed more lung edema than wild-type animals after influenza infection, even at a lower dose of the virus (32 HAU); importantly however, there was no increase in lung edema in TNFR1−/− mice infected with the virus and subsequently exposed to S. aureus (Figure 6D). Absence of further leak was not due to lung edema already being maximal, as mice infected with a higher dose (128 HAU) of influenza virus displayed increased lung edema.

Discussion

The phenomenon of bacterial superinfection causing death after influenza has been recognized for decades (2), yet the mechanism remains unclear. Although many studies invoke bacterial overgrowth,
epidemiological data suggest almost all patients receive antibiotics (12, 43) and many lung cultures at autopsy are negative (10). The fact that superinfection is associated with worse outcomes, despite universal prescription of antibiotics (12), suggests that superinfection-related disease might be associated with an aberrant host response (44), in addition to a role for immunosuppression by the virus.

A severe form of respiratory failure, known clinically as ARDS (14), is a sequela of influenza and bacterial superinfection (6). ARDS is characterized by alveolar edema and severe hypoxemic respiratory failure; despite mechanical ventilation edema and severe hypoxemic respiratory failure; despite mechanical ventilation and supportive care, the mortality rate approaches 40% (45). In this work, we report a mechanism by which influenza primes the lung endothelium to respond to PAMPs from S. aureus, leading to endothelial activation, apoptosis, and vascular leak. Influenza induced TNFR1 up-regulation on lung microvascular endothelium both in vitro and in vivo. Engagement by protein A from S. aureus, a known TNFR1 ligand (26), was necessary and sufficient for endothelial leak even days after viral exposure. Thus, our data demonstrate that prior infection with influenza virus predisposes to ARDS upon exposure to S. aureus.

We found that in vitro priming of lung microvascular endothelium occurs with exposure to low levels of virus (MOI = 0.1), with a greater effect at higher MOIs. Our animal data, in which influenza virus–infected mice are later injected with purified protein A and develop pulmonary edema, strongly argues that endothelial priming also occurs in vivo. Although the virus is known to be capable of infecting human lung endothelial cells in vitro (38), and we detected evidence of murine lung endothelial infection in vivo (Figure E1), it is impossible to know the degree of endothelial exposure to the virus in vivo. This likely varies with the degree of lower respiratory tract involvement, which is, in turn, determined by virus-specific factors (46) and by underlying patient health (11). However, it is well established that the virus infects the alveolar epithelium (18), causing epithelial cell death, denudement of the alveolar septum, and release of new virions. The compact structure of the alveolar–capillary membrane (16) ensures some degree of endothelial access for the virus. Our findings thus highlight an unintended consequence of the proximity of the epithelium and endothelium in the distal lung, an essential quality for gas exchange, but which permits a novel mechanism of injury.

Our data do not preclude an effect of the influenza virus on the persistence or growth of S. aureus, despite conflicting literature about whether this occurs or its pathological significance (9, 47–49). Indeed, bacterial persistence in the setting of up-regulation of lung endothelial TNFR1 is more likely to trigger enhanced vascular leak. Protein A is a known virulence factor for S. aureus that is produced in large quantities on the bacterial surface (23), and has recently been shown to be released by live bacteria (27). In our model, circulating protein A from live or nonviable bacteria or even bacterial fragments could trigger endothelial apoptosis and activation. Although it is not certain whether the bacteria are resident (e.g., in the nares) (50) or are newly acquired during postviral superinfection, our data predict a similar outcome. Although soluble TNFR1 levels were not measured in our study and might be expected to compete with the intact plasmalemmal receptor, it is interesting to speculate that saturation of the soluble receptor with circulating protein A occurs, allowing TNFR1 signaling.

The phenomenon of viral infection predisposing to secondary bacterial infections is not unique to influenza virus or S. aureus. Others have described a role for viral-mediated up-regulation of TLR3 (51) and the NOD-like sensors and their effect on bacterial lethality (52). Although we and others observed induction of NOD2 after influenza virus infection (24), NOD2/RIP2 signaling did not account for endothelial leak in response to intact S. aureus (Figure 3B). The ability of purified peptidoglycan to induce in vitro endothelial leak after influenza virus may reflect more rapid internalization or relative intracellular concentration of the purified ligand relative to the intact bacterium. Unlike the transmembrane localization of TNFR1, NOD2 is cytoplasmic; lack of immediate access to PAMPs may limit the relevance of NOD2 to the endothelial priming response.

Activation of the NLRP3 inflammasome by both influenza virus and S. aureus has been reported, suggesting the possibility that the inflammasome might contribute to priming. The hemolysin toxins from S. aureus in combination with bacterial lipopeptides are essential for NLRP3 activation (33). However, the agr mutant of S. aureus, which lacks expression of hemolysins (but retains protein A [53]), continued to induce endothelial leak and we detected no IL-1β in the supernatant of infected cells, ruling out inflammasome activation. Thus, our data using the protein A–deficient S. aureus strain and the TNFR1-deficient mouse indicate that the protein A–TNFR1 interaction is the dominant cause of priming-induced leak in our model. However, further research will be necessary to quantify the potential contribution(s) from other components of the bacterium and host endothelium.

The observation that priming can be recapitated by poly I:C implies that other viruses could induce the same effect. Such pathogens would require access to the lower respiratory tract and the ability to penetrate the alveolar endothelial barrier. Although most common viruses are limited to the upper respiratory tract, respiratory syncytial virus, an important pediatric lung pathogen, is capable of infecting alveolar epithelium; such infections are often complicated by bacterial pneumonia and respiratory failure (54). Whether lung endothelial priming also occurs in this setting remains to be determined.

It is possible that the relative contributions of endothelial apoptosis versus NF-kB activation to increased leak depend on the dose of influenza virus to which the endothelium is exposed. This, in turn, depends on the efficiency of viral replication in the overlying epithelium. We demonstrated that priming occurs across a range of MOIs, with higher doses exerting an increasing endothelial cytotoxic effect (38). Both endothelial apoptosis (55, 56) and NF-kB activation (39) are known to induce vascular leak; although the specific pathways downstream of NF-kB that cause leak remain unclear, there are numerous candidates, of which the most obvious is the induction of leak-inducing cytokines (57). The up-regulation of TNFR1 by influenza suggested the possibility that elevated TNF levels might contribute to leak. However, sequentially infected lung endothelium did not produce TNF.
in vitro and, more importantly, TNF levels did not differ between mice with priming-induced pulmonary edema and those without. One caveat is that we did not measure serial TNF levels over time in the two groups of mice, and our assay could not distinguish between biologically active and inactive TNF. In aggregate, however, our data suggest that protein A rather than TNF is the dominant TNFRI ligand contributing to priming-induced leak. Although other inflammatory mediators might contribute to leak, cytokine levels in sequentially infected cells in vitro were very low. This lack of cytokine production is consistent with literature demonstrating that lung microvascular endothelium makes almost no inflammatory mediators unless first stimulated by cytokines (58, 59).

In summary, we describe a novel mechanism by which influenza virus predisposes to ARDS and contributes to respiratory failure after bacterial superinfection with S. aureus. Our results reinforce the growing appreciation of the lung endothelium in the pathogenesis of influenza (38, 60), a relatively unexplored area of research. Therapies that selectively enhance the lung endothelial barrier or modulate its activation (61) may represent a useful approach for the treatment and prevention of bacterial superinfection after influenza.

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