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CAUGHT THE FLU?
Exploring the novel role of high mobility group box 1 protein in severe influenza infection

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ABSTRACT
Severe influenza infections are a common cause of acute respiratory distress syndrome (ARDS). ARDS is characterized by acute lung injury as well as pulmonary edema or "leakage," which is caused by endothelial dysfunction and subsequent alveolar flooding from circulating blood vessels. High mobility group box 1 (HMGB1) is a protein released from lung endothelial cells that acts as a proinflammatory cytokine during bacterial and viral infections. However, the effect of HMGB1 on the endothelium during influenza viral infection is unknown. This study demonstrates that HMGB1 released from primary human microvascular lung endothelial cells (HLMECs) during influenza infection increases endothelial activation and apoptosis, while also disrupting adherens junctions between cells. Sequestering extracellular HMGB1 using the small molecule glycyrrhizin (GCZ) attenuates these effects, suggesting that HMGB1 signaling occurs in a paracrine manner. Inhibition of the Toll-like receptor (TLR) 4 pathway, a downstream effector of HMGB1, with peptide P5779 did not substantially decrease endothelial apoptosis. Therefore, HMGB1 likely elicits its effects on the endothelium in a TLR4-independent manner. These results will elucidate a partial mechanism that potentially explains ARDS symptoms seen in patients with severe influenza infections.

INTRODUCTION

Severe respiratory infections cause increased morbidity and mortality in vulnerable populations including infants, elders, and patients with chronic diseases.[1] Influenza viruses are common causes of respiratory infections, producing an estimated 200,000 hospitalizations and 36,000 deaths within a typical "flu season" in the U.S.[2] Previous studies have shown that patients with novel swine-origin influenza A (H1N1) virus infection had pulmonary inflammation, acute lung injury, and acute respiratory distress syndrome (ARDS) – a syndrome characterized by increased microvascular permeability that can cause respiratory failure.[3] Due to the mutability of influenza viruses and their development of resistance to antiviral medication, there is a need to monitor influenza response to existing drugs and develop novel therapeutic approaches.[4] Better understanding of the molecular players and mechanisms underlying ARDS may decrease morbidity and mortality and improve outcomes in affected patients.

Influenza A virus can infect multiple cell types in the respiratory tract. It primarily binds the respiratory epithelium, which lines the respiratory tract and functions as a physical barrier to potential pathogens. However, in ARDS patients, the virus is hypothesized to infect the lung endothelium – a polarized monolayer that lines blood vessels. ARDS patients have pulmonary edema, hypothesized to be caused by a 'leaky' endothelium that allows liquid from the capillaries to move into the alveolar space.[5] In this case, the virus is suggested to contribute to endothelial dysfunction, microvascular leakage, and lung injury.[6,7]

Since resistance to antiviral therapies continues to increase, novel treatments targeting host responses may be better at preserving lung function. A commonly damaged host pathway involves high mobility group box 1 (HMGB1), a nuclear protein present in eukaryotic cells that stabilizes chromatin.[8] Recent studies have revealed its role as a key regulator of pathological immune responses, such as unrestrained inflammation.[8] HMGB1 is secreted as a delayed mediator of inflammation and transduces signals via receptor for advanced glycation end-products (RAGE), Toll-like receptor (TLR) 2, and TLR4.[8,9] Because systemic inflammatory responses are mediated through these receptors, HMGB1 is now recognized as a cytokine.[9]

RESEARCH DESIGN
Hypothesis

HMGB1 is released from lung endothelial cells following influenza infection, compromising the endothelial barrier and promoting subsequent cell injury.

Cell culture and influenza infection

Primary human lung microvascular endothelial cells (HLMECs) were grown to confluency to establish an intact monolayer in cultures of endothelial growth medium-2 (EGM-2) and fetal bovine serum at 37°C.

A mouse-adapted human influenza strain A (H5N1) was used for influenza infection. Prior to infection, cells were washed with phosphate buffered saline, followed by the addition of serum-free media. Influenza particles were then added to the cells in serum-free media, facilitating viral entry. Following 1-hour incubation at 37°C, EGM-2 with serum was added to establish normal culture conditions. Cells were incubated with influenza virus for 24 hours along with any drug co-administration before further experimentation. Specifically, glycyrrhizin (GCZ), a direct inhibitor of extracellular HMGB1, and peptide P5779, an inhibitor of lymphocyte antigen 96 which is required for TLR4 signalling, were used.

Immunoblotting

Levels of intercellular adhesion molecule 1 (ICAM-1) and cleaved caspase-3 expression, which are markers of endothelial activation (a proinflammatory state) and apoptosis, respectively, were measured using standard immunoblotting techniques.

Immunofluorescence

To visualize the distribution of vascular endothelial cadherin (VE-cadherin), which are cell-cell junction proteins, standard immunofluorescent techniques were used. Images were acquired with the Quorum Diskovery Multi-Modal Imaging System.
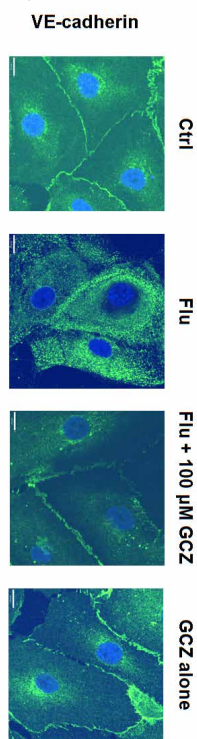
RESULTS
HMGB1 inhibition decreases endothelial activation of ICAM-1 expression during influenza infection

The endothelium can be activated by influenza infection to increase expression of cell adhesion proteins such as ICAM-1.[6] Circulating leukocytes adhere to the activated endothelium and are recruited to proximal tissues. This enables immune cells to mediate antiviral or antibacterial responses and resolve infection, but excessive leukocyte infiltration in the lung can lead to injury.[8] Via western blotting, we observe that HLMECs infected with influenza have increased ICAM-1 expression, suggesting endothelial activation. In infected cells treated with GCZ, a direct inhibitor of extracellular HMGB1, ICAM-1 expression is decreased closer to basal levels, demonstrating significant attenuation of activation (Figure 1).

Figure 2: Inhibition of HMGB1 partially restores VE-cadherin integrity in influenza infected HLMECs.

HLMECs were grown to confluency on coverslips, infected with influenza, and then treated with either GCZ (100 μM) or nothing at all. Through immuno-fluorescence, VE-cadherin was stained to visualize endothelial cell barrier integrity. In uninfected cells, there is continuous VE-cadherin (green) surrounding the cells. In cells infected with influenza, there is induced thinning of VE-cadherin borders and dissociation of junctions, leading to increased intercellular gap formation. This would suggest increased endothelial permeability and an impaired endothelial barrier. With influenza infection and GCZ treatment, there is partial restoration of VE-cadherin surrounding cells and less intercellular gap formation. "GCZ alone" is a control that shows that treatment alone does not affect the cells and the integrity of VE-cadherin (n = 2).

VE-cadherin

Ctrl

Flu

Flu + 100 μM GCZ

GCZ alone

Inhibition of HMGB1, but not TLR4 signalling, decreases influenza-induced endothelial cell apoptosis

Influenza infection of endothelial cells induces apoptosis.[7] Cleavage of caspase-3 is among the final steps of the apoptotic pathway, enabling cleaved caspase-3 to be used as a marker of apoptosis.[7] As seen using western blotting, infected endothelial cells treated with GCZ had significantly reduced cleaved caspase-3 compared to non-GCZ-treated infected endothelial cells (~50%). As GCZ is an inhibitor of extracellular HMGB1, this suggests that paracrine HMGB1 signalling may promote apoptosis in influenza-infected endothelial cells. Treatment of infected endothelial cells with peptide P5779 did not substantially reduce cleaved caspase-3 levels. This suggests HMGB1 may elicit its effects independent of TLR4.

HMGB1 inhibition partially restores VE-cadherin integrity during influenza infection

Infection of the endothelium by influenza viruses enhances endothelial permeability, reflecting endothelial apoptosis or remodeling of endothelial cell-cell junctions such as tight junctions and adherens junctions.[11] To visualize changes in junction integrity, immunofluorescence was used to assess the distribution of VE-cadherin. This endothelial-specific adherens junction protein plays a key role in barrier function and paracellular permeability at the plasma membrane.[7,12] Fluorescent staining showed that VE-cadherin in control endothelial cells were continuous and had tight cell-cell contacts without intercellular gaps. However, endothelial cells infected with influenza had discontinuous VE-cadherin, which was partially rescued through extracellular HMGB1 inhibition with GCZ treatment (Figure 2).

DISCUSSION

Severe influenza infections cause lung damage and affected patients often require admission to intensive care for respiratory failure. There, they exhibit a mortality rate of ~20% despite mechanical ventilation and antiviral therapies.[13] The respiratory deterioration is often caused by virus-induced acute lung injury, which leads to flooding of the alveolar com-

partment, development of ARDS, and death in some cases.[14] Specifically, influenza infection can affect the endothelial barrier – a key component of the alveolar-capillary membrane that normally prevents vascular leakage into the alveolar compartment.[14] During disease states, endothelial leakage is characterized by pulmonary edema, where fluid reduces gas exchange and can result in respiratory failure.[15] Influenza infection can also upregulate the expression of endothelial adhesion molecules, enabling recruitment of leukocytes to the alveolus for an immune response.[16] However, if the response is too extensive, lung injury can result.[16]

This study examines the role of extracellular HMGB1 during influenza infection of the pulmonary endothelium. Inhibiting extracellular HMGB1 with GCZ was shown to decrease the resulting endothelial activation by reducing ICAM-1 expression on influenza-infected endothelial cells. Interestingly, multiple groups have shown that recombinant HMGB1 induces a proinflammatory phenotype by increasing expression of leukocyte adhesion molecules, such as ICAM-1, in a dose-dependent manner.[17,18] These results corroborate our finding that HMGB1 inhibition decreases ICAM-1 expression in influenza-infected endothelial cells.

During influenza infection, endothelial cell apoptosis increases permeability of the endothelium.[8] We show that influenza-infected cells treated with GCZ had attenuated cleaved caspase-3. These results show that inhibiting extracellular HMGB1 attenuates influenza-induced endothelial cell apoptosis, suggesting that, when not inhibited, HMGB1 acts in a paracrine manner to induce endothelial cell apoptosis. In unpublished work, our group has observed that HMGB1 knockdown (i.e. blocking intracellular HMGB1) also yields similar effects, suggesting that HMGB1 is released by influenza-infected cells to elicit its effects on neighbouring cells. Immunoblotting revealed that peptide P5779 does not attenuate cleaved caspase-3 levels, suggesting HMGB1 acts through a TLR4-independent pathway. Studies have demonstrated that recombinant HMGB1 can cause endothelial apoptosis by eliciting stress signaling pathways other than TLR4, which

Influenza infection
Nucleus
Cell junction maintained

HMGB1 release
Binding to RAGE
RAGE signalling
NF-kB

Cell apoptosis
Cell junction disrupted

Adhesion molecule expression

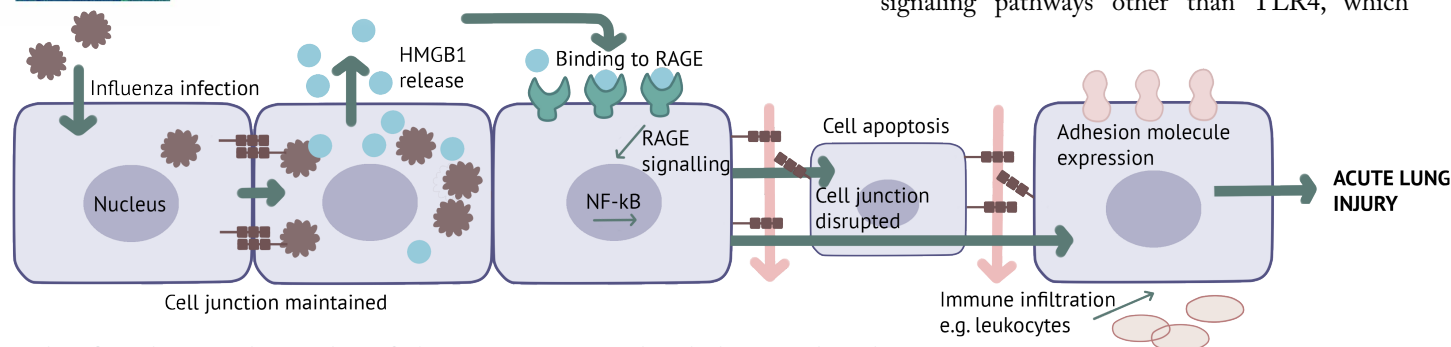**ACUTE LUNG INJURY**

Immune infiltration e.g. leukocytes

Figure 3: Possible mechanism by which HMGB1 acts to modulate endothelial activation, apoptosis, and junction disruption during influenza infection.

Influenza virus infects lung endothelial cells and causes release of HMGB1 into the extracellular space. HMGB1 then acts on a receptor, possibly RAGE, to cause downstream signalling and activate stress signaling pathways, thereby causing the observed effects of increased endothelial activation, apoptosis, and junction disruption. The consequences of these effects in vivo would be vascular leakage, which would lead to pulmonary edema, and greater immune infiltration. These combined effects would then contribute to the observed lung injury during severe influenza infections in ARDS patients.

further supports the lack of effect of P5779.[19,20] Thus, inhibiting the ability of HMGB1 to elicit these pathways may attenuate the apoptosis of lung endothelial cells during influenza infection.

We also investigated how influenza infection of endothelial cells affects adherens junctions such as VE-cadherin, which regulates paracellular permeability to circulating leukocytes.[21] Immunofluorescence staining revealed that influenza infection of endothelial cells disrupts VE-cadherin, and that GCZ treatment partially prevents this disruption. Interestingly, a study shows that a recombinant Slit protein fragment, Slit2N, is able to improve microvascular barrier integrity and decrease vascular leakage in a murine model of influenza infection. Slit2N acts to promote association between p120-catenin and VE-cadherin, thereby stabilizing adherens junctions and improving barrier integrity during inflammatory states.[22] These results highlight the importance of improving destabilized junctions in infected states as the influenza-infected mice that received Slit2N had reduced mortality. This is consistent with the loss of VE-cadherin integrity during influenza infection as well as vascular leakage seen in our results and ARDS patients, suggesting that therapies rescuing endothelial barrier integrity may be effective.

In summary, the presented data provides evidence that HMGB1 plays a role in endothelial dysfunction during influenza infection. When HMGB1 is released from an infected cell, it likely binds to its receptors on neighbouring cells in a paracrine fashion to induce endothelial activation, increase apoptosis, and redistribute adherens junction proteins. These effects present a partial mechanism that provides insight into the causes of ARDS symptoms (Figure 3).[23]

Future investigations should elucidate the mechanism by which HMGB1 modulates endothelial apoptosis, activation, and barrier function. Our study suggests that HMGB1 induces these effects independently of TLR4. Using inhibitors or siRNA treatment, we can explore whether HMGB1 acts through other receptors, such as RAGE or TLR2. Furthermore, although the data suggests that HMGB1 affects adherens junctions during infection, future experiments should address whether it affects claudins, which are tight junction proteins that contribute to the paracellular barrier. Future work should also progress to a mouse model of influenza infection to determine the contributing role of HMGB1 to lung injury and pulmonary edema. A better understanding of HMGB1's mechanism of action in ARDS patients may establish it as a therapeutic target in severe influenza infection.
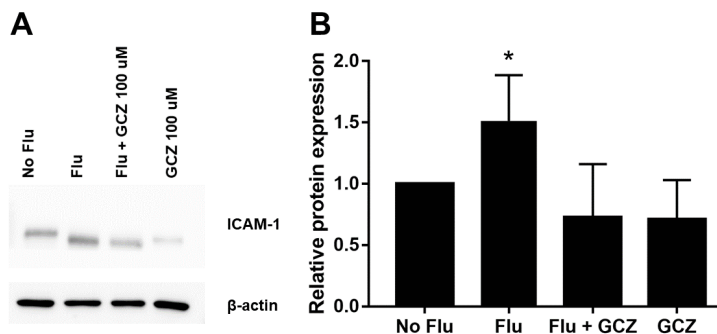
CONCLUSION

During severe influenza infections, HMGB1 can compromise the endothelium, leading to increased leakage of circulating fluid into the alveolar space, as well as lung injury from the immune response to surrounding tissues. Although our findings require confirmation *in vivo*, they imply that endothelial dysfunction is critical to the vascular leakage that contributes to mortality among ARDS patients. Understanding the mechanisms by which influenza impedes endothelial barrier function would allow for the further development of therapeutics targeting harmful host pathways. Our study highlights how HMGB1 signalling may serve as a novel target for influenza therapeutics to reduce endothelial activation and apoptosis, and enhance cell junction continuity.

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Figure 1: Inhibition of HMGB1 causes lower expression of endothelial activation marker ICAM-1 in influenza-infected HLMECs:HLMECs were grown to confluency, infected with influenza, and then treated with either GCZ (100 µM) or nothing at all. Through western blotting, ICAM-1 was probed to detect endothelial activation. A: Visual representation of one of the western blots. B: Analysis of blots shows that treatment with GCZ to influenza-infected HLMECs decreases influenza-induced endothelial activation of ICAM-1 closer to basal levels. n = 3; *, P < 0.05 versus control and Flu + GCZ groups.

REVIEWED BY VICTORIA MINTSOPOULOS

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