

## Influenza lung injury: mechanisms and therapeutic opportunities

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**Gregory DJ, Kobzik L.** Influenza lung injury: mechanisms and therapeutic opportunities. *Am J Physiol Lung Cell Mol Physiol* 309: L1041–L1046, 2015. First published September 25, 2015; doi:10.1152/ajplung.00283.2015.—In this Perspectives, we discuss some recent developments in the pathogenesis of acute lung injury following influenza infection, with an emphasis on promising therapeutic leads. Damage to the alveolar-capillary barrier has been quantified in mice, and agents have been identified that can help to preserve barrier integrity, such as vasculotide, angiopoietin-like 4 neutralization, and sphingosine 1-phosphate mimics. Results from studies using mesenchymal stem cells have been disappointing, despite promising data in other types of lung injury. The roles of fatty acid binding protein 5, prostaglandin E2, and the interplay between IFN- $\gamma$  and STAT1 in epithelial signaling during infection have been addressed in vitro. Finally, we discuss the role of autophagy in inflammatory cytokine production and the viral life cycle and the opportunities this presents for intervention.

acute lung injury; autophagy; influenza; signaling

WE REVIEW HERE RECENT DEVELOPMENTS in the pathogenesis of acute lung injury from influenza. Epithelial biology offers new clues to mechanisms and risks, while translational efforts targeting endothelium identify promising therapeutic candidates. Whether you lean toward basic or applied research, there is good news to share.

### *Damage to the Alveolar-Capillary Barrier During Influenza Infection*

Sanders et al. (48) have performed a longitudinal study of epithelial barrier breakdown in mice infected with virulent PR8 or less virulent x31 strains of influenza A virus (IAV). They compared arterial blood oxygenation, respiratory gas exchange, alveolar epithelial cell survival, and mouse mortality and concluded that infection is survivable until about 10% of type I cells are lost. Notably, they find continued inflammation in survivors even 30 days after infection, by which time acute markers of infection, including viral presence, have resolved. Others have also reported sustained lung damage, including Gotts et al. (19) who found increased barrier permeability to radiolabeled albumin even 3 wk after nonlethal infection. The more commonly used measure of total protein in bronchoalveolar lavage fluid returns to normal within about 3 wk, indicating that improved protein clearance compensates for the sustained permeability (19). This is reflected in the slow recovery of lung capacity measured by plethysmography (29).

A possible beneficial effect of this sustained permeability is to allow accumulation of mediators such as plasma gelsolin, which we have shown to protect against postinfluenza bacterial

pneumonia by enhancing macrophage function (59). However, earlier interventions to preserve the alveolar-capillary barrier during the acute phase of primary influenza infection (or other insults) also may have beneficial effects. Daily administration of a synthetic angiopoietin-1 mimic improves survival of mice infected with PR8 or x31 (50). The mimic, called vasculotide, activates the cell-cell adhesion protein TEK (49, 50) and has previously been shown to maintain barrier function of human microvascular endothelial cells treated in vitro with lipopolysaccharide and to improve alveolar-capillary barrier function and survival in mice during experimental endotoxemia (14). Similarly, using a monoclonal antibody to neutralize angiopoietin-like 4 (ANGPTL4) improves both barrier function and infiltration following experimental infection with PR8 (33). In contrast to angiopoietin-1, ANGPTL4 interacts with integrin  $\alpha_5\beta_1$ , VE-cadherin, and claudin-5 to weaken cell-cell interactions and loosen the epithelial barrier (23). Its expression is elevated in both experimentally infected mouse lungs and human postmortem lung tissue, suggesting a maladaptive response (33).

Sphingosine 1-phosphate (S1P) is a physiological lipid signaling intermediate involved in both immunoregulation and epithelial barrier function (7, 41). The S1P analog 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol [AAL(R)] has shown some success in protecting mice from infection with either 2009 pandemic or mouse-adapted H1N1 strains (55). Mice treated with AAL(R) 1 h after infection show improvements in lung damage and survival compared with vehicle-treated controls. This is accompanied by reduction in proinflammatory cytokine production, including type I interferons and macrophage chemokines (55). AAL(R) is phosphorylated in vivo by sphingosine kinase 2 to AFD(R) ([[(2R)-2-amino-4-(4-heptoxyphenyl)-2-methylbutyl] dihydrogen phosphate), which stimulates S1P receptors (S1PR) 1, 3, 4, and 5 (27, 42). A more selective S1PR1 agonist, CYM5442, also reduces cytokine production by acting on endothelial cells rather than lymphocytes (52), although its effect on mortality has not, to our knowledge, been reported. Given that excessive cytokine production is correlated with poor prognosis (6, 34, 42, 44), this leads to a model in which pathology is mediated by an endothelial cell-driven cytokine storm, which is suppressed by S1P analogs (42). However, suppression of the cytokine response has so far failed to match the therapeutic benefit of S1P analogs or angiopoietin (ant)agonists (47). In addition to regulating immune cell activity and recruitment, S1P is a regulator of endothelial cell adhesion (41, 46), raising the possibility that mortality and cytokine storm are both effects of the permeabilized alveolar-capillary barrier function, rather than the cytokine storm being directly causative of mortality. Clearly, further research is necessary to establish the order of events.

The loss of type I epithelial cells may be accompanied by differentiation of type II cells into type I cells, as a repair mechanism (21). Two groups have recently attempted to en-

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hance this repair by administering mesenchymal stem cells (MSCs) intravenously (13, 19). However, this achieved only a modest reduction in viral load and thrombosis 1 wk after infection (19), with no discernable effects on other markers of lung injury even in combination with oseltamivir (13). Furthermore, porcine MSCs have been shown to support infection and replication of influenza virus (30, 53), raising the possibility that MSC treatment could exacerbate viral replication. In contrast, activation of the signaling kinase AMPK with metformin or AICAR improves the recovery of pulmonary microvascular endothelial cell barrier function from lipopolysaccharide injury in vitro and halts the increase in permeability in rat lungs during experimental endotoxemia (28), suggesting that enhancing endothelial repair mechanisms may be a more productive approach than reseeding the epithelium.

The failure of MSCs to substantially improve outcome of influenza infection is disappointing, since they have shown considerable promise in other models of lung injury (reviewed in Ref. 4) and have been subjected to Phase 1 clinical trial for acute respiratory distress syndrome (56). The benefit apparently comes from paracrine influence on epithelial cells rather than direct repopulation, since conditioned medium can often substantially replicate the effect (18, 24, 31, 32, 38, 63). Prior siRNA knockdown of keratinocyte growth factor (KGF) expression in MSCs reduces the ability of conditioned medium to restore alveolar fluid clearance in an ex vivo model of endotoxin injury in human lungs, and this can be rescued by addition of recombinant KGF (31). Similarly, the ability of MSC-conditioned medium to restore alveolar fluid clearance in human lungs rejected for transplant is inhibited by a KGF blocking antibody (38), and recombinant KGF restores the fluid clearance in *Escherichia coli*-injured lungs to a similar extent as MSC-conditioned medium (32). In vitro, KGF depletion inhibits the ability of concentrated MSC-conditioned medium to rescue inflammatory cytokine-mediated inhibition of sodium transport (18) or to enhance A549 scratch wound repair (9). Interestingly, MSC-conditioned medium promotes an anti-inflammatory, M2 macrophage phenotype, and this may be due to other factors such as insulin-related growth factor-I (IGF-I), rather than KGF (24, 32). Although this may be beneficial in the context of sterile or *E. coli*-mediated lung injury, it may be less appropriate during influenza infection. Alternatively, growing MSCs under hypoxia and in the presence of proinflammatory cytokines to mimic the environment of an injured lung alters the cytokine content of their conditioned medium, including increasing IL-1Ra and prostaglandin E<sub>2</sub> and reducing KGF secretion (18). Thus the environment of an influenza-injured lung may itself inhibit the ability of MSCs to repair the injury. In either case, more detailed identification of the protective soluble mediators secreted by MSCs may enable more effective intervention than use of MSCs themselves.

### Role of Ion Transport

Many of the studies cited above focus on the ability of MSCs to restore fluid transport by regulating the activity of sodium channels. The ability of influenza virus to inhibit the epithelial sodium channel has been long established (5). More recent data show that infection also causes ubiquitin-dependent degradation of the cystic fibrosis transmembrane conductance regular protein (CFTR) (35, 36, 54). This inhibition may contribute to

pathology, since agonist stimulation of alveolar fluid clearance reduces pulmonary edema in influenza-infected mice (57). However, mice heterozygous for the  $\Delta F508$  cystic fibrosis-causing CFTR mutation, which decreases expression of active CFTR, show reduced edema and hypoxemia and delayed mortality following influenza virus infection, without any effect on viral replication (1). Macrophage depletion and adoptive transfer experiments show that the protective effect is mediated by macrophages, and it is inhibited by blocking antibodies to IL-6 and TGF- $\beta$  (1, 58). Further studies are required to determine whether the degradation of CFTR by influenza virus is an adaptation to increase viral transmission by limiting host pathology or whether the benefit seen in  $\Delta F508$  heterozygotes results from host compensation for reduced CFTR expression.

### Signaling Events in the Epithelium

An inflammatory response, including reactive oxygen species and other toxic molecules, cytokine secretion, and recruitment of immune cells, is clearly characteristic of the host's process to eliminate the virus. But much of the damage that occurs during severe infection, both to the lung epithelium and to endothelial tissues, can be attributed to this very same mechanism. Understanding control of the inflammatory response to influenza infection may present opportunities for therapeutic intervention. This is not news: the role of inflammation and opportunities for therapeutic targeting have been extensively reviewed elsewhere (10, 12); here we will focus on some recent developments and their significance.

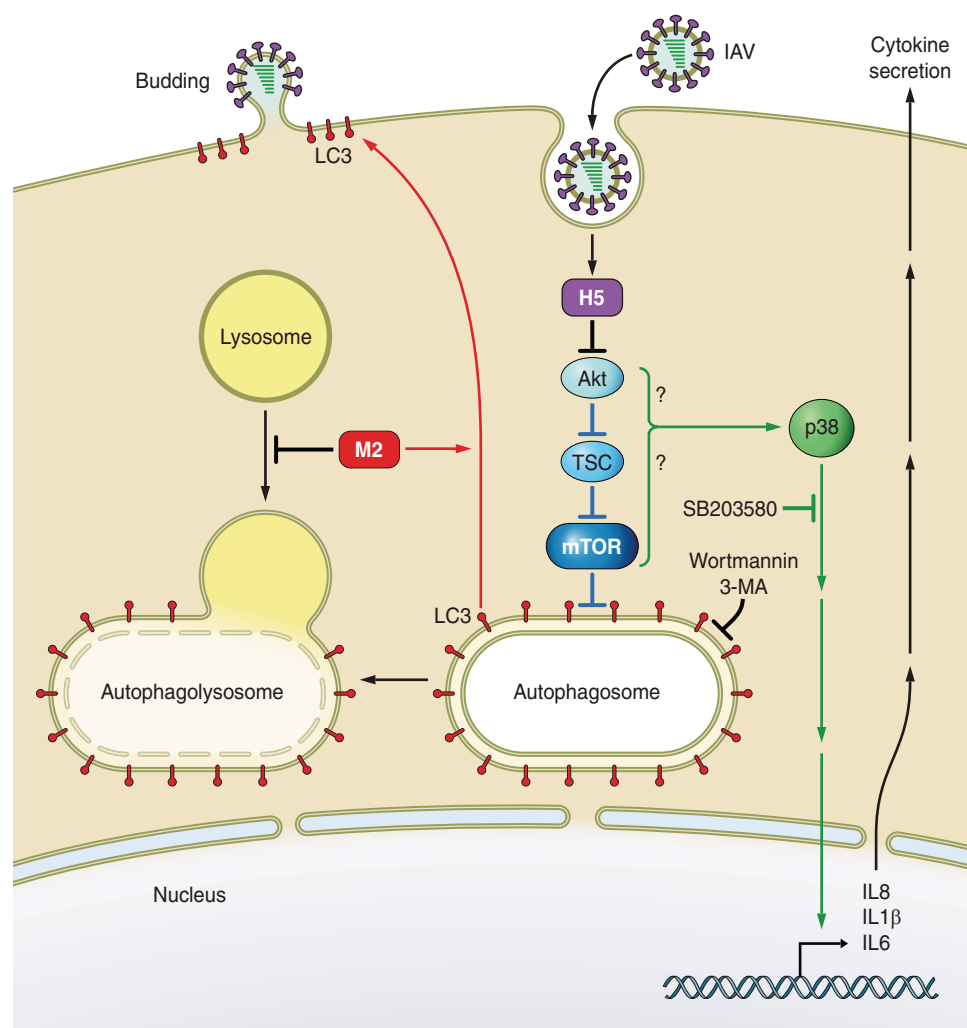
The contrasting beneficial and pathological effects of inflammation are illustrated in a study of fatty acid binding protein 5 (FABP5) (16). *Fabp5* knockout mice have a greatly improved ability to prevent replication of the mouse-adapted H1N1 strain PR8, but at the price of enhanced and sustained infiltration that affects the lungs even after pathology has resolved in wild-type mice. The infection dose in this study was survivable, but one might expect that a higher initial viral dose would lead to both further enhanced inflammation and excess mortality in the knockout mice. Although direct inhibition of FABP5 may be a risky approach to improving viral clearance, the study does provide some interesting mechanistic details. The authors propose that FABP5 acts in part as an activator of the anti-inflammatory transcription factor peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ). A prostanoid PPAR $\gamma$  agonist has been previously shown to reduce inflammation and mortality during influenza infection (8). Gally et al. (16) report a physical interaction between the two proteins that is reduced by infection in vitro, and that *Fabp5* expression is repressed in wild-type lungs following infection. They propose that this reduced FABP5 expression results in weakened PPAR $\gamma$  activity and enhanced inflammation. However, they observe reduced *Fabp5* mRNA expression as late as 14 days after infection, by which time the inflammation has largely resolved in wild-type mice. This suggests that a mechanism other than FABP5-mediated PPAR $\gamma$  activity is responsible for resolution but leaves open the possibility that intervening to restore FABP5 activity may be beneficial later in infection.

The complex interplay between virus and different components of the epithelium, endothelium, and immune system

Ito et al. (25) used a simpler cell coculture system supported by conditioned media, recombinant cytokines, and inhibitors to

### *Role of Autophagy in Proinflammatory Signaling*

Autophagy (strictly, macroautophagy) is an evolutionarily ancient mechanism for nutrient homoeostasis and organelle turnover that also forms an important innate defense mechanism against intracellular pathogens. Characteristic double-membraned vesicles form within the cytoplasm, engulfing targets that can include cytoplasmic aggregates, mito-



**Fig. 1. Role of autophagy in cytokine induction and viral budding.** H5 protein activates autophagy by inhibiting the repressive Akt cascade and induces cytokine production via p38 MAPK. M2 prevents fusion of autophagosomes with lysosomes and relocates LC3 to the plasma membrane. IAV, influenza A virus; mTOR, mammalian target of rapamycin; 3-MA, 3-methylacetate; TSC, tuberous sclerosis complex. See text for further details.



chondria, bacteria, or viral particles into what is termed an autophagosome (reviewed in Refs. 22, 40). This then fuses with lysosomes, and its contents are degraded (Fig. 1, *bottom right*). The process has been shown to be beneficial for controlling many different pathogens; other pathogens have incorporated various aspects of autophagy into their life cycles (22, 26).

IAV infection has been shown to induce autophagosome formation in A549 and other epithelial cell line models (17, 62). However, fusion with lysosomes is inhibited (17), indicating that, like other pathogens, IAV evades autophagy as a defense mechanism. Instead, it appears to subvert the process to promote viral budding by a mechanism that involves relocating the classic marker of autophagic vesicles, LC3, to the plasma membrane (2) (Fig. 1, shown in red). Chemical inhibition of autophagy using 3-methylacetate or wortmannin in MDCK cells, or siRNA knockdown of LC3 or Beclin-1, which is required for induction of autophagy, in A549 cells reduces viral titer (62), indicating the importance of this subversion to viral replication. Moreover, a FRET-based screen for inhibitors of autophagy during infection identified evodiamine as an inhibitor of IAV replication in A549 cells (11). Interestingly, the vacuolar H<sup>+</sup>-ATPase inhibitor bafilomycin A1 also reduces abundance of LC3 and inhibits viral replication in A549 cells, but at concentrations insufficient to interfere with lysosome acidification (60). It is likely that low-dose bafilomycin A1 is interfering with autophagosome maturation or trafficking, but the mechanism is unknown.

Signaling through autophagy has also been linked to proinflammatory cytokine induction and may contribute to the cytokine storm. Inhibition of autophagy, either chemically or by siRNA knockdown, improves mouse survival following infection of mice with an H5N1 strain (51). This is associated with reduced inflammation in the lung, although it is not clear whether this is a result of inhibited viral replication or modulation of the host response (51). Some mechanistic details come from a study that used murine leukemia virus particles pseudotyped to express H5N1 hemagglutinin and neuramidase antigens (43). Inhibiting of autophagy by 3-methyladenosine or by siRNA knockdown of ATG5 or Beclin-1 also inhibited induction of proinflammatory cytokines, including IL-1 $\beta$ , IL-6, and IL-8, in A549 cells (43). Proinflammatory signaling is not inhibited by chloroquine (43), which blocks autophagosome acidification, showing that the induction is not

sensitive to the inhibition of autophagosome maturation observed by Gannagé et al. (17). The induction of autophagy by IAV involves the Akt-TSC-mTOR (tuberous sclerosis complex - mammalian target of rapamycin) pathway (11, 51) (Fig. 1, shown in blue). 3-Methylacetate treatment in vitro reduces activation of the mitogen-activated protein kinase (MAPK) p38 on infection with H5N1 pseudotyped particles, and inhibition of p38 with SB203580 inhibits cytokine induction (43) (Fig. 1, shown in green). Interestingly, the effect may not be restricted to epithelial cells, since avian H5N1 and H7N1 infection strongly induces p38 signaling in HUVEC and Vero cells, and p38 inhibition reduces inflammation and mortality in vivo (3). It is also possible that mitochondria play a role in the inflammation. Mice deficient in MAPK kinase 3 (MKK3, a p38 upstream kinase) show improved survival and reduced lung injury during acute endotoxemia (37). MKK3 is proposed as an inhibitor of autophagic turnover of mitochondria, and its deletion improves mitochondrial integrity and resistance to stress. In contrast, inhibition of autophagy with 3-methylacetate reduces mitochondrial integrity in endothelial cells (37).

Both inhibition of vesicle fusion and LC3 location have been attributed to the viral M2 protein (2, 17) (Fig. 1, shown in red). However, M2 is not included in the pseudotyped viral particles used by Pan et al. (43), indicating a role for viral hemagglutinin and/or neuramidase as well. HA has been linked to activation of LC3 in CS-1 monkey fibroblasts and 293T cells (61). Interestingly, HA serotypes that are more susceptible to protease cleavage (H5, H7) are more potent activators of LC3 than protease resistant serotypes (H1, H3, H9). This suggests that the severe inflammatory pathologies seen in avian H5 and H7 strains may in part be due autophagy-dependent proinflammatory signaling strains (61). H5N1 infection produces a stronger autophagic response than H1N1 in A549 cells and greater inflammation is seen in H5N1-infected mice than following H1N1 infection (51). Thus induction of autophagy by different HA subtypes may represent a critical step in the severe outcomes of highly pathogenic influenza virus infections.

### Summary

This brief tour of recent developments can finish with two main observations. First, painstaking dissection of the cell and molecular biology of lung infection by influenza is paying off enormously. The field is benefiting from abun-

Table 1. Summary of experimental and translational interventions against influenza-induced acute lung injury discussed in text

Intervention	Target	Proposed Biological Function	Reference
Vasculotide	TEK cell-cell adhesion protein	Maintains barrier function	50
Monoclonal antibody	Angiopoietin-like 4	Maintains barrier function	33
Metformin, AICAR	AMPK	Maintains barrier function	28
Mesenchymal stem cells		Attempted repair of endothelial barrier	13, 19
AAL(R)	S1PR family	Reduces proinflammatory cytokines; may regulate barrier function	55
CYM5442	Endothelial S1PR1	Reduces proinflammatory cytokines; may regulate barrier function	52
15-Deoxy- $\Delta$ 12,14-prostaglandin-j2	PPAR $\gamma$	Reduces inflammation	8
Genetic knockout	<i>Fabp5</i>	Reduces viral replication; increases inflammation	16
siRNA knockdown	Beclin-1, LC3, ATG5 (autophagy)	Reduces viral replication; proinflammatory cytokine production	43, 62
3-Methylacetate, wortmannin	PI3-kinase (autophagy)	Reduces viral replication	62
Evodiamine	Autophagy	Reduces viral replication	11
Bafilomycin A1	? Autophagosome maturation	Reduces viral replication	60
Plasma gelsolin	NOS3	Enhances macrophage function	59

dant new insights into the response of epithelial, endothelial, and inflammatory cells to influenza. Second, many of these advances point directly to potential translational strategies for influenza and other serious lung infections, as summarized in Table 1.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

## AUTHOR CONTRIBUTIONS

D.J.G. and L.K. interpreted results of experiments; D.J.G. prepared figures; D.J.G. and L.K. drafted manuscript; D.J.G. and L.K. edited and revised manuscript; D.J.G. and L.K. approved final version of manuscript.

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