How the Respiratory Epithelium Senses and Reacts to Influenza Virus

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Abstract

The human lung is constantly exposed to the environment and potential pathogens. As the interface between host and environment, the respiratory epithelium has evolved sophisticated sensing mechanisms

as part of its defense against pathogens. In this review, we examine how the respiratory epithelium senses and responds to influenza A virus, the biggest cause of respiratory viral deaths worldwide.

Keywords: respiratory epithelium; influenza A virus; viral sensing

Influenza viruses belong to the *Orthomyxoviridae* family. They are enveloped viruses that contain eight negative sense single-stranded RNA (ssRNA) segments encoding 11 proteins, including the hemagglutinin, neuraminidase, and nucleoprotein (1). Influenza A virus (IAV), which causes pandemic and seasonal influenza infections, is one of the four genera of the *Orthomyxoviridae* family (the other three are influenza B and C viruses and *Thogotovirus*) and a major public health burden worldwide (1–3). IAV is responsible for an estimated 645,000 deaths worldwide annually (4, 5). Mutations in the surface proteins (hemagglutinin and neuraminidase) result in antigenic drifts and seasonal epidemics, whereas reassortment of genetic material of two or more strains in a common host forms novel IAV strains and pandemics. The World Health Organization provides annual recommendations for the composition of

influenza virus vaccines before upcoming influenza seasons, but mismatches between the predicted and actual circulating seasonal variants occur as frequently as every 3–4 years (6, 7). Therefore, IAV infection remains a threat to humans, and there is a continued need to understand how the lung protects itself from IAV infection to harness this understanding for new strategies against IAV infections.

IAV preferentially binds α 2,6-linked sialic acid (α 2,6-SA) receptors expressed by airway epithelium from the nose to respiratory bronchioles (8–10). The first epithelial defense takes the form of cell–cell junctions, including tight junctions (located in the apical portion of the lateral epithelial membrane), adherens junctions, desmosomes and gap junctions (11, 12), that are linked to the cellular cytoskeleton via numerous adaptor proteins. These proteins form an impermeable mechanical barrier that is highly resistant to damage during IAV infections (13). The

pseudostratified columnar epithelium in the trachea, bronchi, and bronchioles is interspersed by a further diverse group of cells: serous, club, neuroendocrine, and goblet cells that secrete mucin (not club cells) and a variety of enzymes, protease inhibitors, oxidants, and antimicrobial peptides (14). Among these, mucin, β -defensins, and LL37 have been shown to have specific anti-IAV roles (15–22).

The respiratory epithelium is therefore the first point of defense in the lung's response to IAV. It works closely with the innate immune cells, including monocytes, neutrophils, and natural killer (NK) cells, to deliver an optimal first-line protection system. It also provides a bridge between the innate and adaptive immune responses by producing cytokines and chemokines that attract, mature, and differentiate T and B cells. It communicates with the principal antigen-presenting cells (dendritic cells [DCs]) via secretion of granulocyte-macrophage colony-stimulating factor

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(GM-CSF), thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 and provides a focal point for lymphoid aggregates such as inducible bronchus-associated lymphoid tissue (iBALT) that surround the airways after infection (23). In addition to the infiltrating innate immune cells, there are also lung-resident immune cells— intraepithelial conventional dendritic cells (cDCs), intraepithelial CD8 T cells, innate lymphoid cells (ILCs), and unconventional T-cell populations such as the invariant natural killer T (iNKT) cells, mucosal-associated invariant T cells (MAIT), and $\gamma\delta$ T cells that may play important roles in the “hyper-early” immune activities during influenza. Together with the infiltrating innate immune cells, the respiratory epithelium and resident immune cells form the first innate immune response to IAV infection, and they reduce the viral burden (often clearing the virus altogether) and determine the shape of subsequent T- and B-cell responses, the health of the tissue, and the ensuing immunological memory. In this paper, we examine the first point of this process: how the respiratory epithelium senses and reacts to IAV infection.

Sensing the Influenza Virus

Mucins in the mucus layer are widely regarded as the first defense against IAV. Not only do they form a physical barrier against the virus, but one isoform, Muc5ac, contains α 2,3-linked sialic acid motifs that can act as decoys for the sialic acid receptors on the respiratory epithelium. Thus, the IAV is kept in the mucinous phase and prevented from accessing the airway's surface, a necessary process to internalize the virus (18). More recently, a study suggested that Muc5ac can also relay the presence of viruses (24). Iversen and colleagues showed that airway epithelial cells can sense herpes simplex virus via the mucin layer, triggering the earliest release of CXCL10 from epithelial cells and recruitment of neutrophils independently and even before the early type I IFN responses (24). However, direct studies with IAV have not been performed.

Once the IAV gets past these early barriers, it attaches its surface glycoprotein, hemagglutinin (HA), to sialic acid expressed on sialic acid receptor found on the epithelial cell surface and is endocytosed. In

the endosome, the viral HA is acidified and undergoes a conformational rearrangement, enabling viral and endosomal membranes to fuse (reviewed by Reference 25). The H^+ ions in the acidic environment of the endosome then permeates the virus via its M2 ion channel, disrupting the viral envelope (25). The viral RNA is then released into the cytoplasm of the host cell and imported into the nucleus to facilitate transcription of the viral genome and translation of viral proteins (25). The host cell senses IAV as soon as it is internalized, using pathogen recognition receptors (PRRs). In respiratory epithelium, these are primarily the Toll-like receptors (TLRs), retinoic acid-inducible gene 1-like receptors (RLRs), and NOD (nucleotide-binding oligomerization domain-containing protein)-like receptors (NLRs).

TLRs

TLRs are the first PRRs to confront IAV in the respiratory epithelium. Three TLRs are implicated in IAV recognition by human respiratory epithelial cells: TLR3, TLR7, and TLR8 (2, 26–32). Upon recognition of the relevant protein-associated molecular patterns (PAMPs), these TLRs recruit Toll/IL-1 receptor domain-containing adaptor proteins such as MyD88 (myeloid differentiation primary response 88) and TRIF (Toll/IL-1 receptor domain-containing adapter-inducing IFN- β), which then initiate signal transduction pathways that culminate in the expression of types I and III IFN via the transcription factors NF- κ B and IRFs (IFN-regulatory factors) (Figure 1) (2). IAV-infected MyD88 $^{-/-}$ and MyD88/TRIF double-deficient mice display markedly reduced cytokine production in the lung compared with wild-type mice, indicating the importance of this pathway during IAV infection (26, 31, 32). TLR3 is constitutively expressed by both bronchial and alveolar epithelial cells and recognizes viral double-stranded RNA (dsRNA) (intermediate RNA species produced during replication of IAV), siRNAs, and self-RNAs derived from damaged cells generated during infection (33–36). Unlike retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation-associated protein 5 (MDA5), and NLRP3 (NACHT, LRR and PYD domains-containing protein 3), TLR3 is expressed on both the cell surface and the endosome, allowing the epithelial cells to sense IAV PAMPs both externally and internally (in

the endosome) (27, 36–39). A distinctive feature of TLR3 in IAV infection is its link to secretion of proinflammatory cytokines, including IL-6, IL-8, and RANTES (regulated upon activation, normal T cell expressed and secreted) (36), and to immunopathology (36, 39, 40). *Tlr3* $^{-/-}$ mice showed better survival after lethal IAV infection, despite higher viral loads in the lungs (40). They also have lower chemokine and CD8 $^{+}$ T-cell concentrations after IAV infection, but antibody and CD4 $^{+}$ and CD8 $^{+}$ T-cell responses to sublethal doses of IAV infection are unaffected (41). *Tlr3* $^{-/-}$ mice also showed greater lung pathology after H5N1 infection, but not after infection with pandemic H1N1 infection, possibly because of the greater innate immune response generated by the H5N1 virus (26, 40). In all these studies, no differentiation was made between TLR3 expressed by respiratory epithelial cells and that by myeloid cells; thus, the actual significance of epithelial TLR3 *in vivo* is still unknown.

TLR7 and TLR8 are expressed in endosomes and bind ssRNA ligands, leading to recruitment of the adaptor molecule MyD88 and subsequent activation of NF- κ B and IRF7. TLR7 is highly expressed in certain immune cell types, such as plasmacytoid dendritic cells, which secrete type I IFNs upon infection with IAV (42, 43), and is also found in human bronchial epithelial cells (44). Sykes and colleagues reported that stimulation of healthy primary human bronchial epithelial cells in *ex vivo* undifferentiated culture conditions with R848, a TLR7/8 ligand, did not induce IFN- β secretion (45); rather, it induced secretion of IL-6, IL-8, and epithelium-specific IFN- λ proteins. In a separate study by Ioannidis and colleagues, TLR7 activation by a thiazoloquinolone derivative (CL075) failed to induce secretion of IFN- α , IFN- β , IFN- λ , and IL-6 from polarized, well-differentiated human alveolar epithelial cells *in vitro* and only slightly increased IL-8 secretion (46). These somewhat contradictory findings suggest that epithelial cell differentiation could influence antiviral and proinflammatory responses to TLR7 activation. In an *in vivo* study, TLR7 $^{-/-}$ mice infected with IAV were found to have poor formation of germinal center B cells in lungs and spleen and subsequent poorer B-cell response upon rechallenge, but the viral titers in TLR7 $^{-/-}$ mice during the acute period were not different from those of control animals (47).

Figure 1. Overview of pattern recognition receptors and inflammasome sensing mechanism during influenza A virus (IAV) infection. IAV infects cells by binding of the virion surface glycoprotein hemagglutinin to sialic acid that is expressed by a cell surface receptor and then is endocytosed. The viral hemagglutinin fuses with the endosomal membrane; the virion particle unravels; and the viral RNA is released into the cytoplasm. TLR3 and TLR7 sense viral double-stranded RNA and single-stranded RNA (ssRNA), respectively, within the endosome, whereas retinoic acid-inducible gene 1-like receptors (RIG-I and MDA5) recognize cytosolic ssRNA or viral RNA containing 5'-triphosphate. Via their C-terminal caspase-recruitment domains, RIG-I and MDA5 attach to the signaling adaptor protein (MAVS, found in the mitochondria), causing activation of IRFs and NF- κ B and transcription of proforms of IL-1 β , IL-18, caspase 1, and the types I and III IFNs. As a result of IRF and NF- κ B activation (also acting as signal 1 of the inflammasome pathway), other components essential to formation of the inflammasome complex, such as ASC and NLRP3, are upregulated. NLRP3 forms one of the inflammasome second signal receptors, and when activated (e.g., when sensing ssRNA from IAV and IAV-associated DAMPs), it nucleates ASC, which bridges the inflammasome sensor to oligomerize pro-caspase 1, causing caspase 1 activation. Active caspase 1 cleaves the pro-IL-1 β and pro-IL-18 proteins, generating the biologically active cytokines IL-1 β and IL-18. ASC = apoptotic speck protein containing a C-terminal caspase recruitment domain; DAMPs = damage-associated molecular patterns; IFNAR = IFN- α β receptor; IRF3/7 = IFN-regulatory factor 3/7; ISGs = IFN-stimulated genes; MAVS = mitochondrial antiviral signaling protein; MDA5 = melanoma differentiation-associated protein 5; MyD88 = myeloid differentiation primary response 88; NLR = nucleotide-binding oligomerization domain-like receptor; NLRP3 = NACHT, LRR and PYD domains-containing protein 3; RIG-I = retinoic acid-inducible gene 1; STAT1/2 = signal transducer and activator of transcription 1/2; TLR = Toll-like receptor; TRIF = Toll/IL-1 receptor domain-containing adapter-inducing IFN- β . Adapted by permission from Reference 22.

RLRs

The RLR family of innate receptors are the main cytoplasmic PRR and comprises three cytosolic virus recognition receptors: RIG-I, MDA5, and Laboratory of Genetics and Physiology 2 (LGP-2) (48). All three are RNA helicases and act as viral dsRNA sensors (49). RIG-I and MDA5 also possess C-terminal caspase recruitment domains (CARDs), allowing them to engage with the CARD-containing adapter mitochondrial antiviral signaling protein (MAVS), which then allows activation of IRF and NF- κ B (Figure 1). LGP-2, which is devoid of CARDs, functions as a negative regulator

of RIG-I-induced IFN signaling (50) by sequestering dsRNA (49–51). IAV RNA species found in the cytoplasm are rapidly detected by RIG-I and MDA5 (51, 52), aided by an increase in RIG-I and MDA5 levels in IAV-infected epithelial (including alveolar epithelial) cells (36, 53, 54). The primary consequence of RLR signaling is activation of IFN genes. This occurs via interaction with MAVS, which in turn engages the IRF3/7 transcription factor signaling pathway (55) and NF- κ B, leading to type I IFN induction (36, 49).

RIG-I, at least in murine bone marrow-derived dendritic cells (BM-DCs)

and human HEK293 cells, has also been reported to detect IAV ssRNA that carries phosphorylated 5'-ends (56). Unlike mammalian mRNA, the IAV viral genome comprises uncapped RNA segments with phosphate groups at their 5'-termini (57, 58). The ability of RIG-I to recognize these 5'-phosphate groups on ssRNA and the dsRNA domains makes it a superior sensor. There are also several other distinctive features of RIG-I induction of an antiviral state. First, engagement with the virus occurs almost immediately. It was observed that RIG-I, IAV polymerases, and MAVS associate with each other and with the

mitochondria as early as 3 hours after infection (59). In response, IAVs have established built-in methods to attenuate this pathway. The most prominent is the virus's nonstructural protein 1 (NS1), which suppresses the type I IFN response by interacting with RIG-I (60, 61) and TRIM25 (62). IAV's polymerases (PB1 and PA) also associate very early with MAV in the RIG-I signaling pathway and the mitochondria to inhibit type I IFN expression (59).

The second noteworthy feature of RIG-I signaling is that it induces an antiviral state in several ways. It not only causes IFN induction but also directly inhibits virus replication by binding to the 5'-triphosphate dsRNA end and promoting the disassembly of the viral polymerase complex (63). It also promotes the assembly of an inflammasome complex. RIG-I stimulates NF- κ B-mediated expression of pro-IL-1 β expression via MAVS and CARD9 (2). In parallel, RIG-I can also directly activate the inflammasome complex by binding to the inflammasome's adaptor, ASC (apoptotic speck protein containing a C-terminal caspase-recruitment domain) (64, 65). IFN responses are reduced in RIG-I or MDA5 deficient alveolar epithelial cell lines (A549) (54).

In vivo studies on RLRs have been limited because RIG-I-null mice are not viable on common laboratory background strains (66). However, recent studies have shown that RIG-I-knockout mice on an ICR background (which are viable) demonstrated delayed IAV clearing and display higher viral loads (67). Within RIG-I-like receptor family, RIG-I may be the primary and MDA5 a secondary contributor to viral sensing (68). Between cell types, there may also be a differential contribution of these sensing pathways to the generation of an IFN response. For example, Crotta and colleagues found that in alveolar epithelial cell lines, knocking out TLR7 or MyD88 had minimal effect on type I or III IFN production, but ablation of MAVS greatly reduced IFN levels (69). However, there are currently no *in vivo* studies that localize the effect of RIG-I knockout to respiratory epithelial cells.

NLRs

NLRs are cytoplasmic PRRs that contain central nucleotide and oligomerization (NACHT) domains; these domains

determine the families of NLRs. NLRs contribute to the inflammasomes, which are multiprotein complexes that assemble in the cytosol after exposure to PAMPs or damage-associated molecular patterns (DAMPs), with resultant activation of caspase 1 and subsequent release of the proinflammatory cytokines IL-1 β and IL-18 (70) (Figure 1). These inflammasome complexes usually consist of a cytosolic pattern PRR, pro-caspase 1, and an adaptor protein (ASC) (71). A number of distinct inflammasome complexes have been identified, each with unique PRR and activation triggers (72). The most widely studied is the NLRP3 complex, which contains the NLR, NLRP3, ASC, pro-caspase 1, and the NEK7, a serine-threonine kinase (71, 72). The complete activation of the inflammasome pathway requires two separate signals (73). Signal 1 involves the priming of cells by detection of PAMPs by cytoplasmic PRRs (e.g., TLRs 3, 7, and 8; and RLRs described earlier). This results in increased expression of caspase 1, pro-IL-1 β , and pro-IL-18 (priming step, signal 1) (71–73). Subsequently, indirect activation of NLRP3 occurs through a number of diverse signals (whole pathogens, PAMPs/DAMPs, potassium efflux, lysosomal damaging factors [e.g., silica, uric acid, and alum] and endogenous factors [amyloid- β and cholesterol crystals], as well as by mitochondrial damage), leading to complex assembly and activation of caspase 1 (signal 2) (71–73). Active caspase 1 then cleaves the precursor cytokines pro-IL-1 β and pro-IL-18, leading to generation of the biologically active cytokines IL-1 β and IL-18, respectively, which are then secreted from the cell (71–73). Secretion of the mature IL-1 β and IL-18 inflammatory cytokines then leads to recruitment of neutrophils, monocytes, and macrophages to the site of infection (74, 75). Other inflammasomes can be activated by more directly; double-stranded DNA activates the absent in melanoma 2 (AIM2) complex; anthrax toxin activates NLRP1, and bacterial flagellin activates NLR family CARD-containing protein 4 (NLRC4) (76).

NLRP3 is the most studied NLR family member in IAV infection. Unlike the TLR and RLR, NLRP3 is activated by both PAMPs (ssRNA) and DAMPs generated during an IAV infection (77–81). Intracellular molecules (i.e., ATP and high-mobility group box 1 protein [HMGB1])

released during infection-induced apoptosis, necrosis, or pyroptosis (82), can serve as DAMPs during IAV infection. They accumulate in the extracellular space at a high concentration and act as signal 1 for inflammasome activation (83–85). However, recognition of DAMPs does not always result in an enhanced immune response and viral clearance. For example, recognition of HMGB1 through the DAMP receptor, receptor for advanced glycation end products (RAGE) appears detrimental during IAV infection, as both ligand and receptor become highly expressed in endothelium and bronchial epithelium (86). NLRP3 is required for a successful defence against IAV-human airway epithelial cells express NLRP3 and secrete IL-1 β when challenged with IAV (87, 88) and mice without NLRP3 (or inflammasome pathway components) showed reduced survival and impaired innate immune responses (87). However, the majority of the NLRP3 response is linked to myeloid cells rather than from respiratory epithelial cells in this murine model (87); hence, there is still little to indicate the importance of epithelium-expressed NLRP3. Interestingly, a recent study in bacterial infection suggested that NLRP3 expressed by epithelial cells has a role during infection but via the maintenance of alveolar barrier integrity, independent of involvement of the inflammasome, IL-1 β , and IL-18 (89). Nonetheless, the importance of the inflammasome pathway during IAV infection is suggested by presence of viral mechanisms that interfere with inflammasome activation. For example, the viral NS1 protein can block caspase 1 activation, IL-1 β maturation, and apoptosis (90). The caspase 1-inhibitory effect of NS1 demonstrates strain-specificity H5N1 NS1 does not appear to activate caspases, but instead induces apoptosis of the epithelial cells (91).

Consequence of Viral Sensing

IFN Response

The central consequence of PRR activation during IAV infection is the induction of the IFN response. There are three classes of IFN: type I (IFN- α and IFN- β), type II (IFN- γ), and type III (IFN- λ), each with distinct expression patterns and roles in the antiviral response. In epithelial cells, the key consequence of intracellular recognition of IAV infection by TLRs and RLRs is the

activation of type I and type III IFN pathway. Type I IFN responses are ubiquitous; almost all nucleated cell types are able to secrete type I IFNs and express the IFN- $\alpha\beta$ receptor (92). Type II IFN (IFN- γ) is secreted by specific immune cell populations including CD8⁺ T cells, CD4⁺ T-helper type 1 cells (93), invariant natural killer T cells (94), type 1 ILCs (95), and NK cells (96). Type III IFNs are primarily secreted by epithelial cells, and type III IFN responses are restricted to barrier sites linked to high exposure to pathogens (e.g., airway or intestinal epithelium) (97, 98). The expression patterns and the levels of types I and III IFNs are dependent both on the site of infection and on the pathogen (99, 100).

Although distinct receptor complexes bind types I and III IFNs, many of the intracellular signaling molecules and pathways are shared between type I and type III IFNs. Activation of these pathways by either IFN class results in broadly similar biological activities. Types I and III IFNs rapidly induce an antiviral state by inducing the expression of hundreds of genes, grouped as IFN-stimulated genes (ISGs). These genes carry IFN-responsive elements in their promoters and suppress transcription of other genes, including viral genes. These ISGs both limit viral replication and spread, and they induce immune responses in neighboring cells. In contrast, type II IFNs have limited direct antiviral effects but promote innate and adaptive immune responses (92).

Recent studies have highlighted type III IFNs (rather than type I IFNs) as the dominant IFN response in the airway epithelium (100) and are potentially the superior of the two IFNs in host defense. Robust type I IFN responses are associated with effective viral clearance, but at the cost of bystander immunopathology (101). Generation of type III IFN responses at barrier surfaces, on the other hand, induces an antiviral state with limited damage to the host (97). The bias toward type III IFN responses is restricted to tissues continually exposed to pathogens, such as the lungs and gut. There is also evidence to suggest that type III IFN is generated as a first line of defense before potentially more damaging type I responses (100) and is critical for control of influenza virus dissemination in the upper airways (102). IFN- λ receptors deficient mice release significantly more infectious virus particles and transmit the virus much more efficiently to naive

contacts than did wild-type mice or mice lacking functional type I IFN receptors (102).

Downstream from IFNs, ISG-derived proteins perform two key functions. First, they directly limit viral replication by shutting down protein synthesis (2) and triggering apoptosis (103). Second, ISGs activate key components of the innate and adaptive immune systems, including antigen presentation and production of cytokines involved in activation of T cells, B cells, and NK cells (2). Thus far, several hundred ISGs have been identified, and three key genes—myxovirus resistance protein (Mx), IFN-induced transmembrane protein 3 (IFITM3), and IRF7—have been shown to have important roles in IAV infections. Mx proteins are a family of GTPases with broad antiviral activities against RNA viruses (104–107). They interact with the IAV nucleocapsid and its entry into the nucleus, thereby inhibiting viral genome transcription and replication (108–110). IFITM3, a small, conserved transmembrane protein, was among the first ISGs identified and restricts the proliferation of a broad range of viruses (111). By modulating endosomal cholesterol, IFITM3 blocks pH-dependent fusion in the late endosome and so impairs viral entry to the cytoplasm (112–114). Mice lacking *Ifitm3* had higher viral titers, morbidity, and mortality than wild-type mice when infected with IAV (112, 113). A functional IFITM3 SNP (rs12252) has been linked to severe influenza infection in UK and Chinese patients (115). Although, the antiviral role of IFITM3 is well established, the contribution of epithelial IFITM3 to viral control has been little studied. However, IFITM3 is constitutively expressed by the respiratory epithelium and type II alveolar cells, which suggests that this is a potential mechanism by which respiratory epithelium might reduce viral dissemination (116). The transcription factor IRF7 is a master regulator of type I IFN induction and ISG expression (117). Although specific immune cell populations, including plasmacytoid dendritic cells and macrophages, constitutively express IRF7 (118–120), levels in respiratory epithelial cells are low but induced in response to IFN signaling (121). The critical role of the positive feedback loop generated by IFN and IRF7 is demonstrated by the near ablation of IFN production and increased mortality and morbidity observed in

influenza-infected IRF7-knockout mice (122, 123). Furthermore, life-threatening influenza infection in an otherwise healthy child was linked to IRF7-null mutation and failure to generate an IFN response in respiratory epithelial cells isolated from the child (124).

Overall, it seems highly likely that PRRs in epithelial cells, including alveolar epithelial cells (125), are involved in host defense. However, there is not enough evidence to place their importance in the context of the overall host defense against IAV, because PRRs are also expressed on and in myeloid cells. Indeed, loss of TLR3 (40) and the RIG-I signaling adaptor MAVS and IPS-1 does not affect viral clearance and the adaptive immunity to IAV infection (126).

Cytokine and Chemokine Production

In addition to the initial IFN-mediated antiviral response, epithelial cells secrete various cytokines and chemokines, such as IL-6, TNF- α , IL-8/CXCL8, CXCL10, CCL2, and CCL5 (127–131). Activation of these pathways is rapid, and increased concentrations of cytokines and chemokines can be detected within a few hours of IAV infection (127, 132–136). These soluble mediators further activate and attract other immune cell populations into the lung (127, 132–135). However, both lung-resident and recruited immune cell populations are also able to secrete many of these cytokines. In this review, we highlight work that has specifically established the role of the epithelium-derived cytokines and chemokines during IAV infection.

CCL2 is secreted by both airway epithelium and activated monocytes and acts as a monocyte chemoattractant (137, 138). The CCL2–monocyte recruitment pathway has both beneficial and detrimental roles during IAV infection. The induction of a robust monocyte response is associated with immunopathology (139, 140). Several studies have demonstrated that mice lacking CCL2 receptor (CCR2) expression had improved outcomes in severe influenza infection (141–143), although these mice also showed delayed viral clearance (144). One study was able to show that CCL2 produced by epithelial cells rather than myeloid cells is linked to immunopathology. Maelfait and colleagues showed that deletion of the immune regulatory factor A20 (or TNF- α -induced

protein 3 [TNFAIP3]) specifically in respiratory epithelial cells resulted in a better outcome from IAV infection, and this correlated with a strongly suppressed expression of the chemokine CCL2 during later stages of infection (145).

IL-1 β , an early proinflammatory cytokine, is released in response to viral triggering of the NLRP3 inflammasome (146, 147). IL-1 β both stimulates release of other cytokines and chemokines by the epithelium and increases adhesion molecule expression, enhancing immune cell recruitment to the lungs (148, 149). In mice, IL-1 signaling is necessary for survival during IAV infection, but conversely, IL-1 β also drives lung immunopathology (150, 151). Ablating IL-1 signaling in IAV-infected mice impaired neutrophil recruitment and CD4 T-cell activation and recruitment with subsequent reduction in pulmonary inflammation (150). Respiratory epithelial cells have a key role in both the generation and regulation of the IL-1 β response. At homeostasis, they secrete IL-1 β inhibitors (IL-1RA and soluble IL-1RII) that prevent spontaneous IL-1 β signaling. Therefore, by controlling IL-1 β signaling at steady state, the respiratory epithelium promotes an antiinflammatory environment, preventing IL-1 β -driven pathology; however, this respiratory epithelial cell tolerogenic state can rapidly be broken by proinflammatory cytokines, including TNF- α (152).

Transforming growth factor (TGF)- β is a pleiotropic cytokine with an integral role in regulation of immune responses. Many immune and nonimmune cells can produce TGF- β , but secretion is specifically increased in the airway epithelium early after IAV infection (135, 153, 154). Overexpression of TGF- β in the lungs reduced inflammatory responses but also viral clearance (155). The regulatory properties of TGF- β have been exploited by IAV to control the early immune responses in the lung. Neuraminidase viral protein can cleave and activate latent TGF- β (153, 156), regulating type I IFN production and so generating a degree of viral tolerance in the host. When TGF- β is ablated specifically in the airway epithelial cells, weight loss, airway inflammation, immunopathology, and viral titers are all reduced (135). This protective phenotype was due to an enhanced early IFN- β release from the respiratory epithelium and was amplified by alveolar macrophages rather

than an altered lymphocytic immune response (135). Therefore, IAV infection generates a viral permissive state via neuraminidase cleavage and activation of epithelium-derived TGF- β , which then suppresses early protective IFN responses. The different roles of epithelial and myeloid cells in the generation of the TGF- β responses during IAV infection are further highlighted by Meliopoulos and colleagues. Mice that lacked the β_6 -integrin on the respiratory epithelium and so had impaired activation of TGF- β also showed improved survival after IAV infection due to constitutively activated airway macrophages with increased IFN responsiveness (157).

A key issue with the role of sensors on epithelial cells is the use of whole-organism responses as an endpoint, so that the segregation of epithelial versus hematopoietic cell contributions is not possible (exceptions being specific studies highlighted in the paragraphs above). In addition, experiments were often based on simple *in vitro* cell culture systems that utilize undifferentiated, nonpolarized respiratory epithelial cells. Moreover, there are significant interspecies differences between rodents and humans for certain aspects of IAV-triggered pathogenesis (e.g., induction of Mx proteins). Different responses of undifferentiated versus well-differentiated mucociliary epithelial *in vitro* cultures have been reported (e.g., TLR7-induced IFN production). Complementary approaches that combine better *in vitro* models (158), such as three-dimensionally reconstituted pseudostratified human epithelial innate responses or lung organ-on-chip approaches, will significantly enhance the studies. Animal models in which counterparts of human genes exist and can be selectively manipulated in the respiratory epithelium are needed in this area. However, defining the exact contribution of epithelial cells to the generation of the influenza immune responses *in vivo* is challenging. Several genetically modified models have been developed that allow epithelial cell targeted gene overexpression or deletion, although important caveats must be acknowledged (159, 160). Epithelial cell promoters in common use are Scgb1a1 (club cell secretory protein [CCSP]) and SFTPC (surfactant protein C or SPC) targeting the proximal and distal airway epithelial cells, respectively, in mice (160). These promoters can be used to drive gene

overexpression or deletion of genes flanked by LoxP sites (floxed) via constitutive Cre recombinase (CRE) expression or an inducible expression system (i.e., tetracycline [doxycycline]-responsive element). However, constitutive CRE expression models can result in CRE toxicity (161) and may have limited utility when genes have key roles in development. Using an inducible tetracycline (doxycycline)-responsive element allows excision of a gene at a specific time point (i.e., shortly before infection). However, deletion occurs only at the level of genomic DNA, and pools of both protein and RNA from the targeted gene can remain in the cell for some time (162, 163). It is important to note that neither system offers a complete deletion of the gene of interest in the target population (i.e., some cells will be unaffected), and CRE expression and gene deletion can occur in off-target cell types (164). Despite limitations with genetically modified models, specific targeting of genes in the epithelium provides important insight into the contribution of the epithelium to host defense during influenza infection.

Conclusions

This review is focused on how epithelial cells sense and respond to IAV during the first critical hours of infection. If successful, this first response triggers a process that can eradicate the virus and prevent infection. If not, IAV takes hold and replicates, and the host engages bigger waves of innate immune cells and subsequently the adaptive immune response. The comparative lack of studies localizing the specific role of the respiratory epithelium during IAV infection means that this large defense mechanism has been relatively untapped in its ability to prevent infection and to regulate immunopathology. Understanding the specific part that epithelial PRRs (e.g., the NLRP3 inflammasomes, which sense more triggers than TLR and RLR) play in immunopathology could be particularly useful, because modulating this pathway directly could potentially reduce inflammation in severe disease. ■

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