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## Review Article: Special Edition

# The role of respiratory epithelium in host defence against influenza virus infection<sup>☆</sup>

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## ARTICLE INFO

## Article history:

Received 6 June 2018

Accepted 3 August 2018

Available online 10 September 2018

## Keywords:

Influenza

Airway epithelium

Defence

Sensing

Injury and repair

## ABSTRACT

The respiratory epithelium is the major interface between the environment and the host. Sophisticated barrier, sensing, anti-microbial and immune regulatory mechanisms have evolved to help maintain homeostasis and to defend the lung against foreign substances and pathogens. During influenza virus infection, these specialised structural cells and populations of resident immune cells come together to mount the first response to the virus, one which would play a significant role in the immediate and long term outcome of the infection. In this review, we focus on the immune defence machinery of the respiratory epithelium and briefly explore how it repairs and regenerates after infection.

## The respiratory epithelium

The respiratory epithelium starts as the ciliated pseudostratified nasal epithelial lining and continues as the trachea, bronchus, bronchiole (collectively known as the airway epithelium) and finally, the alveolar duct which feeds into the alveolus. The alveolar epithelium comprises a mix of type I and II alveolar epithelium, and has the added distinct adaptation for gas exchange. Both the airway and alveolar epithelial cells co-exist with numerous other specialised cells [Fig. 1] to form the first barrier to the outside world.

Different parts of the respiratory epithelium possess different methods of initial defence against pathogens and foreign substances. The airway epithelium utilizes structural and barrier defence provided by the muco-ciliary escalator and their incumbent anti-microbial proteins, and intra- or epithelial-associated immune cells like resident dendritic cells, invariant natural killer T (iNKT) cells,  $\gamma\delta$  T cells and intra-epithelial lymphocytes [1–4]. In the alveolar space, a unique environment of surfactant-related antimicrobial proteins and resident immune cells, chiefly, the resident yolk-sac derived, self-renewing alveolar macrophages prevent debris deposition, pathogen colonisation or infection, and generally

<sup>☆</sup> How the respiratory epithelium responds to influenza A virus. The review will explore the role of the airway and alveolar epithelial cells in protection against, sensing and eradication of the virus, and briefly on how they regenerate after infection.

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Peer review under responsibility of Chang Gung University.

<https://doi.org/10.1016/j.bj.2018.08.004>

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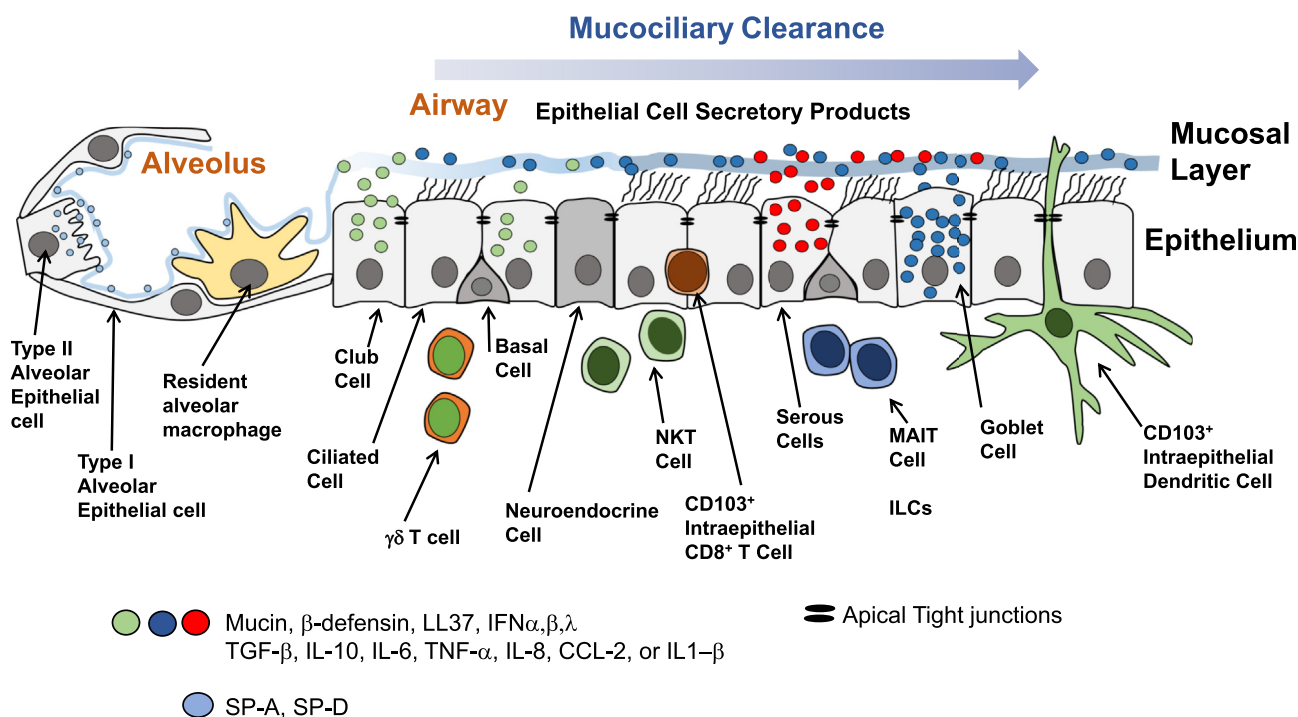


Fig. 1 The respiratory epithelium, and relevant epithelial-associated immune cells and soluble factors involved in IAV infection.

The human respiratory epithelial layer is composed of mostly ciliated cells with some secretory cells and basal cells. Secretory cells including serous, neuroendocrine and goblet cells, generate mucus components and anti-microbial factors including oxidants, enzymes, anti-microbial peptides and protease inhibitors. Ciliated cells facilitate the removal of foreign particles and debris via the mucociliary elevator, sweeping mucus and trapped particles upwards and helping to expel them from the respiratory tract. Club cells (also known as bronchiolar non-mucous secretory cells or Clara cells), are found in distal bronchioles, are not ciliated and may be progenitors to other ciliated cells. Basal cells can also differentiate into other cell types found within the epithelium. Several immune cell populations are resident in epithelium including CD103<sup>+</sup> CD8<sup>+</sup> T cells and CD103<sup>+</sup> conventional dendritic cell populations which act as sentinel cells. Other immune cell populations including innate lymphoid cells (ILCs), mucosal associated invariant T cell (MAIT), natural killer cells (NKT) and  $\gamma\delta$  T cells are in close proximity to the epithelium. The alveolar epithelium is composed of type I and type II alveolar epithelial cells—type I alveoli epithelial cells enable gas exchange and type II alveolar epithelial cells secrete surfactant lipids and proteins helping to maintain the function and structure of the alveoli. Alveolar macrophages are also resident in the alveolar space.

maintain a comparatively immunologically quiescent tone at homeostasis [5,6].

The airway epithelium is the major point of defence during influenza A virus (IAV) infection, specified by the tropism of IAV for these structural cells. Human influenza viruses preferentially bind  $\alpha$ 2,6-linked sialic acid receptors ( $\alpha$ 2,6-SA) which are predominantly located on airway epithelium from the nose to respiratory bronchioles [7–9]. Dominance of  $\alpha$ 2,3-linked SA receptors in the lower respiratory tracts - alveolar ducts and alveoli, prevents initial infection of these sites by established IAV strains. In addition to this distribution of sialic acid receptors, the upper respiratory tracts have also evolved sentinel mechanisms to prevent pathogens from reaching the alveolar space. Failure to do so leads to disruption of the surface tension in alveoli, and the integrity of the closely apposed alveolar capillaries, causing serous leak and collapse of the airspace. Autopsies of fatal cases from all IAV pandemics showed diffuse alveolar damage in the lungs of these individuals [10] characterised by damage of the endothelial-epithelial barrier of the alveoli, fluid leakage and

accumulation of cells and blood in the alveolar lumen [11]. In a case of fatal IAV infection with an H5N1 strain, a key cause of pathology was a mutation in the virus which changed the hemagglutinin (HA) binding preference from  $\alpha$ 2,3 to both  $\alpha$ 2,3 and  $\alpha$ 2,6 linked moieties [12,13]. Thus, a major task of the airway epithelium is to protect pathogens from reaching or infecting the alveolar epithelium. In the following paragraphs, we explore how the airway epithelium and to a lesser extent, the alveolar epithelium, detect and eradicate IAV infection, and what happens to the epithelium after this defence exercise and infection.

## The first line of defence against IAV

### Tight junctions and polarity of airway epithelial cells

Epithelial cells are attached to their neighbours by cell–cell junctions, including tight junctions (found in the apical part of the lateral epithelial membrane), adherens junctions, gap

junctions and desmosomes. These structures link with the cellular cytoskeleton via numerous adaptor proteins and form an impermeable mechanical barrier. They also maintain an apical to basal ionic gradient created by  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  which facilitates directional secretion of many substances [14], enable communication between adjacent cells and regulate intercellular transport of soluble factors [15]. Interestingly, a high concentration of RIG-I, a key activator of type I interferon (IFN) antiviral signalling (see later) is found around tight junctions, suggesting that these junctions could act as apical foci for antiviral sensors [16]. These tight junctions are also resistant to change during infections - in well differentiated airway epithelial cell models, the trans-epithelial resistance conferred by intact tight junctions and the tight junction protein expression were retained throughout the infection period [17].

### **Antimicrobial proteins secreted by airway and alveolar epithelium**

The pseudostratified columnar epithelium [Fig. 1] in the trachea, bronchi and bronchioles are interspersed by a further diverse group of cells - serous, club, neuroendocrine and goblet cells which secrete mucin and a variety of enzymes, protease inhibitors, oxidants, and antimicrobial peptides [1]. Not all of these proteins are relevant to influenza, but mucin,  $\beta$ -defensins and LL37 have roles.  $\beta$ -defensins are secreted by airway epithelial cells and bind to the membrane of pathogens forming a pore-like membrane defect through which essential ions and nutrients efflux. The  $\beta$ -defensin 1 (BD1) isoform is expressed constitutively by human airway epithelial cells whereas expression of BD2, 3 and 4 is inducible [18,19]. Influenza infection increased expression of BD3 and BD4 in murine airway epithelium [20] and mice lacking BD-1 had a worse outcome after IAV infection [21]. LL37, a cathelicidin, which is released in neutrophil granules and by epithelial cells [22] seems also important in IAV infection. Administering LL37 to mice greatly improved outcome in severe influenza infection - mice had lower mortality, morbidity, viral titre and inflammatory cytokine levels in their lungs [22,23].

Epithelial cells are covered by a layer of mucus which forms a physical barrier and serves as biophysical 'rafts' to bring pathogens in contact with antimicrobial proteins. Mucins make up this mucus layer and are secreted by specialised goblet, club and less abundantly, alveolar epithelial cells. Several isoforms exist. Most mucins are either tethered to epithelial cells (for example, MUC4, MUC13, MUC16 and MUC21) or secreted (MUC5B, MUC5AC and MUC2). In the context of IAV infection, MUC5AC has been shown to be induced in airway epithelial cells after infection [24,25]. IAV infection of mice over-expressing MUC5AC in their epithelial cells resulted in significantly lower viral titres and neutrophil infiltration in the lungs compared with control mice [26]. Intriguingly, the sialic acid motifs on Muc5AC appear to act as a decoy, binding to SA receptors and so prevent binding of IAV to cell surface sialic acid receptors, and subsequently viral internalisation and infection [26]. Mucins can also communicate the presence of viruses. Airway epithelial cells can detect virus (e.g. herpes simplex virus) via perturbations in the mucin layer, triggering 'hyper-early' innate responses, including the release of CXCL-10 from epithelial cells and recruitment of neutrophils, independent of

the early Type I IFN responses. This has not been demonstrated with IAV infection.

In the alveolar space, surfactant proteins contribute to the structure, regulation, and function of pulmonary surfactant and also have intrinsic host defence properties [27]. Pulmonary surfactant is mainly made up of lipids, as well as surfactant proteins (at lower concentrations), that together serve critical roles in reducing surface tension in the alveolus during the dynamic changes of the ventilatory cycle [27]. The surfactant proteins SP-A and SP-D are highly evolutionarily conserved members of the collection family of innate host defence proteins and highly expressed by type II alveolar epithelial cells. The carboxy-terminal lectin-like domains of SP-A and SP-D bind with varying specificity to a diverse range of pathogen-associated molecular patterns (PAMPs), including complex carbohydrate surfaces of common respiratory viruses, bacterial and fungal pathogens, and associated toxins to enhance their opsonization and phagocytosis by alveolar macrophages [28,29]. During IAV infection,  $\alpha(2,3)$ -linked sialic acid motifs on SP-A bind viral HA, inhibiting viral entry into cells [30–32]. SP-D can also bind to HA proteins on IAV and cluster the viral particles forming large aggregates of viral particles which reduce optimal functioning of the virus [33]. Both SP-D and SP-A increased viral particle binding and phagocytosis by neutrophils [30,31,33,34]. Mice that are SP-D-deficient had reduced viral clearance, increased pro-inflammatory cytokines and elevated neutrophils after influenza infection [35]. Administering exogenous SP-D mutant protein deficient in a specific collagen domain did not correct the surfactant properties in the alveolar space but was able to reverse the reduced viral clearance in IAV-infected SP-D-deficient mice [36], indicating that SP-D had intrinsic anti-viral properties separate from their surface tension function. There is also evidence in humans that surfactant proteins have antiviral functions, fatal IAV infection appear associated with lower transcript levels of SP-D in lung tissue, although it is unclear whether this is the primary defect or consequence of infection [37]. The former is probably more likely, as SP-A, B and D gene polymorphisms were shown to be associated with severe Influenza in humans [38,39].

### **Sensing the influenza virus**

Three families of innate PRRs including the Toll like receptors (TLRs), RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) are known to cooperate for early recognition of IAV.

#### **TLRs and epithelial cells**

Once IAV crosses these first barriers to reach the airway epithelial cells, there are several key functions that the epithelial cell must perform to limit the spread of infection. They must first rapidly recognize their infected status through sensing viral factors. It then needs to limit viral replication and finally, primarily via soluble factors, they will initiate and influence the local and systemic immune response.

Upon binding sialic acid receptors on the epithelial cell surface, IAV are internalised via receptor-mediated endocytosis [40]. The low pH of the endosome triggers

conformational changes and fusion of the viral and endosome membranes, allowing viral RNA and proteins to enter the cell cytoplasm (reviewed by Ref. [41]). Using nucleus localization signals, viral proteins then use the host cell's machinery to translocate into the nucleus where transcription and replication of the viral genome occurs [41]. The host cell begins sensing IAV as soon as it is internalised, utilising pathogen recognition receptors (PRRs), primarily the Toll-like receptors (TLRs) and RNA-sensing RIG-I-like receptors (RLRs), such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) [Fig. 2].

TLRs are the first PRR to encounter IAV [Fig. 2]. Multiple TLRs - TLR3, TLR7 and TLR8 (human) have been implicated [42–49]. Upon recognition of the relevant pathogen-associated molecular patterns (PAMPs) – double stranded RNA for TLR3 and single stranded RNA for TLR 7 and 8, these TLRs recruit TIR

domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF- $\kappa$ B, IRFs, or MAP kinases to regulate the expression of cytokines, chemokines, and type I IFNs. IAV-infected MyD88<sup>-/-</sup> and MyD88/TRIF double-deficient mice show a marked reduction of pulmonary cytokine production compared to wild type mice [42,48,49], indicating the important role of these TLR signalling pathways in disease.

TLR3 and TLR7 are probably the most relevant respiratory tract TLRs during IAV infection. TLR3 is constitutively expressed by both bronchial and alveolar epithelial cells and recognises viral double-stranded RNA (dsRNA), small interfering RNAs, and self-RNAs derived from damaged cells generated during infection [50–53]. Unlike other PRRs, TLR3 is expressed on both the cell surface and the endosome, allowing the epithelial cells to sense IAV both externally and internally

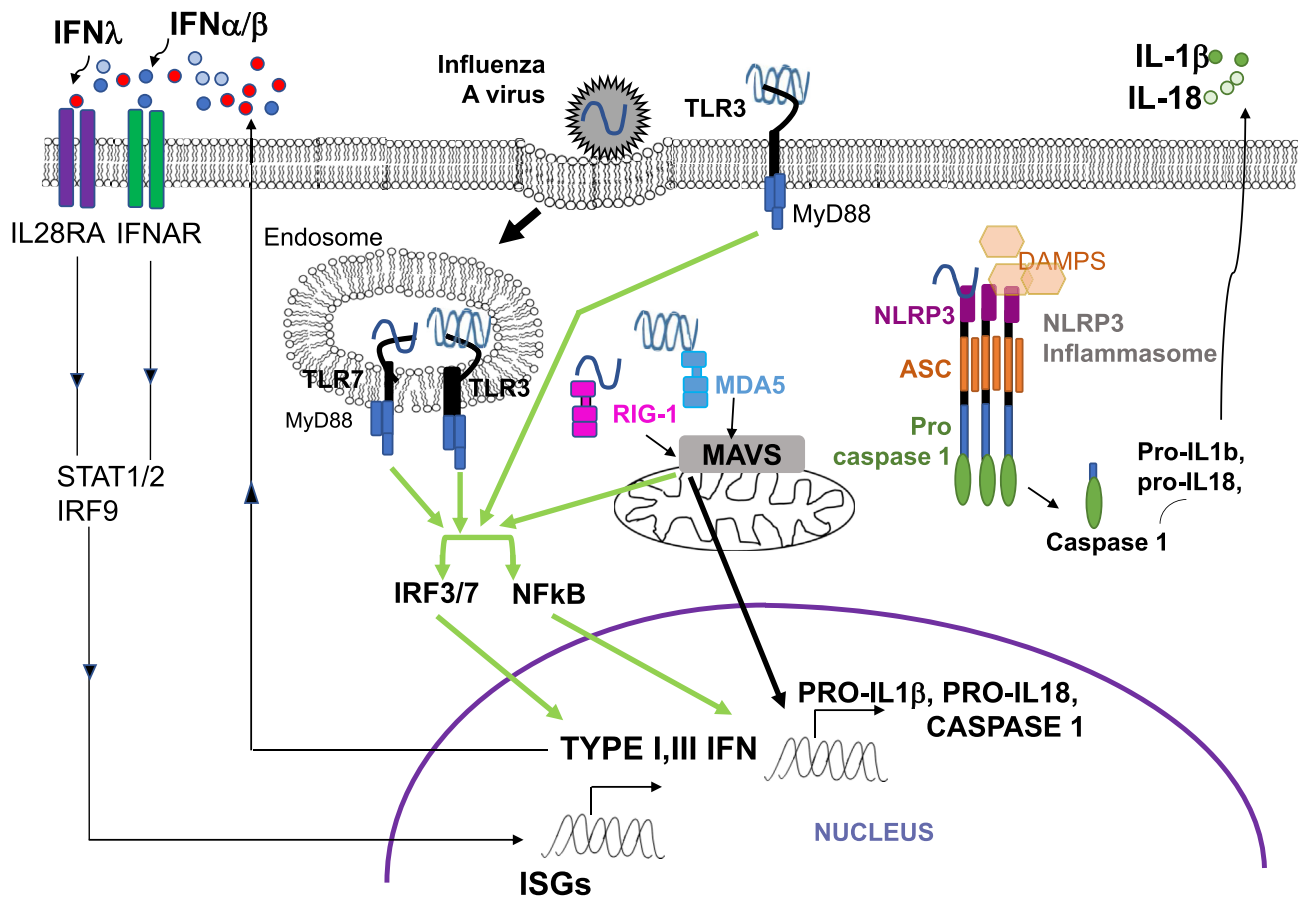


Fig. 2 Key sensors of IAV and dominant outcomes of viral sensing pathway activation.

IAV infects cells by first binding of the virion surface glycoprotein hemagglutinin (HA) to sialic acid that is expressed by a cell surface receptor, then is endocytosed. Upon acidification of the endosome, the viral HA undergoes a conformational rearrangement, enabling viral and endosomal membrane fusion. The H<sup>+</sup> ions in the acidic environment of the endosome then translocate to the virion interior via the virion's M2 ion channel, causing the viral envelope to permeabilize. As a result, the virion particle opens, the viral RNA is liberated into the cytoplasm of the host cell and then imported into the nucleus to enable transcription of the viral genome and translation of viral proteins. TLR3 and TLR7 sense viral ds and ssRNA respectively within the endosome while RIG-I recognizes cytosolic ssRNA or viral RNA containing 5'-triphosphate and by interacting with MAVS, induces type I and III IFN responses through the transcriptional factors NF- $\kappa$ B and IRF. These cytokines bind to the epithelial's own IFNAR receptors or that expressed by myeloid cells causing expression of ISGs and an antiviral state. In parallel, cytosolic ssRNA and DAMPs can also interact with NLRP3 of the inflammasome complex to cause cleavage and activation of caspase-1 and induction of IL-1 $\beta$  and IL-18.



in the endosome [43,53–56]. However, its contribution to host defence is not straightforward. Recognition of IAV by TLR3 leads to secretion of pro-inflammatory cytokines including type I IFNs and IL-6 [53] which can also lead to pathology [53,56,57]. Indeed, *Tlr3*<sup>-/-</sup> mice survive longer than wild-type mice following lethal IAV infection, despite sustaining higher viral loads in the lungs [57]. In the absence of TLR3, chemokine expression in the lungs, as well as the infiltration of leukocytes and CD8<sup>+</sup> T cells, is reduced after IAV infection. However, *Tlr3*<sup>-/-</sup> mice generate normal antibody, CD4<sup>+</sup> T cell and CD8<sup>+</sup> T cell responses to sublethal doses of influenza virus infection [58], which indicates that this sensor is dispensable for generating T cell immunity. Studies have also shown that TLR3 signalling is detrimental to survival and pathology after H5N1 infection, but not after infection with pH1N1 infection, suggesting that the virus itself may utilise TLR3 signalling to subvert detection [42,57]. However, in all these studies, no differentiation was made between TLR3 expressed by respiratory epithelial cells against those by myeloid cells, and therefore the actual significance of epithelial TLR3 in vivo is still unknown.

Another TLR expressed in human bronchial epithelial cells (in endosomes), TLR7, has also been reported to have a role in viral sensing (recognising single stranded genomic RNA) and immunopathology [59]. Here, its role appears somewhat greater than sensing alone. Activation of TLR7 in primary human bronchial epithelial cells, but not alveolar epithelial cells, in vitro, resulted in an increase in type III IFNs and IL-6 [60,61]. Mice lacking TLR7 infected with IAV had similar viral titres to controls, but failed to generate germinal centre B centres and mount a B cell response upon re-challenge [62]. Mice deficient in the MAVS adaptor, which is central to RIG-I-like receptor sensing (see later), and TLR7 showed increased mortality independent of viral load [63]. Instead, these mice had increased bacterial burden, caspase-1/11, and neutrophil-dependent tissue damage, suggesting a key role for MAVS and TLR7 in control of immune response and immune pathology during IAV infection [63].

Therefore, viral sensing via TLR7 may have limited effect on viral control, but rather helps shape immune memory responses. However, lack of in vivo studies localising absence of TLR7 to epithelial cells limits interpretation of these studies.

#### RIG-I-like receptors (RLRs)

During their cytoplasmic phase, IAV RNA species exiting the endosome are detected by RIG-I-like receptors (RLRs) [64,65]. The RLR family – including MDA-5 and RIG-I, are cytosolic double stranded RNA sensors [64,66]. RIG-I is the prototypical member, and together with MDA-5, is constitutively expressed by human bronchial epithelial cells. Protein levels of RIG-I and MDA-5 in epithelial cells are relatively low but viral infection greatly increased this expression [53,67]. The primary function of RLR signalling is to activate interferon genes by interacting with the adaptor mitochondrial anti-viral signalling (MAVS), which in turn engages the interferon regulatory factor (IRF) 3/7 transcription factor signalling pathway [68]. RIG-I signalling is important for activation of the inflammasome via the MAVS–CARD9– nuclear factor  $\kappa$ B signalling pathway. In parallel, RIG-I can also directly activate

the inflammasome complex by binding the adaptor, ASC [69,70]. These viral sensing pathways are used by alveolar epithelial cells which upregulate RIG-I and MDA-5 upon IAV infection [71]. IFN responses are diminished when expression of RIG-I or MDA-5 is silenced in alveolar epithelial cell lines (A549) [71]. Thus far in vivo studies have been limited as RIG-I knockout mice are not viable on common laboratory background strains [72]. However, recent studies by Kandasamy et al. demonstrated that RIG-I knockout mice (which are viable when bred on ICR background) have prolonged morbidity due to higher viral loads and failure to clear IAV as quickly as controls [73]. Work by Benitez et al. suggested a hierarchy within the RIG-I-like receptor family with RIG-I the primary and MDA5 a secondary contributor to viral sensing [74]. The relative contribution of these sensing pathways to the generation of an IFN response may vary according to cell type. Crotta et al. demonstrated that in alveolar epithelial cell lines, knocking out TLR7 or MyD88 had little effect on type I or III IFN production, but MAVS ablation greatly reduced IFN levels [75]. Therefore, the MAVS sensing pathway may function as the dominant mechanism of viral sensing and IFN induction in alveolar epithelial cells.

#### NOD-like receptors and activation of the inflammasome during influenza

NOD-like receptors (NLRs) form part of the multi-protein inflammasome complexes that process caspase 1 and induces the cleavage of pro-IL-1 $\beta$  into its active form IL-1 $\beta$  [76,77]. Activation of the inflammasome requires two signals; signal 1 results from detection of PAMPs and damage-associated molecular patterns (DAMPs) by PRRs which then induce signaling cascades related to the NF- $\kappa$ B transcription pathway; this leads to upregulation of the multiprotein factors that form the inflammasome complex and immature forms of IL-1 $\beta$ , IL-18 and caspase-1. Signal 2 from a multitude of different factors, leads to complex assembly and activation of caspase-1.

In the setting of IAV infection, NLRP3 is the most studied NLR family member. NLRP3 is expressed in the cytosol and recognises single stranded viral RNA [78–82]. Human airway epithelial cells have been shown to express NLRP3 and secrete IL-1 $\beta$  when challenged with IAV [83,84]. Mice lacking NLRP3 (or inflammasome pathway components) showed reduced survival and impaired innate immune responses compared to control mice [83]. However, the majority of the NLRP3 response was derived from myeloid rather than respiratory epithelial cells in this murine model [83]; here too, there is still little to specify the importance of epithelial-expressed NLRP3. The exception is recent work by Kostadinova et al. in bacterial infection which suggests that NLRP3 expressed by epithelial cells could maintain the integrity of the alveolar barrier during *Streptococcus pneumoniae* infection by enhancing cellular adherence independent of the inflammasome, IL-1 $\beta$  and IL-18 [85].

Along with microbial products, such as viral nucleic acids and proteins, IAV infection results in the release of host cell constituents from both damaged or dying cells and from intact cells. Intracellular molecules (ie, ATP and HMGB1) serve as DAMPs during IAV, are released from infected epithelial cells, most often as a consequence of infection-induced

apoptosis, necrosis, or pyroptosis [86], and accumulate in the extracellular space at a high concentration to act as signal 1 for inflammasome activation [87–89]. Recognition of DAMPs usually, but does not always result in an enhanced innate host response and accelerated viral clearance. For example, recognition of HMGB1 through the DAMP receptor known as receptor for advanced glycation end-products, reduced the host resistance to IAV infection [90].

The contribution of the inflammasome pathway, particularly in epithelial cells during IAV infection, has not been fully explored, but its importance is suggested by the presence of viral mechanisms that interfere with inflammasome activation. For example, the NS1 protein of the H1N1 IAV subtype (eg, A/PR/8/34) is capable of blocking caspase-1 activation, IL-1 $\beta$  maturation, and apoptosis [91]. The caspase-1 inhibitory effect of NS1 seems specific to certain strains, since NS1 from the highly pathogenic avian H5N1 appears not to activate caspases and induces apoptosis of epithelial cells instead [92].

### IFN response and interferon stimulated genes in epithelial cells during influenza

Activation of type I interferons is the key consequence of intracellular recognition of IAV infection by TLRs and RLRs. These cytokines bind to the IFN- $\alpha/\beta$  receptor (IFNAR) on infected as well as neighbouring cells and induces the transcription of a large group of genes (interferon stimulated genes or ISG) whose main task is to limit spread of infection. Although plasmacytoid dendritic cells (DCs) are recognized as the cell type specialized for the production of large amounts of type I interferons [93] during IAV infection, there is clear evidence that generation and detection of IFN signals also occur in airway epithelial cells. In epithelial cells, type I IFN has the additional task of acting as an early warning system, communicating viral threat between infected and uninfected cells. Another group of interferons, type III interferons, consisting (in humans) of four IFN- $\lambda$  molecules called IFN- $\lambda_1$  (IL-29), IFN- $\lambda_2$  (IL-28A), IFN- $\lambda_3$  (IL-28B) and IFN- $\lambda_4$ , have been recently identified [94,95]. IFN- $\lambda$ s signal through a receptor heterodimer complex consisting of IL-10 receptor  $\beta$  and IFN- $\lambda R_1$  (also known as IL-28RA). Despite the distinct receptor complexes used by type I (ie, IFNAR-1 and IFNAR-2) and type III interferons, they trigger similar intracellular signaling pathways in a wide variety of target cells, resulting in many of the same biological activities. However, unlike type I interferon receptors, which are widely expressed on many cell types, including leukocytes, the receptors for IFN- $\lambda$ s are largely restricted to cells of epithelial origin. Moreover, although type I IFN responses are global and can be generated in almost all nucleated cell types, type III responses appear restricted to areas exposed to pathogens like the airway or gut epithelium [96,97]. There is growing evidence that type III IFNs are the dominant IFN response in the airway epithelium [98–106] and one specialized for defence against infection at the mucosal interface [107]. Recent studies by Klinkhammer et al. demonstrated that IFN- $\lambda$  was critical for control of influenza virus dissemination in the upper airways. Mice lacking functional IFN- $\lambda$  receptors shed significantly more infectious virus particles and transmitted the virus much more efficiently to naïve

contacts compared with wild-type mice or mice lacking functional type I IFN receptors [108].

While initiation of Type I IFNs responses can be accompanied by severe immunopathology [109], the generation of type III IFN responses at barrier surfaces generates an antiviral state with limited damage to the host [96]. In humans, mucosal epithelial cells both produce and respond to type III IFNs [61,110,111]. In vivo, type III IFNs, rather than type I, are the primary IFNs found in the airways after influenza A virus infection [112]. There appears to be a degree of functional redundancy between type I and III IFNs in the airway epithelium [113,114]. However, only when both pathways were ablated did mice become highly susceptible to respiratory infections [75]. There is also evidence to suggest chronology in the induction of IFN responses in the lung with type III induced prior to type I [103].

Activation of both type I and III IFN results in induction of hundreds of ISGs. ISGs trigger apoptosis [115], shut down protein synthesis [47] and activate key components of the innate and adaptive immune systems, including antigen presentation and production of cytokines involved in activation of T, B, and natural killer (NK) cells [47]. The most prominent products of ISGs that have an impact on IAV are IFITM3, Mx, PKR and the 2'-5'-oligoadenylate synthetase (OAS)/RNase L system. IFITM3 blocks viral entry by impairing virus-host cell membrane fusion [116,117]. IAV-infected mice lacking *Ifitm3* had higher viral titres, morbidity and mortality compared to control mice [116,117]. An association between an IFITM3 (rs12252) SNP which causes truncation of IFITM3 and severe influenza infection was also found in UK and Chinese patients [118]. The role of IFITM3 as a restriction factor in IAV infection seems clear though it is not known which IFITM3-expressing cells contribute most to curb IAV spread.

The anti-viral activity of Mx, a family of GTPases, has been recognised for several decades [119–122]. Its expression is tightly regulated by IFNs although induced rapidly upon IFN pathway activation with kinetics similar to that of type I IFN mRNA expression [123]. The human MxA protein interacts with the IAV nucleocapsid, interfering with nuclear import of viral nucleocapsids as well as with genome transcription and replication, but can be counteracted by the viral NP [124–126]. Many common inbred laboratory mouse strains which are susceptible to influenza infection have an inactive Mx1 gene due to exon deletion or nonsense mutation which causes termination of translation to protein [120,122,127]. A mouse strain with functional Mx protein, the A2G mouse strain, has been shown to be highly resistant to IAV infection [120,122,128]. However, the Mx family may be less critical than other ISGs since mice with intact Mx1, but deficient Mavs and TLR7 were found to show increased mortality independent of viral load [63].

The dsRNA-binding kinase, PKR, promotes translational arrest, especially early in IAV infection [129]. PKR also activates NF- $\kappa$ B [130] and recruits RLRs and other antiviral ISG products to optimize antiviral signalling [131].

Another ISG worth mentioning in IAV infection is IRF7 (or interferon regulatory factor 7), an interferon regulatory transcription factor. Knockdown of IRF7 leads to greatly increased viral replication and, conversely, loss of IRF7 repressors substantially lowers influenza replication [132,133]. Mice lacking IRF7 generated almost no IFN $\alpha\beta$  response to IAV infection and

exhibited significantly more weight loss and mortality than controls. IRF7 mutations have been linked to severe IAV disease in human, a heterozygous null IRF7 mutation with defects in type I and III IFN response was detected in an otherwise healthy child who suffered life-threatening influenza infection [134]. This study is of particular interest as the investigators generated pulmonary epithelial cells from this patient, and found that IFN responsiveness was impaired and levels of viral protein higher in the infected patient's cells compared to controls [134].

In summary, it seems clear that PRRs in epithelial cells are involved in host defence, but there is not enough evidence to confirm an absolute requirement of epithelial-expressed PRRs for successful protection. Neither the absence of TLR-3 [57], nor the RIG-I signaling adaptor MAVS/IPS-1 [135] diminishes viral clearance and adaptive immunity to IAV infection.

There is likely to be considerable redundancy among different PRRs in their ability to support the early antiviral sensor roles and their presence in epithelial cells may not add greatly to that offered by other sentinel cells in terms of further activity of the innate immune cells. However, conceptually, epithelial TLRs and RLRs and consequent ISG expression are likely to be particularly important in IAV, since sensing and then inducing an anti-viral state in the cells that IAV infects and hijack for propagation, would seem vital to halting infection before spread. There is also a possibility that these PRRs may contribute to immunopathology, be critical determinants of the balance between eradication and immunopathology, and therefore the outcome from IAV infection.

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### Epithelium-derived cytokines and chemokines during IAV infection

The airway and alveolar epithelium can secrete a variety of cytokines and chemokines that activate and attract other immune cells to the lung. This response is rapid – expression of IL-1 $\beta$ , IL-6, CXCL10, TGF- $\beta$ , TNF- $\alpha$ , IL-8 and CCL2 are upregulated within 3–24hrs after IAV infection [136–140]. Expression is hierarchical and synergises with IL-1 $\beta$  and TNF- $\alpha$ , stimulating expression of other cytokines and chemokines [141]. However, many of these cytokines and chemokines are also secreted by resident and recruited immune cells in response to influenza infection and so delineating the specific contribution of epithelial cells to the prevailing cytokine milieu is difficult. Additionally, temporal effects compound the situation. Epithelial cells are likely to be the main early source of cytokines, but within 24–48hrs this balance shifts with recruited and resident immune cells becoming the dominant producers [140–142]. Here we discuss the roles of some key epithelial-derived cytokine and chemokines IL-1 $\beta$ , IL-6, CCL2 and TGF- $\beta$  in shaping the immune responses to influenza.

As outlined above, viral triggering of the NLRP3 inflammasome leads to release by epithelial cells of IL-1 $\beta$  a key early pro-inflammatory cytokine [143,144]. Together with IL-1 $\beta$  produced by resident immune cell populations, chiefly, alveolar macrophages [142], this pluripotent cytokine stimulates secretion of other cytokines and chemokines by the epithelium, and increases adhesion molecule expression, thus enhancing immune cell recruitment to the lungs [145,146].

Upon infection with IAV, human airway epithelial cells secrete IL-1 $\beta$  from their apical surface, suggesting an auto-crine signalling role [83]. In mice, IL-1 signalling is necessary for survival during IAV infection, but this beneficial effect is at the cost of IL-1 $\beta$  driven lung immunopathology [147,148]. Mice lacking IL-1 receptors had reduced pulmonary inflammation due to impaired neutrophil recruitment, and diminished CD4 T cell activation and recruitment [147].

IL-6 can function as both an anti- and pro-inflammatory cytokine, with diverse roles in the innate and adaptive immune response during IAV infection [149–152]. Both bronchial and type II alveolar epithelial cells secrete IL-6 after challenge with H1N1 and H5N1 viruses in vitro [137]. IL-6 was elevated in the serum of IAV- infected patients and was a hallmark of disease severity [153,154]. A recent study by Yang using IL-6 knockout mice demonstrated a role for IL-6 in recovery from lung injury by controlling extracellular matrix turnover and suppressing TGF- $\beta$  production to prevent fibrosis [155]. IL-6 also reduced virus-mediated apoptosis of airway epithelial cells and improved IAV clearance by macrophage phagocytosis [155].

CCL2 is a monocyte chemo-attractant, secreted by both airway epithelium and activated monocytes [156,157]. Monocytes recruited to the lung are required for host defence as CCL2 deficient mice do not survive IAV infection. However, they are also a major determinant of immune driven pathology [158,159]. Mice lacking CCL2 receptors have a better outcome from severe IAV infection [160–162]. Several studies have linked high levels of CCL2 and inflammatory monocytes in the lungs to severe disease in influenza infected patients [159,163–166].

TGF- $\beta$  is a multifunctional cytokine with important immune regulatory roles. Although most cells can produce TGF- $\beta$ , its expression is increased in the airway epithelium after IAV infection [140,167,168]. IAV has evolved to exploit the immune regulatory properties of TGF- $\beta$  as the NA protein can cleave latent TGF- $\beta$  into its active form [167,169], which then regulates type 1 IFN production. A recent study showed that overexpression of TGF- $\beta$  in the lungs resulted in reduced inflammatory responses, but impaired viral clearance [170]. When TGF- $\beta$  was specifically deleted from the airway epithelium, there was a marked reduction in IAV-induced weight loss, airway inflammation and pathology, and viral titres [140]. This protection was not associated with a heightened lymphocytic immune response, but an enhanced early IFN- $\beta$  release [140]. Therefore, IAV infection induced TGF- $\beta$  expression in epithelial cells and its subsequent cleavage to the active form, which in turn restrains early IFN- $\beta$  responses, and resulting in a viral permissive state.

These studies show that the four key cytokines and chemokines secreted by the airway epithelium during IAV infection act to recruit neutrophils, monocytes and CD4 T cells, impacting on repair and anti-inflammatory signals like TGF- $\beta$ . Their actions are also strongly associated with lung immunopathology.

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### Epithelium-associated immune cells and IAV infection

The airway epithelium supports niches for several specialised tissue-resident immune cell populations including intra-

epithelial conventional DCs (cDCs), innate lymphoid cells (ILCs), unconventional T cell populations (natural killer T cells, NKT; mucosal-associated invariant T cells, MAIT and  $\gamma\delta$  T cells) and intra-epithelial CD8 T cells. The cDCs and CD8<sup>+</sup> T cell population occupy specialised surveillance positions within the epithelium which allow them to sample both the luminal environment and detect perturbations in the epithelial layer. Both cDCs and CD8<sup>+</sup> T cells express CD103 (Integrin  $\alpha$ E) which binds to integrin  $\beta$ 7 expressed by epithelial cells, anchoring them between epithelial cells [2,3]. There is substantial cross-talk between epithelial and immune cells sequestered in the epithelium [171]. CD103<sup>+</sup>cDCs continuously sample the airway via extended dendrites and respond to chemokines and cytokines (including type I and III IFNs) and DAMPs secreted by IAV- infected epithelial cells [171–173].

Intra-epithelial dendritic cells are essential to generate protective IAV-specific CD8<sup>+</sup> T cells; mice lacking this DC subset succumb to severe disease and impaired viral clearance (reviewed in Ref. [174]).

Intra epithelial CD8<sup>+</sup> T cells differ phenotypically from peripheral blood CD8<sup>+</sup> T cells [175]. Piet et al. demonstrated that in human lung tissue, intra-epithelial resident CD8<sup>+</sup> T cells function as a local memory population [3]. At homeostasis these resident CD8<sup>+</sup> T cells are non-cytotoxic, protecting epithelial integrity [3]. However, recognition of their specific antigen or contact with epithelium derived type I IFNs rapidly increase both cytokine production and cytotoxicity [3].

In addition to the intra-epithelial niche, other immune cell populations are located in close proximity to the epithelium and able to respond to epithelial changes. These include invariant natural killer T (iNKT) cells, a CD1d-restricted lymphocyte with semi-invariant T cell receptors recognizing glycolipid antigens. iNKT cells have immunoregulatory roles [176] and have been shown to be 'held' within the intravascular space of the lungs, next to airway epithelium [177]. In this position iNKT cells can be activated rapidly and move into the lung interstitium and airway lumen where they may be involved in enhancing the immune response and regulating the size of myeloid responses [177–179]. There is evidence that human airway epithelium can express CD1d [179,180], but it is unknown whether epithelial CD1d is involved in activating iNKT cells; and the cognate glycolipid which activates iNKT cells during IAV infection is also unknown. However, IL-12 produced by intra-epithelial CD103 DC may activate iNKT cells in an indirect pathway [181–183]. In the mouse, presence, and exogenous activation, of lung iNKT cells by  $\alpha$ -GalCer, protects against lethal H1N1 and H3N2 influenza in prophylactic settings [158,178] [224]. Recent work by Gaya et al. demonstrated that iNKT cells resident in the interfollicular areas of lung draining lymph nodes are the earliest source of IL-4, prior to activity of T follicular helper cells in this site. This IL-4 seems crucial to the formation of germinal centres and in the ability of antibody-secreting cells to class-switch to IgG1 during IAV infection [184]. It is thought that CD1d-bearing resident macrophages activate iNKT cells in this process, though it is possible that CD1d-expressing epithelial cells may also contribute.

Another group of innate immune cells, the MAIT cells are also associated physically with the airway epithelium and

have recently been shown to be protective in influenza infection [185]. Classical activation of MAIT cells is via small molecule ligands, metabolic products generated by microbes, and presented by an MHC-I-like molecule, MR-1; however, during viral infection MAIT cells can be activated via epithelial cytokines [185]. ILC2 populations are enriched at airway epithelial surfaces and respond to epithelial derived cytokines including IL-33 secreted in response to damage or IAV infection [186]. ILC2 can also contribute to epithelial repair via secretion of amphiregulin, observed during IAV infection [187].

There is a group of resident natural killer (NK) cells, now classified as a group 1 ILC together with ILC1s [188,189], which express a mature and quiescent phenotype with high levels of inhibitory receptors and low levels of activating receptors, migration/adhesion-associated molecules and co-stimulatory molecules [190]. Cells with this phenotype (like intra-epithelial CD8 cells) protect epithelial integrity at homeostasis, but are rapidly activated and express functional molecules to respond to infection [190]. NK cells are one of the first innate cells to be found during IAV infection, but it is unclear whether these are resident NK cells or those recruited from the circulation.

In the above capacity, the airway epithelium provides a platform for the interaction of several innate immune cells which act as early cellular responders to IAV infection.

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### The respiratory epithelium – injury and repair during IAV infection

The epithelial cells' attempt to clear IAV results in inevitable tissue injury, in part because of collateral damage from the accompanying innate immune response and direct induction of apoptosis by IAV [92,191,192], but also because cytotoxic T cells will eventually kill cells with IAV peptides presented on their MHC class I molecules. If epithelial cells are not killed they undergo apoptosis or de-differentiation. If IAV reaches the alveolar epithelium, various injurious events can occur. The type 1 alveolar epithelial cells are closely apposed to the endothelium forming a barrier [11], and are bound to each other by tight junctions. The alveolar epithelium also keeps the alveolar lumen free of fluid through the action of ion channels and membrane proteins, which includes the epithelial sodium channels (ENaCs), the cystic fibrosis transmembrane conductance regulator (CFTR), and many different aquaporins. ENaC, is found on the apical surface of both type 1 and type 2 alveolar epithelial cells [193,194]. Very early after infection, IAV causes fluid accumulation in the alveolar lumen by direct inhibition of ENaCs [195]. The M2 ion channel of the virus might also inhibit the activity of ENaCs on epithelial cells by triggering the production of reactive oxygen species and subsequently activating protein kinase C [196]. All these result in compromise of the endothelial-alveolar epithelial barrier, and oedema fluid fill the airspace, causing respiratory insufficiency.

Airway epithelial injury leaves immediate and longer term consequences. One major immediate sequel is co- or secondary bacterial infections. Most influenza deaths are associated with histologic changes or microbiological evidence of bacterial infections [197–199]. Viral denudation of the airway



epithelium provides increased attachment sites for bacteria, resulting in invasive disease and greatly increased mortality. In mouse models, secondary bacterial infection correlates most closely to the point of greatest IAV-induced lung tissue damage [200]. The effect of IAV infection on susceptibility to bacterial infection extends to epithelium-associated innate cells. Alveolar macrophages, a major defence against bacterial infection have been shown to be completely depleted in severe infection [159] although, these were rapidly replaced by monocyte-derived macrophages, and there was no direct evidence that this contributed to secondary infection. However, defects in alveolar macrophage-mediated phagocytosis following IAV infection have been widely reported [201–203], possibly mediated by  $\text{IFN-}\gamma$  [204,205]. More recent studies investigating the causes of enhanced secondary bacterial infection after IAV infection have implicated disruption of innate immune defences (NK cells, neutrophils) as equally important factors in secondary bacterial infections.

As for longer term sequelae of airway epithelial injury, there is evidence that the anti-inflammatory immune responses upregulated to restore airway epithelial integrity may also leave a mark on susceptibility to future infections. For example, Hussell and colleagues propose that IAV can cause long-term modification of the lung microenvironment through an increase in the myeloid cell negative regulator CD200R (CD200 receptor) and de-sensitization to bacterial products [206,207]. This may limit subsequent inflammatory damage while the lung is healing, but is likely to predispose to bacterial colonization which worsens outcome.

Multiple mechanisms come into play in the early stages of infection to repair and regenerate after IAV infection. During infection, attempts are also made early on by lung epithelial cells to contain cell damage. This happens in several ways and simultaneously. For example, the airway epithelium expresses CD200, which can engage its receptor (CD200R) displayed by inflammatory macrophages during IAV infection, inhibiting the proinflammatory activity of this cell population [208]. The presence of DAMPs signals can also induce the production of the anti-inflammatory  $\text{TGF-}\beta$  and  $\text{IL-10}$  from epithelial cells. An intriguing study showed that alveolar macrophages can also establish immunosuppressive communication links with each other through synchronized  $\text{Ca}^{2+}$  waves in the epithelium [209] and direct reduction of alveolar neutrophil recruitment and diminished secretion of proinflammatory cytokines in the lungs.

After IAV infection, the clearance of apoptotic cells and cellular debris from the airways is essential to regain tissue homeostasis [210,211]. Although macrophages are the primary player in this clearance process, they can also enhance phagocytosis in other professional or nonprofessional phagocytes via the release of factors and extracellular vesicles [212]. For example, phagocyte-derived microvesicles and exosomes modulate phagocytic capacity in epithelial cells and insulin-like growth factor-1 (IGF-1) released from macrophages promotes the engulfment of macrophage-derived microvesicles by epithelial cells, leading to reduced inflammatory responses in epithelial cells [212]. Using an epithelial cell specific knockout mouse model, Juncadella et al demonstrated that intracellular signalling via the small GTPase, Rac1, is critical for epithelial cell engulfment [211]. Furthermore

induction of this pathway and phagocytosis by epithelial cell resulted in an altered ‘wound healing’ epithelial cell phenotype with increased  $\text{TGF-}\beta$  and  $\text{PGE}_2$  production [211] as observed during house dust mite and ovalbumin administration. Although there is no evidence for this pathway in IAV infection, the study is one of few which were able to attribute a function specifically to airway epithelium. Potentially, the airway epithelium could also contribute similarly to repair after IAV infection.

Once virus and virus-infected cells are cleared and associated pulmonary inflammation is controlled, the repair process begins in earnest. The lung's ability to regenerate the airway epithelium (in contrast to alveolar epithelium) is rapid. Neighbouring epithelial cells spread into the denuded area and progenitor cells proliferate to reconstitute the epithelium, first as squamous epithelial cells, which then differentiate into their original epithelial type. In the alveolar epithelium, the initial repair process is re-epithelialization of the denuded basement membrane and reassembly of tight junctions. This is accomplished primarily by type II alveolar epithelial cells, which are relatively resistant to injury [213–218]. Type II cells spread, proliferate, and transdifferentiate into type I cells to restore normal alveolar structure and barrier function [213]. Various signaling pathways have been identified that promote type II cell spreading [219,220], proliferation [214,221], and trans-differentiation [218,222]. A recent study shows that a subset of type II alveolar epithelial that is specifically responsive to Wnt and Fgf signalling may be the dominant progenitor cells during injury in mouse and humans [223]. The human lung also contains stem cells capable of forming functional bronchioles, alveoli, and pulmonary vessels after tissue damage, although these cells are less likely than progenitor cells to be relevant to IAV injury [224].

At the same time as epithelial activity, phagocytes - monocyte-derived macrophages and the resident alveolar resident macrophages begin to ingest microparticles released from leukocytes and tissue cells, matrix materials, surfactant components, pathogens (either dead or alive), apoptotic bodies, and intact apoptotic cells [225,226]. All these must be cleared in order to restore function of the alveoli. Overall, mouse studies depleting macrophages during different stages after tissue injury suggest that macrophages are important for the inflammatory, repair, as well as the resolution stages of lung injury [227,228], but the relative importance of resident alveolar macrophages to monocyte-derived macrophages in repair is unclear. IAV infection depletes resident alveolar macrophages that are found scattered along the alveolar epithelium early in infection [159], which may impact on repair of the airway epithelium since these macrophages have pro-repair properties.

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## Conclusion

The respiratory epithelium, comprising the airways and alveolar epithelial cells and their associated resident immune cells and progenitors have evolved a multi-layered defence against influenza A virus. In most individuals the virus is contained within the upper respiratory tract by stringent barrier, sensing and specific anti-viral mechanisms, providing

protection of the alveoli from damage. IAV strains have evolved mechanisms to counter these defence, but as long as the airway epithelium can prevent infection and spillover of the inflammatory process to the alveolar epithelium, the likelihood of a good outcome from IAV infection is high. The repair process for the upper airway is robust, enhancing protection of the lower airways (alveolar duct and alveoli) from infection and over exuberant immune responses, essential to survival of the host.

## Funding

Laura Denney is supported by NIHR UK Oxford Biomedical Research Centre (BRC) and Ling-Pei Ho by MRC and Oxford University.

## Conflicts of interest

The authors declare no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bj.2018.08.004>.

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