

# Innate immune responses to influenza A H5N1: friend or foe?

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**Avian influenza A H5N1 remains unusual in its virulence for humans. Although infection of humans remains inefficient, many of those with H5N1 disease have a rapidly progressing viral pneumonia that leads to acute respiratory distress syndrome and death, but its pathogenesis remains an enigma. Comparison of the virology and pathogenesis of human seasonal influenza viruses (H3N2 and H1N1) and H5N1 in patients, animal models and relevant primary human cell cultures is instructive. Although the direct effects of viral replication and differences in the tropism of the virus for cells in the lower respiratory tract clearly contribute to pathogenesis, we focus here on the possible contribution of the host innate immune response in the pathogenesis of this disease.**

## Introduction

Type A and B influenza viruses cause regular seasonal influenza epidemics but only type A is associated with pandemics. The virus haemagglutinin (HA) and neuraminidase (NA) are the major surface proteins of the virus that induce protective host antibody responses, and they are classified into 16 HA and nine NA subtypes by antigenic analysis. Influenza virus is a single-stranded RNA virus with an eight-segmented genome. As with other RNA viruses, mutations generate genetic and antigenic diversity (genetic drift). The segmented RNA genome allows the virus an additional mechanism for generating diversity through genetic reassortment. The pandemics of 1957 and 1968 arose by the prevailing human H1N1 influenza virus acquiring a novel HA and the polymerase basic 1 (PB1) gene (and in 1957 also the NA) from an avian source, to generate a virus with a novel subtype: H2N2 in 1957 and H3N2 in 1968 [1]. The origin of the H1N1 virus in 1918 remains controversial, with some arguing that it arose from an avian virus directly (i.e. all eight gene segments) and adapted to efficient transmission in humans [2], whereas others contend that it is also derived by reassortment [3].

Avian influenza H5N1 virus continues to zoonotically transmit to humans, which causes severe disease and poses a pandemic threat [4]. However, this virus has so far not adapted to efficient human-to-human transmission. In early 2009, a novel H1N1 variant (H1N1v) virus of swine

origin emerged and has now become pandemic [5]. This confounded the previously held dogma that an influenza pandemic is associated with the emergence of a virus with a novel HA subtype. The novel H1N1 virus is antigenically distant from the prevailing human H1N1 virus, and there is little prior cross-reacting humoral immunity in the population, with the exception of those individuals older than 60 years [6]. Thus, we now have a pandemic virus of an influenza subtype (H1N1) that is already endemic in the human population. It remains to be seen whether the novel pandemic H1N1 2009 virus will replace or co-circulate with the previously endemic H1N1 and H3N2 viruses. Although the infection appears to be comparatively mild, some patients have developed fatal pneumonia with acute respiratory distress syndrome (ARDS) [7], but information on the pathogenesis of pandemic H1N1 is still emerging [8]. In this review, we focus on innate immune responses in the pathogenesis of lung disease caused by influenza, with a particular emphasis on human H5N1 disease.

## Seasonal and pandemic influenza

Influenza is typically a self-limiting upper respiratory disease but may range from an asymptomatic to (rarely) a severe illness, with potentially fatal complications, especially in those with a preexisting underlying disease. Complications of influenza include pneumonia, exacerbation of asthma and chronic obstructive pulmonary disease (COPD) [9]. Influenza is also associated with febrile seizures in children, encephalopathy, which is particularly notable in Japan, and increased risk of myocardial infarction and strokes [10–12]. There is little evidence of systemic spread of the seasonal influenza virus, therefore, the systemic manifestations (e.g. myalgia), as well as some of these complications of seasonal influenza, have been attributed to cytokines and other inflammatory mediators [10,13].

The pneumonia that follows seasonal influenza is generally a rare complication and might be primary viral pneumonia, or more commonly, secondary to bacterial infection. By contrast, primary viral pneumonia is a major manifestation of human H5N1 disease [14,15]. Primary viral pneumonia was seen in the severe pandemic of 1918. A similar diffuse alveolar pattern of disease was also seen in a minority of young patients who died in the 1957 pandemic. Virus antigen has been detected in alveolar epithelial cells and alveolar macrophages. There continues to be controversy over whether the lung pathology of primary

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influenza viral pneumonia is caused solely by a direct viral cytopathic effect, or whether it is contributed to by innate immune responses [16–18]. Primary viral pneumonia is also now occasionally being reported in those with severe pandemic H1N1v disease [8].

### Human H5N1 disease

#### *Virology*

The current lineage of highly pathogenic avian influenza (HPAI) H5N1 virus derives from a group of H5N1 viruses that were first recognised in geese in Guangdong province, China in 1996 [19,20]. These viruses have undergone a series of genetic reassortments with other avian influenza viruses to give rise to a number of different virus genotypes (constellation of eight genes). The virus HA has also undergone genetic mutation over the past 14 years to give rise to a number of recognised virus clades and sub-clades that are antigenically and genetically diverse [21]. However, only limited numbers of the H5N1 genotypes and clades are known to have caused human disease [15].

A number of viral mutations are recognised as potential virulence factors for humans. The non-structural 1(NS1) gene segment is an interferon (IFN) antagonist and plays a key role in evading host innate immune responses [22]. NS1 binds double-stranded RNA, thereby preventing the activation of 2'5'oligo(A) synthetase and the downstream consequences of its activation. NS1 interacts with RIG-1 RNA helicase and suppresses its normal function as a cytosolic innate immune sensor of viral infection [23]. The four carboxy-terminal amino acids of NS1 form a PDZ ligand domain motif that is relevant for mouse virulence [24]. The PB1 gene segment of most human and avian influenza viruses encodes a second open reading frame, PB1-F2. Via its interaction with mitochondrial proteins, PB1-F2 is believed to induce apoptosis [25], enhance inflammation in mice, and synergistically enhance the severity of secondary bacterial infections [26]. In addition, influenza virus infection blocks many features of dendritic cell (DC) maturation (e.g. co-stimulatory molecules CD80, CD86) that are key to T-cell stimulation [27].

Although the basic amino acids at the HA cleavage site are clearly an important virulence factor for chicken, turkeys and mice, their contribution to virulence in humans is less clear. PB2 Lys627 is associated with virulence in mice [28] and contributes to replication competence in mammalian cells at lower temperatures [29]. However, humans infected with clade 2.2 H5N1 viruses that consistently carry PB2 Lys627 do not manifest more severe disease; in fact, the reverse might be true (see below) [30]. Other known viral determinants of pathogenicity include PB2 Asp701Asn, PB1-F2 Asn66Ser, NS1 Asp92Glu [reviewed in Refs. 31 and 32].

#### *Clinical features and epidemiology*

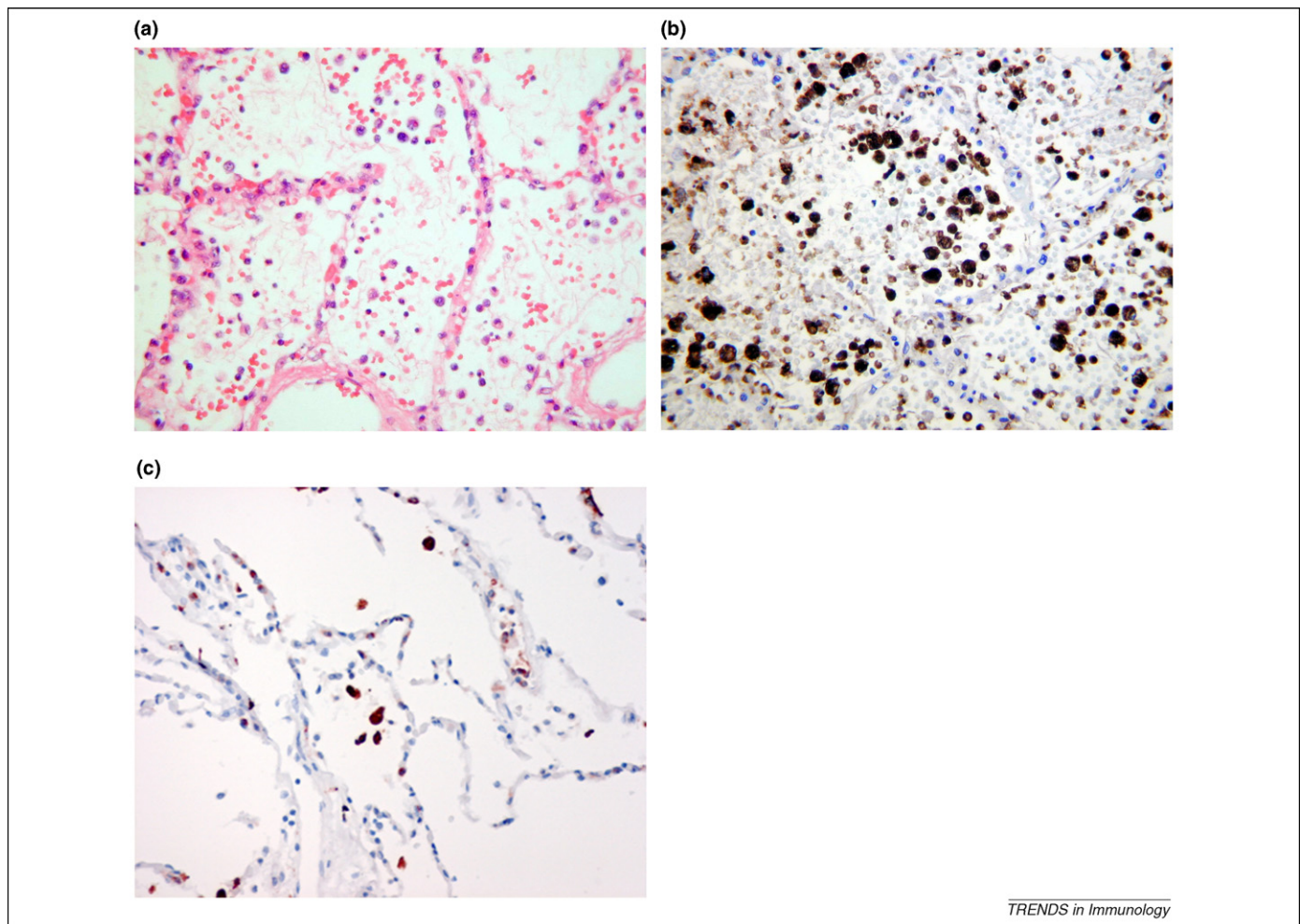
Avian influenza H5N1 is transmitted inefficiently and rarely to humans in spite of repeated and substantial exposure to the virus [33,34]. Underlying immunocompromising factors or other diseases associated with increased risk for seasonal influenza are not observed often in patients with H5N1 disease. Thus, exposure to virus is necessary but not a sufficient explanation for the observed epidemiology;

other factors such as host genetic or immunological susceptibility, or unusual routes of exposure are likely to play a role. H5N1 disease is less common in those aged over 40 years; an observation not explainable by the population age structure or risk behaviour of affected populations [35,36]. It is possible that cumulative hetero-subtypic immunity through repeated exposure to seasonal influenza might contribute to this age distribution. There is increasing evidence of antigenic epitopes that can mediate such cross-subtype immunity [37].

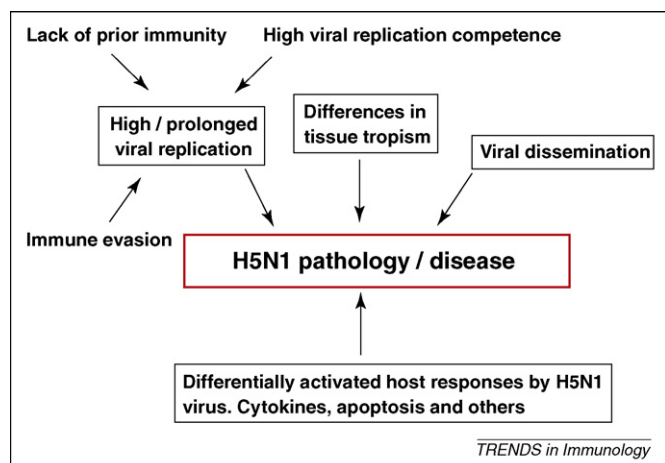
The overall mortality of patients with virologically confirmed H5N1 disease is >60% [15]. Although this observation might be skewed by the selective investigation and diagnosis of patients who are more severely ill, there is no doubt that H5N1 disease in humans is overall associated with a markedly worse clinical outcome. A proportion of patients appear to have a milder disease presentation, and these have been reported most notably in Hong Kong in 1997 (clade 0 virus) and more recently in Egypt (clade 2.2 virus), although even then, overall case mortality was >30%. In these two instances, young children had a milder disease presentation than adults [30,38]. Whether this reflects increased detection of milder cases in Hong Kong and Egypt, or is a reflection of differences in virulence of different virus clades remains unclear. Patients with severe H5N1 disease have a rapidly progressive primary viral pneumonia associated with leukopenia (a finding also documented in the 1918 outbreak), gastrointestinal symptoms, and mild liver and renal dysfunction [15,38]. The key autopsy findings are diffuse alveolar damage with hyaline membrane formation, i.e. the pathology of ARDS. Patchy interstitial infiltrates and pulmonary congestion are seen with varying degrees of haemorrhage (Figure 1A). The cellular infiltrate predominantly comprises macrophages, neutrophils and activated lymphocytes (Figure 1B). Apoptosis of alveolar epithelial cells is noted. Lymphocyte depletion is seen in the spleen and lymph nodes [39–44].

#### *Pathogenesis*

The severe disease associated with H5N1 infection in humans might arise through different mechanisms (or combinations thereof). These include: (i) dissemination of virus beyond the respiratory tract (in contrast with seasonal flu); (ii) higher and prolonged viral replication that leads to direct viral cytolytic damage; (iii) differences in the tissue tropism of the avian H5N1 virus (in contrast to the human seasonal influenza viruses); and (iv) differences in host responses induced by H5N1 virus (Figure 2). Although H5N1 virus appears to have the ability to spread beyond the respiratory tract, that is, the virus can be isolated from the faeces [45], serum [46,47] and very rarely from the central nervous system [46], the lung pathology (ARDS) remains the major cause of mortality in human H5N1 disease. However, at the time of death, immunohistochemistry rarely shows overwhelming viral infection of the lungs and often very few, if any, virus-infected cells are demonstrable [39,41,43,44]. The limited autopsy data arise from patients who have died after prolonged periods of illness and assisted ventilation, therefore, the paucity of virus at autopsy does not preclude a major role for virus in initiating lung injury.



**Figure 1.** (a) Lung histology of fatal human H5N1 disease stained with haematoxylin and eosin, which show increased cellular inflammation within the alveoli compared to normal lung. (b) Immunohistochemistry for the macrophage marker CD68 (brown) show increased numbers of macrophages infiltrating the lung tissue. (c) The histological appearance and alveolar macrophages shown by CD68 immunohistochemistry in a control lung is shown for comparison. Magnification 200 $\times$ .



**Figure 2.** Mechanisms that might contribute to the pathogenesis of H5N1 disease. Arrows indicate the factors that contribute to outcome. Higher levels of viral replication, binding of the H5N1 virus to receptors in alveolar epithelial cells, and spread of the virus beyond the respiratory tract could all contribute to the severity of human H5N1 disease. In this review, we argue that the H5N1 virus differentially activates host responses in macrophages and primary lung alveolar epithelia, and such differences in host responses contribute to disease pathogenesis. This figure is modified from Ref. [3].

The mechanism for ARDS is not fully defined, but cytokine-induced inflammatory responses are believed to play a significant role [reviewed in Refs. 48 and 49]. Scientific approaches to address the pathogenesis of ARDS in human H5N1 disease include clinical studies, investigations in relevant animal models, and *in vitro* investigations in primary human cells. Although each has advantages and limitations, a synthesis of knowledge from all three approaches would be informative.

H5N1 virus load in the respiratory tract remains elevated for much longer than is usually seen with seasonal influenza [50]. This is not surprising because, in contrast to seasonal influenza that infects most of us repeatedly, and leads to development of cross-reacting immune responses, most humans (perhaps with the exception of older individuals, see discussion above) are unlikely to have prior immunity against H5N1. The H5N1 polymerase complex is associated with its virulence in ferrets [51]. The Glu627Lys in the viral gene PB2 determines virulence in mice, as well as efficiency of viral replication in mouse cells [52].

It has been proposed that the avian H5N1, which binds sialic acid (SA) with  $\alpha$  2-3 linkages (typically found in avian cells), preferentially infects cells of the human lower respiratory tract. The alveolar epithelial cells express  $\alpha$  2-3

SA-galactose-N-acetylglucosamine, whereas the upper respiratory tract has a paucity of these receptors. By contrast, the human upper respiratory tract (nasopharynx and trachea) has an abundance of  $\alpha$  2-6 SA, which preferentially binds the human seasonal influenza viruses H3N2 and H1N1. They also have O-linked  $\alpha$  2-3 SA, which binds avian and human influenza viruses [53–57]. Furthermore, within the lung, H5N1 viruses preferentially attach to type 2 pneumocytes and macrophages. These findings have led to the hypothesis that the lung pathology of H5N1 is caused by differential targeting of the virus to the lower respiratory tract. Furthermore, if the H5N1 virus is unable to replicate efficiently in the upper respiratory tract, it would explain why the virus is not readily transmitted to humans. However, there are some key observations that do not fit in with such a hypothesis. H5N1 viruses readily infect *ex vivo* cultures of upper respiratory tissues (e.g. nasopharyngeal and tonsillar tissue) [54] and immunohistochemistry for viral antigen and virus receptors in tracheal tissue of a patient with fatal H5N1 disease has demonstrated that the virus can be found infecting tracheal epithelium [58]. Conversely, some seasonal H1N1 influenza viruses can readily infect the *ex vivo* cultures of lung (lower respiratory tissues) [53,54] but they are not commonly associated with severe lung pathology. There are also patients with H5N1 disease who have mild, self-limited, upper respiratory illness without lower respiratory tract involvement. As part of rapid diagnostic procedures, influenza antigen has been detected in nasopharyngeal epithelial cells from nasopharyngeal aspirates of these patients, which suggests that H5N1 virus can in fact infect the upper respiratory tract [38]. Although tissue tropism could well play a role, we contend that other mechanisms also contribute to the unusual severity of human H5N1 disease (discussed below).

### Host responses to H5N1 influenza

Host responses to influenza are clearly complex and involve humoral and cell-mediated, as well as innate immune responses. Specific antibodies are the best established correlate of protection against infection, whereas, cell-mediated immune responses play a key role in recovery from disease [59]. Adoptive transfer experiments have shown that CD8<sup>+</sup> T memory cells can cross-protect across different subtypes [60]. Furthermore, memory T cells induced in response to seasonal human influenza can cross-react even with avian influenza H5N1 [61]. There are however, limited data on cell-mediated immune responses in H5N1 disease. For the purpose of this review, we therefore focus on innate immune responses, while remaining fully cognizant of the importance of the contribution of adaptive immune responses in the clinical outcome of H5N1 disease.

### Clinical data

When compared with seasonal influenza, patients with H5N1 disease have higher serum levels of macrophage and neutrophil chemoattractant chemokines (CXCL10, CXCL2, IL-8) and both pro- and anti-inflammatory cytokines (e.g. IL-6, IL-10, IFN- $\gamma$ ) [50,62]. Patients who died had higher serum levels of these mediators than those who

survived. However, the levels of these mediators also correlated with viral load in the nasopharynx and might simply be a reflection of increased virus replication and increased pathology[50].

### Animal models

Ferrets, mice, macaques and other mammals have been used as experimental models for influenza pathogenesis, each with their own advantages and limitations [63,64]. The most appropriate animal model for use depends on the purpose, that is, vaccine evaluation, antiviral testing, transmission or pathogenesis. The most challenging of these is the choice of an animal model to study transmission and pathogenesis. Although guinea pigs are used to study virus transmission, influenza causes minimal pathology or disease in this animal model [65]. Relevant features of available animal models for the study of pathogenesis of HPAI H5N1 and human seasonal influenza are summarised in the table. Ferrets most closely mimic humans in the distribution of putative SA receptors in the respiratory tract, whereas mice and macaques do not [57]. For instance, ferrets can be infected with human influenza viruses without prior adaptation, but mice typically cannot. There are however a paucity of immunological reagents and genomic data on ferrets; an issue that currently imposes major limitations on the detailed immunological study of this animal model. Mice have been used widely for studies on influenza virulence and pathogenesis, particularly because of the availability of reagents, however, inbred mouse strains and mice with defined gene defects still have a number of caveats. Firstly, there is sometimes poor correlation between lethality of viruses for mice and for ferrets [63]. For instance, some H5N1 viruses are highly neurotropic in mice, and fatality is therefore more likely related to virus dissemination to the brain rather than related to the lung pathology. Many investigators use H5N1 viruses with rapid lethality for mice because they provide a clear end-point, although such lethality may in fact reflect neurotropism rather than the ARDS-like lung pathology, which is the cause of death in humans (Table 1).

In general, in comparison with seasonal influenza viruses, HPAI H5N1 viruses show increased virulence in mice, ferrets and macaques, with evidence of increased viral replication and dysregulated host responses. Mice infected with H5N1 have cytokine and chemokine responses including the IL-1 $\beta$ , IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , macrophage inflammatory protein (MIP)-1 $\alpha$ , IL-6 and MIP-2, monocyte chemoattractant protein-1, KC protein (equivalent to human IL-8), IL-1 $\alpha$ , at days 3–5 post-infection [66–68]. This is associated with recruitment of macrophages and neutrophils into the lungs, which causes acute lung inflammation [68]. Ferrets infected with H5N1 viruses have markedly stronger induction of the chemokine CXCL10 and IFN response genes in the lungs in comparison with H3N2 subtype seasonal influenza. On the contrary, CD45, GRB2, phosphoinositide-3-kinase gene and the mitogen-activated protein kinase (MAPK) genes associated with B and T-cell signalling are all down-regulated [69]. Blocking of CXCR3, the cognate receptor of CXCL10, with the drug AMG487 in H5N1-infected ferrets results in a reduction of symptom severity and delayed

**Table 1. Animal models used for the study of pathogenesis of HPAI H5N1 and seasonal human influenza (based on data and reviews in Refs. [57,63,64]).**

	Human	Mouse	Ferret	Macaque	Cat	Pig
<b>H5N1 virus</b>						
<b>Binding of H5N1 virus. Anatomical site and cell type</b> <sup>57</sup>	Alveoli> >trachea Type II pneumocyte	Trachea> alveoli Type II pneumocyte	Alveoli> >trachea Type II pneumocyte	Alveoli> >trachea Type I pneumocyte	Alveoli> >trachea Type II pneumocyte	Alveoli> >trachea Type II pneumocyte
<b>Disease readily caused by H5N1 virus without prior virus adaptation</b>	Yes ARDS Hyaline membranes	Yes ARDS with some strains, e.g. A/Ck/Hebei/ 108/02	Yes	Yes ARDS with a few strains A/HK/156/97	Yes ARDS-like Hyaline membranes	Poor
<b>Dissemination beyond respiratory tract</b>	Limited extent	Might be widespread. Depends on virus strain	Might be widespread. Depends on virus strain. Dissemination may not correlate with strains that disseminate in mice	No	Yes	No
<b>H1N1 or H3N2 virus</b>						
<b>Binding of human seasonal H1N1 or H3N2 virus. Anatomical site and cell type</b> [57]	Trachea> >alveoli Type 1 pneumocyte	±alveoli	Trachea> >alveoli Type 1 pneumocyte	Poor binding	Poor binding	Poor binding Type I pneumocyte
<b>Disease following human H1N1 or H3N2 virus without prior virus adaptation</b>	Yes	Very mild and does not recapitulate human disease. Mice-adapted viruses may lead to severe disease.	Yes Mimics human disease.	Mild illness	No	Mild illness. Tracheal inoculation may lead to more severe pneumonia
<b>Other comments</b>	Availability of genetic sequence data, microarrays and immunological reagents	Availability of genetic sequence data, microarrays, knock-out mouse lines and immunological reagents	Paucity of genetic information and immunological reagents	Availability of some genetic sequence data and immunological reagents	Paucity of genetic information and immunological reagents	Paucity of genetic information and immunological reagents

mortality compared to vehicle treatment [69]. Macaques infected with H5N1 virus have more intense and more protracted expression of type I IFN responses, IFN-induced genes IL-1 and 6, TNF- $\alpha$  and CXCL10 than in H1N1-infected macaques. Similarly, H5N1 virus infection is associated with higher and more prolonged viral replication in the lung, more severe lung pathology, as well as a dramatic depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and premature apoptosis of DCs [70]. Surprisingly, although the H5N1 virus induces a more potent IFN response, virus replication remains poorly controlled.

The aberrant host responses induced by H5N1 virus in mice and macaques is reminiscent of those induced by the 1918 H1N1 virus in these animals. In mice, the 1918 virus differentially activates apoptosis pathways, IL-6, type I IFN and Toll-like receptor (TLR) response genes, and these findings are associated with severe pulmonary pathology [71]. Similarly, further studies have found higher viral

titres in the lung and increased lung macrophage and neutrophil infiltration, MIP-1 $\alpha$ , IL-1, IL-6 and IFN in association with the 1918 virus [68]. In macaques, the 1918 virus leads to dysregulated immune responses with higher IL-6 and lower type 1 IFN titres [72]. A recombinant seasonal influenza H1N1 virus with the 1918 HA and NA with or without the NS gene segment has been studied for its pathogenicity in macaques [70]. While these recombinant viruses do not have the virulence associated with the full 1918 H1N1 virus reported by Kobasa and colleagues [72], the 1918 HA and NA appear to increase the virulence of the seasonal influenza H1N1 virus and are also associated with changes in gene expression profile.

#### *Studies with gene-defective mice and the effect of experimental immunomodulator treatment*

When challenged with H5N1 viruses, mice deficient in IL-6 or MIP-1 $\alpha$  have comparable morbidity and mortality in

comparison to wild type controls, although this is not surprising, because most cytokines and chemokines have some redundancy in their effector pathways. Mice with defects in genes for the IL-1 receptor and the type I interferons IFN- $\alpha$  or IFN- $\beta$  have a worse outcome following H5N1 infection, which suggests that these pathways are protective [67,73]. Interestingly, TNF-receptor-1-deficient mice, as well as mice treated with anti-TNF antibody, have less weight loss following infection when compared with controls, although survival is no different [67]. This may reflect a role for TNF receptor signalling in the pathogenesis of influenza-induced lung disease, although mortality (which in these mice is associated with neuro-invasion) is unaffected. In a study in which a number of immunomodulators currently in clinical use were investigated, together with an antiviral agent (zanamivir) in a mouse model of H5N1 disease, zanamivir in combination with a cyclooxygenase-2 (COX-2) inhibitor (celecoxib) and mesalazine led to improved survival when compared to the antiviral alone [74]. The hyper-induction of chemokines by H5N1 virus leads to the accumulation of a particular subset of DCs, which are described as TNF- $\alpha$ /inducible nitric oxide synthase (iNOS) producing DCs (tipDCs) in the lung airways. These tipDCs are important for proliferation of influenza-specific CD8<sup>+</sup> T-cells in the lung. Chemokine receptor 2 (CCR2)-defective mice that lack this chemokine attraction have markedly reduced accumulation of tipDCs in the lung, which leads to delayed virus clearance. However, modulation of the tipDC trafficking by treatment with the peroxisome proliferator-activated receptor- $\gamma$  agonist pioglitazone moderates the deleterious effects of tipDC recruitment, without losing its beneficial effects of cytotoxic-T-cell recruitment [75].

Studies with seasonal influenza viruses have suggested that defects in TLR3 or COX-2 are beneficial to mice challenged with seasonal H3N2 virus [76,77], which suggests that innate immune responses may sometimes be deleterious. On the other hand, ferrets infected with a higher virulence influenza virus induce weaker type I and II IFNs and IL-8 mRNA but more IL-6 mRNA in the nasal fluid washes compared to animals infected with a lower virulence virus. The authors of this study have speculated that the lack of an IFN response allowed the virus to spread to the lung, which led to an increase in virus virulence. However, the lung cytokine levels were not measured [78]. These findings support the therapeutic use of IFN to correct this weaker induction of IFN by the more virulent seasonal influenza virus.

It has been found that mice with inactivating mutations in TLR4 or its downstream signaling molecule TRIF (but not MyD88) are protected from acute lung injury (ALI) by chemical agents as well as inactivated H5N1 virus administered intratracheally [79]. IL-6-deficient mice are also protected from lung pathology in this model. Inactivated H5N1 virus has been shown to induce oxidised phospholipids, which trigger an inflammatory response that leads to ALI via TLR4 and the TRIF/TRAFF6 signalling pathway. Deletion of the *Ncf1* gene, which controls reactive oxygen species production, reduces the severity of H5N1-mediated ALI. Collectively, this suggests that oxidative stress and innate immunity are key injury pathways that control the

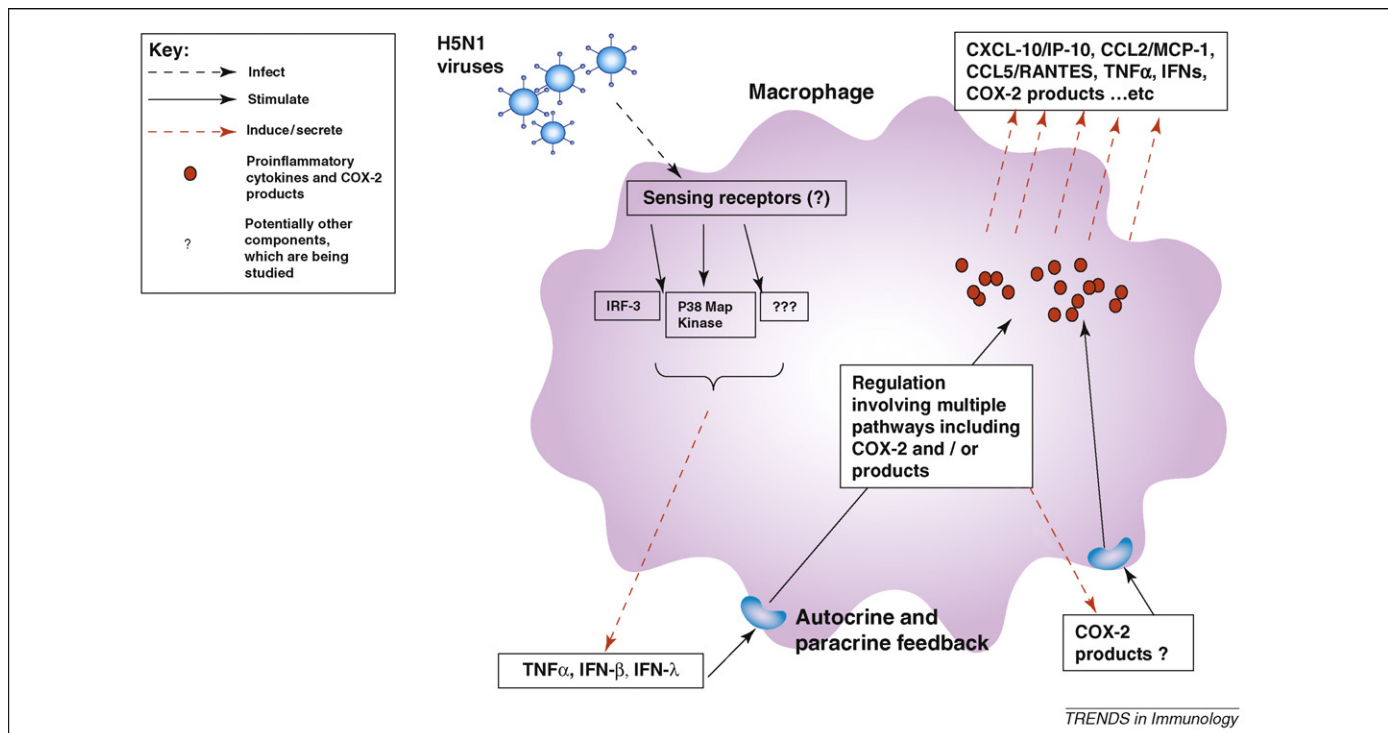
severity of ALI. It remains to be seen if these pathways are relevant to infection with the live H5N1 virus.

Protease-activated receptors (PAR) are activated by extracellular proteases that are abundantly found in the lung. PAR<sub>2</sub> has been shown to be induced by seasonal influenza virus infection, and appears to inhibit virus replication via an IFN- $\gamma$ -dependent pathway. PAR<sub>2</sub> agonists increase survival of A/PR/8/34 (H1N1)-virus-infected mice. The increased survival is associated with reduced viral titres in the lung, reduced neutrophil infiltrates, reduced RANTES (a chemokine) and increased IFN- $\gamma$  secretion [80].

#### *Studies in primary human cells in vitro and ex vivo cultures*

Studies in humans with H5N1 disease, as well those in animals experimentally infected with H5N1 and seasonal influenza viruses have demonstrated that the increased pathology of the H5N1 virus is associated with increased viral replication and enhanced host responses. As these observations reflect the outcome of multiple cycles of virus replication and associated tissue damage in humans or animals, it is not possible to differentiate whether the aberrant host responses are merely secondary to the enhanced pathology caused by H5N1 virus, whether they reflect increased cumulative viral load, or whether there are intrinsic differences between viruses in their ability to induce these host responses. This question can only be addressed by experiments carried out on physiologically relevant, well-defined cell populations with a defined virus inoculum, a synchronous virus infection, and sampling of the host response at defined times. Since alveolar pneumocytes and macrophages are the two key cells infected by H5N1 viruses *in vivo* (see above), we and others have compared the host responses induced in primary human macrophages and type 1 epithelial cells by H5N1 or seasonal influenza (H1N1, H3N2) viruses. Compared with human H1N1 or H3N2 viruses, equivalent infecting doses (approximately two infectious virus particles per cell) of H5N1 virus more strongly induces a range of cytokines including TNF- $\alpha$ , IFN- $\alpha$  and - $\beta$ , IL-1  $\beta$ , as well as chemokines such as CCL2, CCL3, CCL4, CCL5 and CCXL10 from primary human macrophages [68,81]. Similarly, H5N1 viruses differentially upregulate CXCL10, IL-6, IL-8, CCL2, CCL5, and IFN- $\beta$  from alveolar epithelial cells [82]. Thus, many of the cytokines and chemokines found to be differentially elevated in the sera of patients with H5N1 disease (compared to seasonal influenza) are also more strongly induced *in vitro* using comparable challenge doses of the virus (Figure 3). This differential gene expression occurs within the first few hours of virus infection and is dependent on infection with live virus. Furthermore, increasing the infecting dose of H1N1 (low cytokine phenotype) virus to 10 times that of H5N1 virus still does not result in a comparable host response.

Cytokine responses are induced directly by the stimulus but are also amplified by autocrine and paracrine mediator cascades. The primary mediators directly and differentially induced by H5N1 virus are TNF- $\alpha$ , IFN- $\beta$  and IFN- $\lambda$ ; the other mediators being the result of autocrine and paracrine responses [83] (Figure 3). The induction of



**Figure 3.** Cytokine and chemokine induction in macrophages infected with H5N1 virus. Virus infection activates IRF-3 and the p38-MAPK signaling pathways as well as others. Activation of these pathways leads to the expression of primary mediators such as TNF- $\alpha$ , the type I interferons IFN- $\alpha$  and - $\beta$ , which in turn trigger release of other cytokines and chemokines through autocrine and paracrine effects. COX-2 is involved in regulating cytokine expression within the infected cell, as well as those activated by secreted mediators in adjacent uninfected cells.

the primary mediators occurs, at least in part, via the virus differentially activating interferon regulatory factor (IRF)-3 and p38MAPK pathways [83,84]. In an intact organ, there are interactions between different cell types, and we have tried to mimic such interactions between virus-infected macrophages and alveolar epithelial cells *in vitro* [85]. The virus-free supernatants of H5N1-infected macrophages induce mediator cascades in alveolar epithelial cells, and these lead to amplification and broadening of the host responses. For example, TNF- $\alpha$  is not induced directly by H5N1 virus infection of alveolar epithelial cells, but virus-free supernatants from H5N1-infected macrophages do induce TNF- $\alpha$ . COX-2 has been found to be a key controller of this amplifying cytokine cascade, and COX-2 inhibitors can dampen these amplifying mediator cascades [85] (summarised in Figures 3 and 4). Some of these cytokines (e.g. IFNs) are expected to have an antiviral effect (see above), but the overall effects of these cytokine cascades might contribute to pathogenesis. Although we have reported that H5N1 more strongly induces type 1 IFNs in alveolar epithelial cells [82], others have reported that H5N1 induces a weaker type 1 IFN response in differentiated bronchial epithelium than does seasonal influenza H3N2 [86].

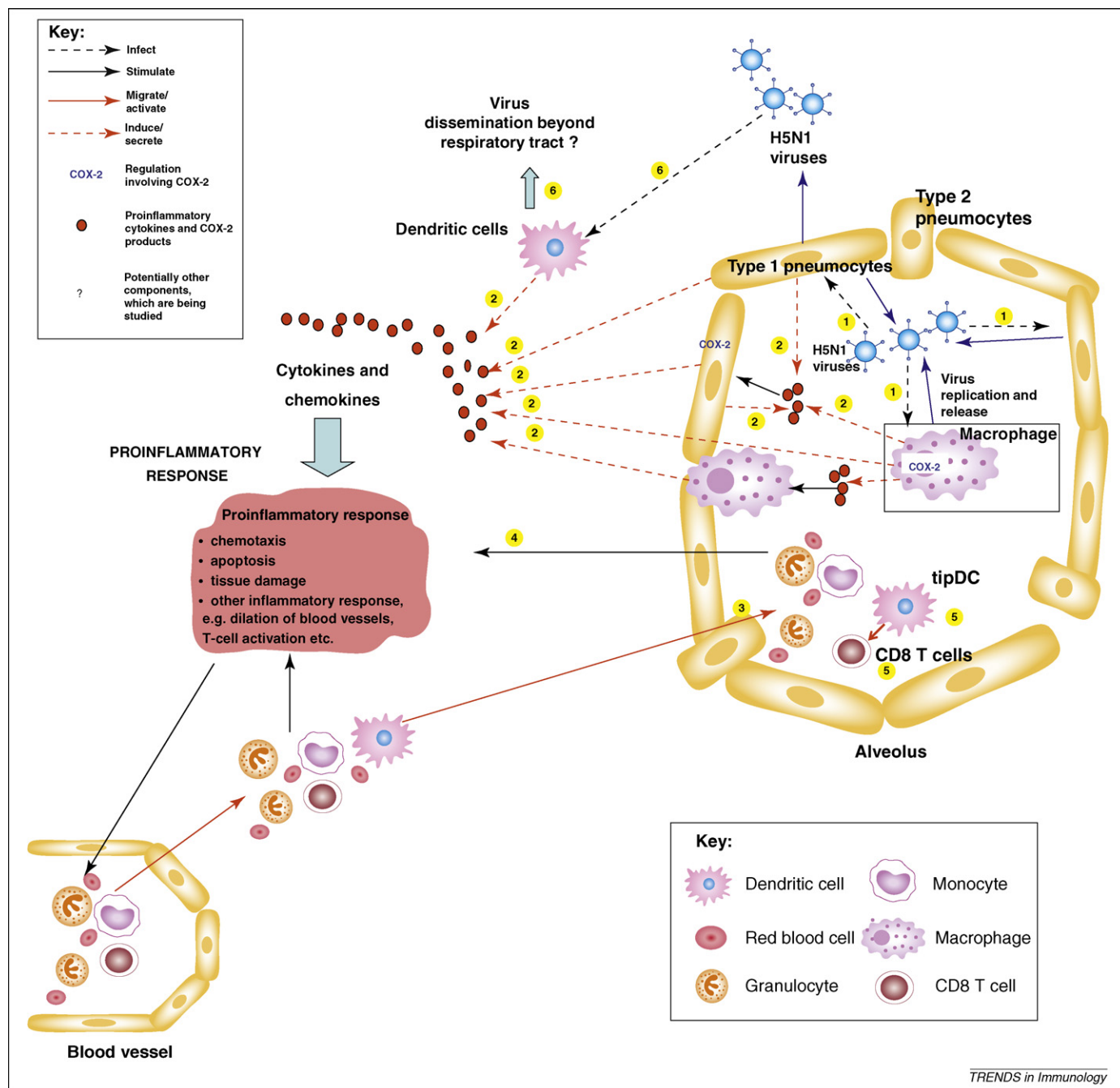
Taken together, these findings support the contention that the differences in host responses seen in animal models and in humans with H5N1 infection, at least in part, reflect intrinsic differences in the virus-induced host response. In contrast with H5N1 viruses, the 1918 H1N1 and seasonal influenza H1N1 viruses induce comparable levels of cytokines in macrophages infected *in vitro* [68]. Thus, the differential host responses seen in animals with

1918 H1N1 infection (see above) might not reflect differential host response at the individual cell level, although further work needs to be done on other cell types (e.g. alveolar epithelium).

It is important to understand the virus genetic factors that determine the high-cytokine phenotype of H5N1 viruses. Using virus reverse genetics, we have established that the H5N1 virus HA and NA are not essential to the high-cytokine phenotype. Rather, the constellation of internal genes, in particular the polymerase genes and NS gene segments play key roles in this [87]. Not all H5N1 virus genotypes manifest the high-cytokine phenotype but those that are associated with human disease appear so to do [87,88].

Cytokine induction is not the only host response pathway that is differentially modulated by H5N1 viruses. Seasonal influenza virus H1N1 induces apoptosis pathways faster than H5N1 viruses [89]. Induction of early apoptosis is potentially a host defence mechanism to limit viral replication, and these differences might therefore have pathogenic relevance. On the other hand, H5N1 viruses appear to enhance expression of TNF-related apoptosis-inducing ligand (TRAIL) and lead to more potent bystander apoptosis of T cells [90]. This may contribute to the lymphopenia seen in H5N1 disease.

H5N1 virus infects other cells of the innate immune system with potentially pathogenic consequences. *In vitro* infection of human myeloid DCs leads to productive virus replication, production of IFN- $\alpha$  and TNF- $\alpha$ , and leads to cell death within 24 h. DCs are present below the respiratory epithelial layer and migrate to draining lymph nodes upon activation. Thus productive infection of these DCs



might contribute to dissemination of the virus, however, depletion of these cells might lead to an impaired immune response to the virus infection. Pretreatment of DCs with IFN- $\alpha$  abolishes virus infection and plasmacytoid DCs, which are naturally high type I interferon producers, are refractory to virus infection, and produce large amounts of IFN- $\alpha$  after co-culture with H5N1 virus [68,91]. H5N1 virus can also infect and replicate in primary human

natural killer (NK) cells, which leads to apoptosis. Targeting NK cells might help the virus evade NK cell innate immune defences [92]. Finally, recent *in vitro* studies have demonstrated that  $\gamma\delta$  T cells (which are chiefly associated with mucosal surfaces) can be activated and expanded to have potent cytotoxic activity against cells infected with a wide range of influenza virus subtypes, including H5N1 [93].

## Review

## Conclusion

The world is currently in the throes of a pandemic caused by a novel H1N1v virus. The disease remains relatively mild, although a small minority of patients appear to manifest with a primary viral pneumonia that may progress to an ALI or ARDS-like clinical presentation. Most of these patients have been healthy young adults and some of them had no underlying illnesses that would predispose to severe influenza disease. Although the clinical presentation in these patients with severe respiratory disease is reminiscent of human H5N1 disease [7], the underlying pathogenesis remains to be explored.

Notwithstanding the current H1N1v pandemic, H5N1 remains endemic in poultry in Asia and parts of Africa and continues to pose a major threat to public health. Its morbidity and mortality in humans is not contained very effectively by antiviral therapy alone. For example, in Indonesia, while earlier commencement of oseltamivir treatment is associated with improved survival, even treatment within the first 4 days of disease is not a guarantee of survival and is still associated with 42% mortality [94]. Alternative therapeutic strategies are needed urgently. The first step towards this is to understand the pathogenic mechanisms that underlie the lung pathology associated with human H5N1 disease, and how it differs from that of seasonal influenza. Here, we have reviewed data that suggest that the innate immune response may be both friend and foe. This points to possibilities of novel therapeutic interventions that deserve further investigation. Preliminary data suggest that the differences in host responses induced by H5N1 appear to be similar to those of seasonal H1N1, albeit of greater intensity [83], but more systematic studies using gene expression profiling and proteomic studies to address this question are warranted. It is relevant also to investigate whether biomarkers that provide early indication of poor prognosis can be identified to guide such immunomodulatory interventions. It is particularly interesting that targeting some signalling pathways that are associated with inflammation (e.g. the RAF-MEK-ERK kinase cascade, nuclear factor- $\kappa$ B activation) can also block viral replication [95]. Thus, such therapeutic interventions might potentially combine antiviral effects with beneficial immunomodulatory effects.

As the pandemic H1N1 virus originated in swine, its global spread might be associated with a panzootic of this novel H1N1 virus in swine. This enhances the opportunity for reassortment between the pandemic virus and other viruses, including with H5N1 virus, which has been reported in pigs [96]. The internal gene cassette of these swine viruses from which the H1N1 pandemic viruses arises appears to have an unusual propensity for reassortment. Given the unprecedented severity of H5N1 disease and its continued threat to human health, it is important that we understand better the biological basis of its virulence and pathogenesis. This would also provide an improved understanding of the pathogenesis of ALI and ARDS caused by influenza, as well as other causes. ALI and ARDS arising from many diverse causes continues to be a major cause of morbidity and mortality [97].

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