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Meeting Report

Report of the 'Mechanisms of lung injury and immunomodulator interventions in influenza' workshop, 21 March 2010, Ventura, California, USA*

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**This meeting was dedicated to the memory of Dr Jerome 'Jerry' A. Donlon, December 21, 1939 to October 12, 2009, Chief Science Officer and Medical Advisor in the Office of the Biomedical Advanced Research and Development Authority (BARDA), US Department of Health and Human Services.*

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The clinical course of influenza and the extent of lung injury are determined by both viral and host factors, as well as sometimes secondary bacterial infections and exacerbations of underlying conditions. The balance between viral replication and the host immune responses is central to disease pathogenesis, and the extent of lung injury in severe influenza infections may be due in part to overly exuberant or dysregulated innate inflammatory responses or sometimes deficient responses. Acute respiratory distress syndrome (ARDS) is the principal cause of respiratory failure associated with severe influenza. ARDS can be triggered by both direct lung insults (e.g. respiratory pathogens) and systemic insults (e.g. sepsis), and the lung damage is exacerbated by the inflammatory response associated with either infectious or non-infectious insults. This workshop aimed to review the current understanding of lung injury in acute influenza and describe cellular and molecular mechanisms of lung injury that are common to influenza and infections by other respiratory pathogens. In addition, therapeutic agents that target host response proteins and pathways were identified and investigational agents in development reviewed. A logical strategy would be to combine antiviral treatment with drugs that modify excessive host responses or supplement deficient ones. However, a better understanding of common cell signalling pathways associated with acute lung injury caused by influenza and other pathogens is necessary to understand immunopathologic causes of lung injury. This will help determine which immunomodulatory interventions might be useful, and to predict the appropriate timing and consequences of their use.

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Introduction

Most influenza virus infections are uncomplicated and self-resolving, but severe, life threatening disease can occur, especially in the very young, elderly or people with underlying health conditions or when novel viruses emerge. Influenza A viruses of subtypes H3N2 and H1N1 have been circulating in the human population since 1968 and 1977, respectively. However, in 1997, the highly pathogenic avian H5N1 virus emerged in China, subsequently spread across Europe and into Africa in 2004–2005 and continues to cause sporadic zoonotic human infection associated with high mortality[1] in multiple countries. In 2009, the first pandemic of the 21st century was caused by a novel H1N1 influenza virus of swine origin. This pandemic H1N1 2009 virus has been associated uncommonly with severe viral pneumonia and excess mortality in children and young to middle-aged adults[2] and continues to circulate in the human population today. These recent events and the continued threat of pandemics caused by other subtypes have lead to renewed influenza research on disease pathogenesis, clinical management and novel therapeutic options.

Whilst oseltamivir antiviral treatment appears to reduce mortality in both H5N1 and severe pandemic H1N1 infections, even early treatment does not always lead to survival.[3,4] Therefore, other treatment options need to be investigated. Disease severity is dictated by both viral and host factors. Respiratory failure is the most common cause of death in severe cases, often caused by the development of acute respiratory distress syndrome (ARDS). Lung

pathology is postulated to arise from a number of factors: high and prolonged viral replication, viral tropism for cells in the lung and a differentially activated host response. In particular, hypercytokinaemia is associated with severe H5N1 influenza infections and thought to exacerbate lung pathology.

This workshop was convened to bring together academic, commercial and public sector researchers to discuss current knowledge in the field and novel therapeutics. The aims of the workshop were to describe the mechanisms of lung injury in acute influenza; to identify the mechanisms of lung injury that are common to influenza viruses and other respiratory pathogens and to identify potential immunotherapeutic targets to mitigate influenza-mediated acute lung injury and review investigational agents in development. Presentations covered observations on the pathology and clinical disease during severe influenza, the cellular and molecular biology of infection in humans and animal models and both novel, prototypic interventions and well-known therapeutic candidates, including TNF-α antagonists, IFN-α and commonly prescribed drugs with immunomodulatory properties like cyclooxygenase 2 inhibitors and statins. The meeting concluded with a discussion on regulatory issues and future directions for studying immunomodulators and novel therapeutics.

This report describes each of the speaker's presentations, commencing with an overview to introduce the topic of lung injury in H5N1 and pandemic H1N1 2009 infections.

Overview of lung injury in human influenza infections

Comparison of the disease spectrum and pathogenesis of avian H5N1 and pandemic H1N1 2009 influenza

Professor Malik Peiris (University of Hong Kong) reviewed and compared the disease spectrum and pathogenesis of avian H5N1 and pandemic H1N1 2009 influenza virus infections in humans.

Since initial detection in humans in 1997, the highly pathogenic H5N1 virus has spread widely and is now endemic in poultry in many countries in Asia and Africa, fuelling the sporadic occurrences of human H5N1 infections with high lethality. As of March 2011, there have been a total of 530 recorded cases with an average case fatality rate of 59% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_03_10/en/index.html). Severe H5N1 infections have usually developed in otherwise healthy, young individuals, predominantly under 45 years of age. Mild disease and asymptomatic infections have been documented in seroepidemiologic studies, but severe illness is predominant.[1,5]

The newly emerged pandemic H1N1 2009 virus was first detected in Mexico in February 2009, then rapidly spread around the globe.[5] Millions of people have been infected with more than 17 000 laboratory-confirmed deaths reported (as of March 2010). However, this underestimates the total number of deaths owing to lack of ascertainment. Furthermore, an accurate case fatality rate is difficult to determine owing to incomplete surveillance data on mild and asymptomatic cases. In Hong Kong, seroepidemiology and investigation into hospitalised cases have allowed good estimates of pandemic H1N1 2009 infection attack rates and disease severity. The overall population attack rate was found to be 10.7%, but infection and mortality rates were highly age dependent. Whilst overall infection rates of those 30–60 years of age were low, case fatality rates increased dramatically in older adults (i.e. 66-fold increase in fatality rates in those 50-60 years of age compared with children).[6] Many mild or asymptomatic infections occurred, especially in children, as reported elsewhere.[6,7] Thus, H5N1 has infected small numbers of people, but the proportion developing severe disease is very high, whereas the disease profile of pandemic H1N1 infections shows that the proportion of severe cases is very small with a far larger proportion of mild and asymptomatic infections. These observations suggest an important role of as-yet-unidentified host genetic factors in predisposing to infection by H5N1 and to severe disease by both H5N1 and pandemic H1N1 viruses.

When severe cases do occur, the clinical features of H5N1 and pandemic H1N1 exhibit many similarities but also some differences. The pathology is characterised by a primary viral pneumonia that can lead to acute respiratory distress syndrome (ARDS) and multiple organ failure (MOF) associated with lymphopenia and renal and liver dysfunction. The virus can sometimes disseminate systemically to cause extrapulmonary disease, but the major cause of death is caused by progressive respiratory failure. Severe pandemic H1N1 disease has been associated in the majority of cases with underlying conditions such as pregnancy, morbid obesity, chronic respiratory disease or cardiovascular problems, but the proportion of severely affected patients who have no recognised underlying condition has exceeded 50% in some reports, therefore many previously healthy persons developed severe disease. Whilst the risk of severe disease in pandemic H1N1 infections was lower than with H5N1, those who did develop severe disease had similar lung pathology and respiratory complications, usually succumbing to ARDS and acute respiratory failure. In contrast to severe H5N1 disease, a significant proportion of patients with severe pandemic H1N1 illness had secondary bacterial infection at presentation (26–55% of fatal cases).

Enhanced viral replication, tropism for cells in the lung and a dysregulated inflammatory response are all thought

to contribute to lung pathology in H5N1 and pandemic H1N1 viral pneumonia. Evidence for a dysregulated host innate immune response in H5N1 infections comes from clinical observations and animal models of infections (reviewed in Peiris *et al.* 2009[8]). H5N1 infections induce high levels of proinflammatory serum cytokines and chemokines (e.g. MCP-1 IL-6, TNF-α and IL-8) which correlate with nasopharyngeal viral loads.[9] H5N1 infection of macaques also shows markedly elevated levels of innate cytokine production that corresponds to prolonged, high-level viral replication.[10] H5N1 preferentially infected type II pneumocytes in the lung that function to maintain lung integrity and repair that are compromised during influenza infection.[10] Of note, fatal cases of pandemic H1N1 virus infection show viral replication in both type I and II pneumocytes.[11] In a novel attempt to reduce disease severity, a CXCR3 antagonist (AMG487) has been used to block an innate signalling pathway in a ferret model. Ferrets infected with H5N1 and treated with AMG487 showed a reduction in weight loss and viral load, although final mortality rates were the same for both treated and untreated ferrets.[12]

Although elevated cytokine levels are associated with enhanced disease in H5N1 infection, the pathogenesis of disease remains incompletely understood. It is unclear whether severe disease and ongoing viral replication cause the dysregulated cytokine patterns observed, or whether and to what extent the cytokine dysregulation causes the severe disease. Correlations exist between increased viral load and increased cytokine levels,[8] so that continued viral replication is a contributing factor to disease. One strategy to elucidate the mechanisms of the early innate responses to infection is to use *in vitro* systems employing primary human epithelial cells and macrophages. Both epithelial cells and alveolar macrophages can be infected with H5N1 virus, and cytokines produced by each cell type act in an autocrine and paracrine manner.[13] Studies to date have shown that primary human macrophages infected with H5N1 differentially express proinflammatory cytokines by stronger activation of the p38MAPK and IRF-3 cell signalling pathways.[14–17] In addition, primary human macrophages infected with H5N1 produce highly elevated levels of TNF-α, IFN-β and IFN-λ1 in comparison with seasonal H1N1 infection.[17] Transcriptomic studies on macrophages infected with H5N1 and seasonal H1N1 influenza infection show that the marked differences between these viruses are quantitative rather than qualitative differences resulting from activating unique signalling pathways.[18] Primary epithelial cells infected with H5N1 also upregulate the expression of proinflammatory cytokines like IFN-β, IL-6, IL-8 and chemokines.[19] The cytokines produced by the epithelial cells and macrophages represent only the start of the immune response, and their paracrine action further amplifies and broadens the host response. As infection progresses, leucocytes are recruited to the infection site, thus enabling adaptive immunity to develop. Because H5N1 infection causes an early rise in chemokines, it is expected that increased numbers of immune cells are also recruited to the lung. Cyclooxygenase -2 (cox-2) has been found to play a central role in amplifying this proinflammatory response and therefore may be a suitable target for therapeutic intervention.[13] In addition, recent *in vitro* studies showed that endothelial cells also upregulate cytokine expression in a manner similar to that seen in epithelial cells after H5N1 infection.[20] Because H5N1 viruses can infect and egress from endothelial cells from either the apical or basolateral aspect, this provides a mechanism by which these viruses may spread systemically.[20]

Upregulation of the cytokine response by H5N1 viruses appears owing to the viral polymerase genes.[21] Whilst the degree of cytokine induction is related in part to polymerase activity, the viral polymerase proteins can also interact with host cell factors involved in signalling cascades[22] This raises the question of how this information could be used to direct therapeutic interventions. One area for study is to determine whether antiviral drugs that inhibit polymerase activity (e.g. favipiravir) could be useful as both antiviral and immunomodulator.

In mild pandemic H1N1 2009 infections, viral shedding and clinical illness profiles appear similar to those caused by seasonal H3N2 infections.[23] Other studies have shown more protracted replication in mild–moderate pandemic H1N1 2009 infections than observed historically in seasonal influenza.[24,25] Furthermore, in those pandemic H1N1 2009 patients with viral pneumonia, higher and more sustained viral loads have been found in tracheal aspirate or bronchoalveolar lavage specimens than in upper respiratory tract ones.[26] The extent of pneumonia seen in hospitalised patients with pandemic H1N1 2009 illness correlates with elevated serum cytokine levels, but it is currently not possible to know to what extent virus replication or lung injury itself might be causing the cytokines' increase. Experimental infections of ferrets have shown that the pandemic H1N1 2009 causes more severe disease than seasonal H1N1 but generally less than H5N1 influenza infection. The pandemic H1N1 2009 virus is also found at higher titres and more widely distributed in the lower respiratory tract tissue as well as sometimes in the gastrointestinal tract in animal models.[27,28] In *ex vivo* human respiratory tissue cultures, comparable viral replication was found for pandemic H1N1 2009 and seasonal H1N1 influenza viruses. Greater pandemic H1N1 2009 replication was observed in primary bronchial epithelial cultures incubated at 33°C, although not at 37°C that may contribute to an increase in virulence and the tracheitis observed in humans. Other studies have found greater replication in human lung specimens at 37°C for pandemic H1N1 2009 than seasonal H1N1 viruses.[29,30]

Pandemic H1N1 2009 can also replicate in the human conjunctiva more efficiently than seasonal H1N1, a finding that reflects a subtle difference in viral tropism that may play a role in pathogenicity.[30] However, unlike H5N1 virus, pandemic H1N1 2009 does not differ from seasonal H1N1 virus in its intrinsic capability to upregulate cytokines in alveolar epithelial cells, macrophages or dendritic cells.[30–32] Global gene expression profiling in infected human type-I pneumocytes re-enforces the similarity of the pandemic and seasonal H1N1 viruses.[33]

Although the pandemic H1N1 2009 virus differs from seasonal influenza in tissue tropism and virulence, these differences are subtle, so that host factors such as comorbidities, genetic susceptibility and pre-existing immunity would appear to be the main factors that drive disease. Patients with severe disease show slow viral clearance and prolonged hypercytokinaemia. In these cases, current antiviral therapy used alone may not be sufficient, and both more potent antiviral regimens (e.g. intravenous antivirals, combinations) and adjunctive treatments (e.g. immunomodulators, passive immunotherapy) may be important for improving outcomes in severe pandemic H1N1 2009 and H5N1 infections.

Pulmonary pathologic findings of fatal pandemic H1N1 2009 influenza viral infections

Dr William Travis (Columbia University, New York City, NY, USA) described the pathologic findings of fatal pandemic H1N1 influenza infections in 34 adults from New York.[34] Autopsy reports, clinical records and histopathological slide specimens were examined to describe and understand pulmonary pathology related to fatal pandemic influenza cases. The main pathologic observations noted in pandemic H1N1 fatalities included tracheitis, bronchitis and bronchiolitis, often accompanied by inflammation, ulceration and squamous metaplasia. These pathologic findings are similar to those observed in autopsies from the 1918 pandemic.[35] In both instances, the lungs frequently exhibited oedema, haemorrhage, infiltrating lymphocytes and diffuse alveolar damage (DAD) with hyaline membrane formation, a hallmark of ARDS. The type of DAD varied between patients; the majority of fatal cases manifesting DAD had acute DAD (64% of cases) associated with a shorter time in hospital before death (mean, 3.4 days), as compared to those with organising DAD (28% of cases; mean, 11.7 days) or fibrosing DAD (8% of cases; mean, 31.5 days). These patterns are similar to those seen in SARS where fatal cases that had a hospital duration or 10 days or less exhibited acute DAD, whereas cases hospitalised for more than 10 days showed organising DAD.[36] It would be important to fully understand the mechanisms behind the very striking pulmonary inflammatory reactions in some patients. Whilst almost all of these patients would have been artificially ventilated with high airway pressures and oxygen levels, interventions that might cause additional damage and associated fibrosis, similar fibrosis was also seen in fatal cases during previous pandemics where such interventions were not used. Therefore, it is thought that these pathologic observations are primarily a function of the disease rather than mechanical interventions.

Influenza virus antigen was demonstrated in all 34 cases examined, most commonly in the tracheobronchial tree epithelium, but also at a lower frequency in alveolar macrophages and in alveolar epithelial cells (both type I and II pneumocytes). A recent macaque model of pandemic H1N1 infection has shown virus-associated damage of both type I and type II pneumocytes[37] that reinforces the reported findings of involvement of these in fatal human cases.[11] Viral antigen detection in the nuclei and cytoplasm of alveolar macrophages indicates productive replication in these cells. Viral antigen often did not colocalise with foci of DAD, which might suggest rapid asynchronous kinetics of virus infection and clearance, as well as substantial heterogeneity in local pulmonary immune responses. Rather than being owing to asynchronous viral replication kinetics, another explanation could be that the pathology results from inflammatory mediators released during infection of other areas of the respiratory tract and not a result of direct virus cytopathic effects.

In the pre-antibiotic era, the 1918 pandemic was characterised by the high proportion of patients who developed secondary bacterial infections. This study found that 55% of fatal pandemic H1N1 cases were positive for secondary bacterial infection, lower than reported in the 1918 and 1957 pandemics.[35] Bacteria most commonly identified were *Streptococcus pneumoniae* and *Staphylococcus aureus* with one case of MRSA. Other pandemic H1N1 reports have found lower rates of secondary bacterial infection in fatal cases with a range of 26–38%.[11,38] However, antibiotics might have been administered to most of these patients with H1N1 2009 illness, thereby confounding bacterial detection upon autopsy. This uncertainty serves to highlight the importance of compiling comprehensive clinical notes that may help assess the usage and impact of antibiotic use in patients with pandemic influenza.

This study highlights the common pathologic features seen in each pandemic of the last century and the value of autopsy pathology employing modern techniques (e.g. antemortem CT scanning, RT-PCR and immunohistochemistry on fixed tissue sections). It seems that the pulmonary pathology remains consistent whilst the sector of the human population that is susceptible to lethal infection changes with each new viral emergence. The current pandemic deaths have occurred disproportionately in young adults, which is in contrast to seasonal influenza where most deaths occur in those over 65 years of age.[39] In

the New York study, the decedents included two infants and 29 adults who ranged in age from 25 to 49 years. The factors that predispose to severe viral pneumonia are incompletely understood, but an important contributing factor appears to be the existence of underlying medical conditions. In this study, 91% of fatalities had a comorbidity, and those with obesity (BMI > 30; 72% of patients), especially morbid obesity (BMI > 39; 47% of patients), were disproportionately represented. The exact mechanism by which these comorbidities contribute to severe disease, in both seasonal and pandemic influenza, remains to be fully understood. Adiponectin, an adipokine that reduces macrophage activity and proinflammatory cytokine production, is produced in decreased amounts in patients with obesity, and this decrease has been postulated to contribute to their increased risk of severe influenza, in addition to obesity-related alterations in lung mechanics and physiology.[40]

Cellular and molecular biology of influenza infections

Role of the NLRP3 inflammasome in innate immunity to influenza

Dr Jenny Ting (University of North Carolina) presented her work on the nucleotide-binding domain and leucine-rich-repeat-containing protein 3 (NLRP3). NLRP3 is a member of the recently discovered nucleotide-binding domain and leucine-rich-repeat-containing NLR family of pattern recognition receptors (PRR). Other well-known PRRs include the RIG-I helicases and the toll-like receptors (TLRs). The NLR proteins detect cytosolic DNA via their nucleotide-binding domain (NBD) which is flanked by regions of leucine-rich repeats. There are 20 NLR proteins discovered to date, but few have been characterised in depth.[41,42]

The NLRP3 protein and its adaptor protein PYCARD (PYD- and CARD-domain-containing protein) serve to control the production of active IL-1β through the formation of a biochemical complex known as the 'inflammasome'.[43] It does this by upregulating the production of active caspase-1 that in turn cleaves IL-1β and IL-18 precursors into their functional forms. Other NLR proteins, such as NLRC4, NLRP1 and NAIP, are also able to activate caspase-1 and produce functional IL-1β and IL-18. Mutations of NLRP3 can be found in the population and cause a rare gain-of-function inflammatory disease, known as cryopyrin-associated periodic syndrome (CAPS). Treatment using an IL-1R antagonist has been found to successfully ameliorate disease.

The NLRP3 inflammasome is involved in the innate response to a wide variety of bacterial, fungal and viral stimuli, including the influenza virus. In a mouse NLRP3 knockout model of A/PR8 virus infection, it was found that NLRP3 is necessary for viral clearance and survival, even though infected NLRP3$^{-/-}$ mice had less pulmonary pathology.[44] Thus, the increase in pulmonary cellular infiltrate that was observed in this model appears necessary for the protection against disease and death. Similar experiments showed that PYCARD and caspase-I are also necessary for the protection against influenza. NLRP3$^{-/-}$ mice also fail to mount detectable serum IL-18, serum or pulmonary IL-1β or pulmonary MIP-2α responses. Whilst other NLR proteins have been linked to IFN production, no effect on type I IFN response was seen in this mouse model.[44]

As well as responding to pathogenic stimuli, NLRP3 can also respond to non-pathogenic stimuli known as DAMPS (danger-associated molecular patterns). DAMPS comprise endogenous cellular or extracellular molecules released upon stress or damage and can include chemicals such as silica crystals and alum salts. DAMPS undergo lysosomal degradation causing the release of lysosomal cathepsin B that has been associated with NLRP3 activation and subsequent IL-1β production and cell death. Lysosomal degradation products can also initiate the formation of reactive oxygen species (ROS) in the cytosol which in turn can also activate NLRP3. *In vitro* and *in vivo* mouse experiments showed that ROS inhibition reduced the IL-1β response after influenza infection, suggesting that ROS formation is necessary for inflammasome activation during influenza infection.[44] Cathepsin B was also found to be necessary for the NLRP3-dependent IL-1β influenza response. These results indicate that the NLRP3 inflammasome response to influenza infection involves lysosomal cathepsin B and ROS that are typical of DAMP detection pathways. However, as discussed below, ROS also contribute to lung damage, and inhibitors like *N*-acetyl cysteine have been used in individual cases of severe pandemic H1N1 influenza.[45] In addition, viral RNA sensing was also found to be important in initiating the inflammasome response: mice exposed to synthetic RNA analogues, both dsRNA and ssRNA, subsequently mediated NLRP3 inflammasome IL-1β production.[44] These observations have the potential to inform therapeutic choices for future study, although much work will be necessary to characterise the magnitude and timing of the cytokine responses to other influenza viruses and in models involving other species before it can be determined that a particular immunomodulator might be of benefit and not retard viral clearance.

Reactivation of local tissue memory T cells and role of IL-22 in acute influenza infection

Dr David Topham (University of Rochester) presented data describing pulmonary-specific adaptive immune responses in a mouse influenza model. Clearance of virally infected epithelial cells is largely mediated by cytotoxic CD8$^+$ T cells. During primary influenza infection in mice, cytotoxic CD8$^+$ cells are recruited to the respiratory tract and peak

8 days post-infection, several days after peak viral titres. During a secondary infection, an accelerated $CD8^+$ cell response and virus clearance occur earlier. In human infections of older children and adults, influenza is almost always a secondary infection in that most people are exposed to influenza early in life. A secondary immune response involves recruitment of memory T cells from mucosal lymphoid tissues, e.g. nasal-associated lymphoid tissue (NALT), the circulating memory pool and extrapulmonary lymphoid tissues. These memory T cells tend to exhibit a tissue-specific phenotype enabling firstly migration into the respiratory tract upon secondary challenge, and secondly appropriate effector function.

VLA-1 and VLA-2 are matrix-binding integrins expressed by resident lung $CD4^+$ and $CD8^+$ T cells. VLA-1 is upregulated on $CD8^+$ T cells after influenza infection.[46] In a VLA-1 mouse knockout model, the development of tissue memory $CD8^+$ T cells was found to be impaired after influenza infection, thereby establishing the role of VLA-1 in lung memory CD8 T-cell generation. VLA-1^+ $CD8^+$ cells can be found for up to 50 days post-infection in the lung and airways in mice.[46]

Fewer lung $CD4^+$ T cells express less VLA-1 than lung $CD8^+$ T cells. In contrast, most $CD4^+$ cells express VLA-2. However, after primary infection in mice, VLA-1 expressing $CD4^+$ T cells increase, thereby producing a unique subset of antigen-specific memory VLA-1^+ $CD4^+$ T cells resident in the lung.[47] These cells are distinguishable from their central and effector-memory counterparts. These memory VLA-1^+ $CD4^+$ T cells appear to be the primary effector cells during secondary virus challenge (about 80% of the IFN-γ-expressing $CD4^+$ T cells are VLA-1^+), are reactivated within hours of secondary infection and account for the majority of the early cytokine production in the mouse lung. In contrast, typical effector memory $CD4^+$ T cells in lymphoid tissues do not proliferate into an effector-memory population until after 2 days post-infection and only migrate into the lung tissue after days 3 post-infection.[47] These data are consistent with a distinct tissue-memory and lymphoid effector-memory subsets sequentially activated during secondary infection in this model.

IL-22 is a homeostatic cytokine produced by NK and $CD4^+$ T cells which stimulates epithelial proliferation. A deficiency in IL-22 can leave animals susceptible to lethal lung and gut infections. Using a microarray gene expression chip, an increase in $CD4^+$ T cell–expressed IL-22 was detected after secondary influenza infection in the mouse, concurrent with a decrease in IL-17 and IL-23 expression. In turn, this may impact NK cell production of IL-22, as lung NK cell production of IL-22 is IL-23 dependent. IL-22 levels in the lung decreased after day 2 of a primary infection, and influenza-infected mice treated with an anti-IL-22 antibody displayed limited weight loss and mortality. Of note, lung viral titres were reduced in the anti-IL-22-treated mice.[48] Together, this suggests IL-22 may be actively regulated during influenza infection to limit epithelial proliferation, effectively decreasing target cell numbers.

Human host factors required for influenza virus replication

Dr Megan Shaw (Mount Sinai School of Medicine, New York, NY, USA) reviewed the recent work in determining the human cellular factors required for influenza replication. The limited coding capacity of influenza virus means that it must hijack a number of host cellular factors to complete its replication cycle. Knowledge of the viral–host interactions offers the opportunity to design novel therapeutics to disrupt or prevent these interactions and limit virus replication.

One approach involved the use of a genome-wide short interfering RNA (siRNA) screening method to elucidate host factors necessary for influenza replication.[49] Briefly, the method entailed transfecting a human epithelial cell line (A549) with more than 98 000 siRNAs covering approximately 19 000 genes, followed by infection with a recombinant WSN virus (A/WSN/33) with the coding region of the haemagglutinin gene replaced by a *Renilla* luciferase gene. Amongst 295 host genes that affected virus replication without the siRNA directly conferring cell toxicity, 177 fell into 11 functional groups, including kinase regulated signalling, ubiquitin pathway, phosphatase activity and transcription factors. By further integrating protein interaction data from available databases, a dense interaction network containing 181 of the confirmed host proteins and influenza virus proteins was formed. Within this network were cellular pathways previously implicated in influenza virus replication as well as novel host pathways or complexes such as the coat protein (COPI) complex and fibroblast growth factor receptor (FGFR) signalling pathway. Of note, this work did not detect any elevation in early viral replication which may have identified antiviral host factors. This is a limitation of the screening method utilised that could not assess the effects of siRNAs on multicycle replication (owing to the lack of the HA coding region in the recombinant virus used for infections during the screen).

Of the 295 host factors identified to affect viral replication, 219 were confirmed to be necessary for wild-type virus growth in multicycle growth assays. One of these factors was CSE1L, which exports karyopherin-alpha proteins out of the nucleus. CSE1L knockdown experiments found that CSE1L was not necessary for viral entry but was required for nuclear import of viral RNPs. Twelve representative host factors were shown to be necessary for WSN replication as well as for pandemic H1N1 virus replication. This indicates that the host factors identified in this paper are likely to be applicable to other influenza viruses.

Four other studies using RNAi screening technology in influenza infection have been published recently.[22,50–52] Perhaps surprisingly, there appears to be modest overlap of the host factors identified from each study. Konig *et al.* and Karlas *et al.*[49,52] share the highest number of matching host factors, perhaps because both of these studies used the same epithelial cell line and virus strain in their experiments. The other studies use different viruses (i.e. A/PR8 or VSV-G enveloped virus) and different cell lines (e.g. HBEC, *Drosophila* cell line). The source of the RNAi libraries may also be a contributing factor to the observed variation. However, when analyses are carried out at the pathway or complex level rather than the gene level, more common pathways can be identified across the published studies for influenza. These include pathways involved in ion transport; nuclear pore function; interferon-related and kinase signalling. In addition, analyses were extended to identify host pathways shared between influenza and other RNA viruses (e.g. HIV, HCV). Many common cellular pathways and complexes were found, for example, certain shared kinase signalling complexes are necessary for influenza, HIV, HCV and WNV replication.[49]

Finally, host factor–directed inhibitors of influenza replication were tested. For example, CAMK2B is a host cell kinase involved in cytoskeletal regulation and CREB-dependent transcription. CAMK2B was found to be needed for optimal influenza virus polymerase activity and postulated to be involved in viral RNA transcription. Using the CAMK2B inhibitor, KN-93, viral replication was reduced in a dose-dependent manner *in vitro*. Diphyllin that inhibits vATPases (several members of which were identified in the screen) was also effective in reducing viral replication. Both of these inhibitors warrant further study in animal models of influenza. Other studies have shown that host factor–directed inhibitors like targeting the Raf/MEK/ERK kinase pathway and the activation of NF-κB[53–55] lead to decreased viral replication.

Innate immune response of human alveolar type II cells and alveolar macrophages during influenza infection

Dr Robert Mason (National Jewish Health Centre, Denver, CO, USA) described the expression of innate immunity genes in alveolar epithelial cells and macrophages during influenza infection *in vitro*. A method to culture differentiated type II pneumocytes and alveolar macrophages from primary human cells has been previously established.[56] Type II pneumocytes predominantly express the α2-3-linked sialic acid receptor which make them preferentially permissible to avian viral subtype infections rather than human seasonal influenza viruses. When cultures of type II pneumocytes and alveolar macrophages were infected with A/PR8 virus and an A/H3N2 reassortant virus (with HA, NA and NP genes from A/Philippines/2/82 and the remaining genes from A/PR8), both viruses were able to infect each cell type but only the pneumocytes were able to support productive virus replication.[56] The host innate immune response was determined by mRNA expression using an Affymetrix gene chip followed by verification by real-time RT-PCR and ELISA. These experiments concentrated on the first 24 hours post-infection, and during this time, no cytopathic effect was observed. Type II pneumocytes displayed a marked increase in the expression of cytokines, chemokines and PRR molecules after infection. In particular, these included IFN-λ1 (IL-29) and IFN response genes; RANTES; IP-10, IL-6 and IL-8 but not TNF-α or IFN-α. Unexpectedly, the ELR-negative group of chemokines (CXCL-9, -10, -11) were found to be substantially upregulated. The two viruses induced the same cytokine types, but overall, the A/H3N2 virus elicited lower levels compared to A/PR8. In particular, A/PR8 induced markedly increased levels of IL-29 compared to infection with H3N2 (up to 10-fold more). The reason for this difference is unclear as both viruses exhibited similar replication levels in this system, but this observation highlights important differences in the host response amongst different influenza viruses. Viral infection of alveolar macrophage cultures mainly elicited similar innate responses to those seen in type II pneumocyte cells, although infected macrophages did produce TNF-α and IFN-α but lower levels of IL-29.

Lung surfactants can both suppress and enhance inflammatory signals. In a resting lung, surfactants can help downregulate inflammation via NF-κB, whereas in a damaged lung, surfactants allow the activation of NF-κB.[57] Human surfactant-D (SP-D) has also been shown to inhibit influenza infection and alveolar macrophage activation.[58] During *in vitro* infection of cultured type II pneumocytes, no consistent change in SP-D expression was detected during short-term experiments. SP-D would be expected to suppress infection and therefore slow down any cytopathic effects, especially for circulating H3N2 viruses that have a highly glycosylated HA known to be inhibited to a greater extent by SP-D than viruses with HA surface proteins that are less glycosylated such as A/PR8 and the recent pandemic 2009 H1N1 virus.

The origins of the hypercytokinaemia associated with severe influenza disease may be due to cytokines produced by cells initially infected or by cytokines produced by neighbouring cells through paracrine action. Work carried out in rat lung epithelial cells infected with coronavirus showed that cells expressing CXC chemokines did not express viral proteins, indicating that in this system, CXC chemokine expression was a result of a paracrine effect. As the addition of an IL-1R antagonist, but not a soluble TNFR, was able to block CXC chemokine secretion from the rat coronavirus (sialodacryoadenitis virus (SDAV)-infected alveolar

epithelial cells, IL-1 is partly responsible for CXC chemokine production.[59] In an A/PR8 influenza infection of alveolar macrophages, the addition of an IL-1R antagonist or soluble TNFR was able to reduce IL-8 (CXCL8) secretion by approximately 50%, or about as much as observed after the addition of inactivated virus.

These observations demonstrate that alveolar type II pneumocytes are able to respond robustly to non-avian influenza virus strains and other respiratory viruses and to play a significant role in initiating innate immune responses and the elaboration of proinflammatory cytokines. Such responses might be especially important in the pathogenesis of severe influenza viral pneumonia.

The role of TLR4 in influenza pathogenesis and ACE2 interventional therapy

Professor Yumiko Imai (Akita University School of Medicine, Japan) presented data elucidating mechanisms of innate immune signalling in the pathogenesis of ARDS and novel interventional therapies. TLRs have previously been observed to play a role in increased lung injury. For example, in a mouse model of acid-induced ARDS used to understand innate immune mechanisms, it was found that TLR4$^{-/-}$ mice exhibited a natural resistance to acid-induced lung injury.[60] TLRs act as pathogen recognition receptors for a variety of extracellular and intracellular pathogens. TLR4 is most commonly associated with extracellular bacterial lipopolysaccharide (LPS) recognition, but interestingly it is also involved in RSV inflammatory responses.[61,62] During infection by the 1918 influenza virus, mice showed an increase in TLR gene expression in lung tissue.[63] The TLR4 signalling pathway involves either MyD88 or TRIF activation, both of these then lead to the activation of TRAF6 and finally to NF-κB-activated transcription of proinflammatory cytokines that are associated with the over-exuberant response associated with severe influenza disease.

To examine the role of TLR4 in responses to influenza, mice were inoculated with an inactivated H5N1 virus. Using immunohistochemistry, viral antigen was observed in lung pneumocytes and more commonly in alveolar macrophages. Virus inoculation of TLR4$^{-/-}$ mice showed a much reduced level of severity of disease characterised by reduced lung elasticity and pulmonary inflammation.[60] TRIF$^{-/-}$ and TRAF6$^{-/-}$ mice exhibited a similar reduction in influenza-induced lung injury, indicating that lung injury is caused through the TLR4-TRIF-TRAF6 pathway in this murine model. The severity of lung injury was correlated with the increased production of various proinflammatory cytokines and chemokines (i.e. IL-6, CXCL10/IP-10, MIP-2, KC) but not with that of TNF-α or IL-1β.[60] In accordance with the previous observations that seasonal H1N1 elicit lower proinflammatory cytokines from alveolar macrophages, similar responses were not seen with inactivated seasonal H1N1 influenza virus administration, a finding that again underscores the importance of viral virulence factors.

Oxidative stress has been previously linked to the inflammatory response and ALI. Influenza viruses expressing the 1918 HA show an increase in reactive oxygen species (ROS) expression in the lungs of infected mice.[64] Oxidative stress has also been seen to recruit TLR4 to lipid rafts in plasma membranes of alveolar macrophages.[65] The oxidised phospholipid, OxPAPC, is generated at the sites of inflammation. Furthermore, OxPAPC has been found in the membranes of apoptotic macrophages in atherosclerosis[66] where it mediates an inflammatory effect through TLR4 or MyD88.[67,68] Anti-inflammatory properties of OxPAPC have also been reported in LPS-induced sepsis and ALI, which is proposed to act by direct antagonism of LPS recognition.[69,70] Based on these prior observations, one hypothesis is that the H5N1 caused the production of ROS that converts PLs to OxPLs, thereby triggering the TLR4–TRIF–TRAF6 pathway. Indeed, upregulation of ROS and TLR4 membrane recruitment in alveolar macrophages was observed in mice given inactivated H5N1 but not inactivated seasonal H1N1 virus, and oxidised PLs were also found in the lungs of both inactivated and live H5N1 virus-inoculated mice. OxPAPC enhanced the expression of IL-6 through the TLR4–TRIF pathway as demonstrated by studies administering OxPAPC to TLR4$^{-/-}$ and TRIF$^{-/-}$ mice. The contribution of ROS to OxPL formation and ALI was assessed by diminishing ROS production in mice mutated in the *ncf* (NADPH oxidase P47^{phox}) gene. Such ncf$^{-/-}$ mice showed less lung disease upon inactivated H5N1 virus inoculation.[60] In H5N1 virus infections in humans, immunohistochemical staining of lung specimens shows positive staining for OxPLs (in collaboration with John Nicholls and Malik Peiris, University of Hong Kong). In summary, H5N1 virus appears to induce the formation of ROS that oxidises PAPC. OxPAPC is then able to trigger TLR4 and lead to a signalling pathway through TRIF-TRAF6 that enables NF-κB activation of proinflammatory cytokine responses, particularly IL-6, that contribute to ALI. Interestingly, some underlying risk factors for severe disease in influenza are also associated with increased oxidative stress and raised ROS levels, for example, obesity and diabetes.

The SARS outbreak in 2003 saw that 10–20% of patients with SARS developed ARDS. SARS virus studies have revealed a potential broad-spectrum therapeutic for ARDS. The cellular receptor for SARS is the angiotensin-converting enzyme 2 (ACE2)[71,72] which is a component of the renin-angiotensin system (RAS; reviewed in Kuba *et al.*[73]). ACE2 serves as a negative regulator, whereas ACE, another component of RAS, exacerbates RAS-associated disease outcomes. An ACE polymorphism in the human population

has been found to be significantly associated with increased mortality in patients with ARDS.[74] Both SARS infection and SARS coronavirus spike protein administration to wild-type mice downregulated the SARS receptor ACE2 that exacerbated ARDS.[72] In contrast, ACE2 knockout mice were protected from a productive SARS virus infection.[72] In comparison with wild-type mice, ACE2 knockout mice were shown to have severely impaired lung function, and increased lung oedema during ARDS caused by both acid aspiration and sepsis.[75] A novel recombinant human ACE2 treatment was shown to protect against acute lung injury in the acid-induced ARDS mouse model,[75] and preliminary studies for ACE2 therapy in experimental influenza infection are currently being carried out in mice.

These observations offer novel rational drug strategies for the treatment of ARDS and ALI caused by influenza, other respiratory viruses and extrapulmonary triggers of ARDS. These include antagonists of the host innate immune response (TLR4; TRIF); interventions in the oxidative stress machinery; modifications in the lipid metabolism pathways (e.g. statins; cox-2 inhibitors); and interventions in RAS (ACE antagonists; ACE2 therapies).

Therapeutic interventions in influenza animal models and human studies

The role of CD200 and OX40 in lung inflammation and potential therapeutics

Dr Erika Wissenger (Imperial College London) presented data from Professor Tracy Hussell's group regarding mucosal innate immunity. Mucosal sites are continually exposed to predominantly innocuous stimuli and therefore must maintain tight regulation of innate immune responses to prevent excessive inflammation. Even in the face of pathogenic infection, it is important that the immune response does not 'over-react' and compromise organ function. In the lungs, alveolar macrophages play a key role in regulating the balance between activating and inhibitory signals of immunity through corresponding receptors that are engaged on alveolar macrophages, the balance of which determines their activation threshold. They have described this as the 'innate immune rheostat', similar to a light controlled by a dimmer switch.[76] Activating molecules on alveolar macrophages include PRRs, CD40 and OX40L. Molecules that are involved in dampening the response include suppressive cytokines (e.g. IL-10), TGF-β, adenosine and CD200L. The balance of both activation and dampening pathways is site specific, such that in mucosal sites, the balance is tipped in favour of the dampening pathways to prevent excessive and potentially damaging immune reactions.

The kinetics of a respiratory infection can be described in three stages: homeostasis, inflammation and resolution. Homeostasis in the lung is maintained via signalling through epithelial TGF-β, IL-10 and CD200 and the corresponding receptors (TGF-βR, IL-10R and CD200R) found on alveolar macrophages. These signals serve to keep macrophages in a relatively inactivated state. After pathogen is encountered, the inflammation stage begins. Inflammation can be triggered by direct damage to the epithelium by infecting virus. Epithelial damage also leads to the loss of 'dampening' ligands that are found on the epithelial cells that express them, e.g. CD200. In addition, OX40L is upregulated on macrophages allowing helper signals to be provided to OX40-expressing T, B and DC cells. During the resolution stage, macrophages upregulate CD200R expression to a level exceeding that seen before infection; therefore, post-infection macrophages are in a stronger 'dampened' state than they were pre-infection with a higher activation threshold and suppressed TLR responsiveness. OX40L is also maintained on alveolar macrophages after resolution.

An ideal immunomodulator would serve to be beneficial at all stages of illness and target cells that were involved in immunopathogenesis but not prevent pathogen clearance. Innate and adaptive immune responses must also be allowed to prevent susceptibility to repeat and secondary infections. Conceptually, the 'inflammation' stage may be an optimal stage for therapeutic intervention. During this stage, macrophages and other APCs interact with T cells to elicit an effector T-cell immune response. A number of costimulatory molecules are necessary for the development of this response, including the T-cell-expressed OX40 interaction with OX40L on APCs. OX40-Ig fusion protein (OX40:Ig) can block the interaction between OX40 and OX40L. The administration of OX40:Ig to mice infected with influenza was found to significantly decrease morbidity and lung pathology, even with the treatment given as late as 3 days post-infection.[77] OX40:Ig treatment did not affect viral titres in the lung, and both treated and untreated mice showed similar ability to clear virus.[77] During inflammation, epithelial cells are damaged leading to the loss of epithelial CD200, thereby contributing to the loss of dampening signals. An alternative therapeutic approach would therefore be to treat with exogenous CD200. Experiments where mice infected with influenza and then treated with CD200:Fc, both at the time of infection and up to 4 days post-infection, showed reduced morbidity and lung inflammation and increased recovery.[78] In addition, a CD200R agonistic monoclonal antibody showed similar beneficial effects. Both the CD200:Fc and agonistic antibody treatment did not affect viral clearance.[78] These data indicate CD200R agonists as promising novel therapeutics.

The resolution of lung inflammation can lead to an excessive dampening of responsiveness with increased levels of TGF-β, IL-10 and CD200R expression, thus leading to a state whereby the lung is actually more susceptible to

secondary infection. The 'innate immune rheostat' appears to be qualitatively different after influenza infection where a sustained increase in basal levels of CD200R is observed on alveolar macrophages.[78] In mice, influenza is also associated with an apparent post-viral desensitization to TLR ligands which can last for up to 6 weeks post-infection. Mice challenged with a TLR agonist 6 weeks after influenza infection showed a decrease in KC, MIP2α and TNF-α expression in both alveolar macrophages and epithelial cells compared to uninfected controls.[79] This effect was not owing to differences in TLR expression levels but instead to reduced levels of NF-κB nuclear translocation in response to TLR ligation in alveolar macrophages.[79] Study of other virus strains, such as influenza X31 (A/Hong Kong/68 H3N2 HA and NA genes on a PR8 backbone) and RSV, in these systems indicate that the severity of the initial infection is related to an increase in CD200R after resolution, such that influenza causes more CD200R expression than RSV infections. This 'innate imprinting' can account for observations of increased susceptibility to secondary bacterial infections following influenza. Mice infected with influenza followed by a *Streptococcus* bacteria up to 2 weeks post-influenza infection show not only an increase in bacterial load in the lung, but also much higher mortality than mice given virus or bacteria only[79,80] (Goulding J, A Godlee, S Vekaria, M Hilty, R Snelgrove, B Askonas and T Hussell, Imperial College London, unpublished observations). Of note, CD200R knockout mice can control respiratory bacterial infections better than wild-type animals. In summary, the position of the 'innate immune rheostat' is specific to person, body site and previous pathogen experience. Each individual begins with a slightly different ''rheostat-setting'' that is further recalibrated each time a new infection is experienced. This concept is also relevant to asthma, complications arising from chronic lung conditions, hypersensitivities, autoimmunity and response to vaccination.

TipDCs-the necessary evil of lethal influenza infection

The exuberant inflammatory responses associated with virulent influenza strains comprise dysregulated cytokine production and enhanced recruitment of innate inflammatory cells into the lung. Dr Jerry Aldridge (St. Jude Children's Research Hospital, Memphis, TN, USA) described a unique subset of dendritic cells (DCs) that contribute to this lung pathology and studies evaluating the alteration in innate cell trafficking hypothesised to occur during severe influenza infection. In mice infected with a lethal A/PR8 or a sublethal X31 virus inoculum, greater increases in a subset of lung DCs that were $Ly6c^{hi}$ and $CD11b^{hi}$ (called tipDCs) occurred in animals infected with A/PR8 compared to X31.[81] Similarly, tipDCs were also increased in lungs of mice lethally infected with an A/H5N1 virus as compared to those infected with a non-lethal H5N1 strain. TipDCs were found to be recruited to the lungs during influenza infection by the chemokine CCR2. However, in $CCR2^{-/-}$ mice with ablated tipDC recruitment, no reduction in morbidity or protection from lethal challenge was observed. In addition, it was found that antigen-specific $CD8^+$ T-cell numbers were significantly reduced in the airways, but not in the mediastinal lymph nodes, of $CCR2^{-/-}$ mice, and this leads to compromised viral clearance. In studies using a virus mutated in two $CD8^+$ T-cell epitopes (in NP and PA) and a wild-type virus of comparable replication competence, tipDCs from mice infected with the mutant virus and then adoptively transferred to X31 virus-infected $CCR2^{-/-}$ mice were unable to elicit an antigen-specific $CD8^+$ T-cell response. In contrast, tipDCs adoptively transferred from the wild-type virus–infected mice were able to rescue this response. Therefore, antigen is likely presented by tipDCs, and this is a necessary prerequisite for the recruitment of antigen-specific $CD8^+$ T-cell responses in the lung.

Complete depletion of tipDCs in the infected lung was detrimental to mouse survival. Therefore, a partial reduction in tipDC recruitment was undertaken to lessen inflammation but retain the recruitment of beneficial antigen-specific $CD8^+$ T cells. Initial experiments used varying doses of MCP-1 neutralising antibodies and $MCP\text{-}1^{-/-}$ mice to reduce tipDC recruitment via the MCP-1 ligand CCR2, but neither of these approaches had any effect. The peroxisome proliferator–activated receptor-γ (PPAR-γ) agonist pioglitazone, already a licensed drug for type II diabetes, has multiple pharmacologic actions including reducing MCP-1 (monocyte chemotactic protein-1 or CCL2), TNF-α and iNOS levels. Pioglitazone pre-treatment of mice given A/PR8 virus reduced morbidity and mortality significantly without affecting viral replication. Protection was found to be correlated with reduced MCP-1 and MCP-3 production and fewer numbers of tipDCs in the lungs after infection. The fact that viral replication was not altered with pioglitazone and yet mortality improved in some experiments suggests mechanisms other than viral clearance might be operative, perhaps the amount of damage caused by CD8 cells, the rate of injury repair or alterations in other cell types within the infiltrating inflammatory population. Interestingly, an early increase in lung neutrophils was observed in pioglitazone-treated mice, whereas some earlier studies have correlated increased neutrophil numbers with worse disease in 1918 virus-infected mice.[63] Another action of pioglitazone is to alter mitochondrial membrane potential, which might lead to other intracellular inflammatory pathways and apoptosis that may enhance inflammation in some circumstances, potential effects that should be explored future experiments. However, pioglitazone has also been observed to enhance mitochondrial biogenesis without altering membrane potential in neurons.[82]

Initial studies suggest that another PPAR-γ agonist drug, rosiglitazone, may offer superior effects to pioglitazone in the mouse influenza model. Further experiments are currently ongoing to address whether such agonists are therapeutically effective and to examine the effects of PPAR-γ treatment combined with antiviral drugs. In aggregate, the results indicated that increased numbers of tipDCs correlate positively with pathology and mortality but that antigen presentation by tipDCs in the lung is necessary for optimal antigen-specific $CD8^+$ T-cell responses in mice. Modulating tipDC trafficking by a PPAR-γ agonist can protect from lethal influenza challenge in mice, and such drugs offer a possible therapeutic intervention for study in severe influenza disease in humans. In this regard, epidemiological studies of populations that take regular PPAR-γ treatment to assess incidence of severe influenza and other inflammatory diseases would be of interest.

Observations from knockout animal models of influenza

Terrence Tumpey (US CDC) presented data from experiments using cytokine knockout mice to assess cytokine-specific effects on disease severity. Individual cytokine and cytokine receptor knockout B6/129 mice (IL-6, MIP-1α, IL-1 receptor (IL-1R) and TNF receptor 1 (TNF-R1)) were used in H5N1 virus experiments to test whether specific cytokines were involved in causing severe disease. Surprisingly, results showed that the absence of IL-6, MIP-1α or IL-1R had no effect on mouse morbidity, mortality or virus titres after challenge with the highly pathogenic A/Hong Kong/483/97 H5N1 virus.[83] The A/Hong Kong/483/97 virus is highly pathogenic in the mouse, and it may be that this virus exerts its lethality so quickly (causes systemic infections including encephalitis) and that any cytokine-mediated effects on lung pathology may have been masked. Therefore, to detect more subtle differences over a longer time frame, a subsequent experiment used the A/Hong Kong/486/97 H5N1 virus that is of lower lethality for mice. Infections using this virus showed that IL-6 and MIP-1α knockout mice continued to show no difference in disease compared to wild-type mice. However, IL-1R knockout mice displayed increased morbidity and mortality and a delay in viral clearance when infected with this H5N1 strain of lesser lethality.[83] In contrast, TNF-R1 knockout mouse exhibited decreased morbidity upon both A/Hong Kong/483/97 and A/Hong Kong/486/97 virus challenge. This did not appear to be related to virus spread as similar virus titres were found in the lungs, brain and lymphoid organs of wild-type and TNF-$R1^{-/-}$ mice. However, although TNF-$R1^{-/-}$ mice exhibited reduced disease progression, mortality rates were ultimately not affected and mice succumbed by day eleven post-challenge.[83]

Triple knockout mice deficient in both TNF-α receptors 1 and 2 (TNF-α-R1 and TNF-α-R2) and the IL-1R (genotype: TNF-$R1^{-/-}$, TNF-$R2^{-/-}$ and IL-$R1^{-/-}$; bred on the C57BL/6J background[84]) may be a more relevant model of pathogenesis because no single cytokine has been observed to be dysregulated in H5N1 virus infections. Moreover, because there is functional redundancy of some cytokines, studying the effect of deleting only one cytokine may be of limited value in this context. Results from the triple mutant mice challenged with A/Hong Kong/483/97 H5N1 virus showed a significant decrease in KC (the mouse equivalent of human IL-8), MIP-1α, MIP-1β, IL-12 and IFN-γ production compared to infection in the wild-type mice.[85] This correlated with a decrease in pulmonary histopathologic changes, a decrease in morbidity and a significant delay in time until death as compared to wild-type mice.[85] However, eventually all mice eventually succumbed to infection by day nine post-challenge. Therefore, the lack of TNF-α and IL-1 signalling does not ultimately protect against death in this model. In line with results from the single IL-1R and TNF-R1 mutant mice challenged with the highly pathogenic H5N1 virus, the reduced morbidity seen in triple mutant mice was not associated with any difference in lung viral titres.[85] This observation suggests that an antiviral drug that sub-optimally reduces viral titres might not produce a concurrent reduction in cytokines that are associated with increased lung inflammation and morbidity. However, studies with antiviral drugs were not reported upon in this model, and earlier studies in experimentally induced human influenza did find reductions in proinflammatory cytokines with the administration of neuraminidase inhibitors.[84,86,87]

Immunomodulator treatment in mice infected with influenza

Dr Patrick Woo (University of Hong Kong) presented data on combination antiviral and immunomodulator treatment of influenza-infected mice.[88] Amongst human cases of H5N1, mortality remains high despite oseltamivir use in many patients, although delayed time to treatment is a major variable in many cases. Patients with severe H5N1 disease suffer from viral pneumonia with multi-organ involvement typically associated with hypercytokinaemia. Early attempts to manage the excess cytokine responses involved administration of systemic corticosteroids. However, such corticosteroid treatment has been associated with adverse side effects and no improvement in survival in severe H5N1 or pandemic H1N1 illness.[1]

The network of inflammatory mediators and cellular signalling cascades is highly complex[89] making the identification of appropriate therapeutic interventions and their timing difficult. An earlier study has showed that

cyclooxygenase-2 knockout (cox-$2^{-/-}$) mice exhibited lower mortality after A/H3N2 virus infection than did cox-$1^{-/-}$ or wild-type mice.[90] The improved survival correlated with lower levels of IFN-γ and higher levels of PGE2. Interestingly, cox-$2^{-/-}$ mice exhibited a higher lung viral load at day 4p.i., but virus was eventually cleared to levels seen in wild-type mice by day 6p.i.[90] Prostaglandins have been found to affect cytokine production during an inflammatory response, and PGE2 can dramatically limit TNF-α production. Following on from these observations, celecoxib (a cox-2 inhibitor) was tested in mouse models of H5N1 virus infection with and without the antiviral zanamivir given intraperitoneally. In addition, mesalazine (an anti-inflammatory drug widely used to treat inflammatory bowel disease) and gemfibrozil (a fibrate with reported beneficial effects in murine influenza[91]) were also tested. Both of these drugs also inhibit cyclooxygenase pathways and NF-κB activation. When treatment was initiated 48 hours after infection, significant improvements in survival rates, a reduction in inflammatory markers, and much less histopathologic change was observed in the group treated with the combination therapy of zanamivir, celecoxib and mesalazine compared to zanamivir alone.[88] Viral titres were similar to those found with zanamivir treatment alone, although several animals in the triple regimen group had protracted viral detection. When the immunomodulator agents were used alone, there was a small, non-significant delay until death. Thus, the triple combination therapy of zanamivir, celecoxib and mesalazine significantly decreases mortality, and this correlates with reduced cytokine levels and cellular infiltrate in the lung. It will be important to extend these studies to other model systems and consider evaluating these therapies in randomized controlled clinical trials in humans with severe influenza, particularly H5N1 illness. Such an approach might also be of benefit to severe systemic inflammatory reactions caused by other insults.

Epidemiologic studies with statins and immunomodulators in influenza

Dr David Fedson (Sergy Haut, France) presented the background to the concept of using generic immunomodulatory agents to treat severe influenza.[92,93] A number of inflammatory conditions induced by different stimuli share common inflammatory response pathways. Both sepsis and severe influenza can lead to the development of ARDS and MOF, and both are characterised by an exuberant or dysregulated cytokine response.[92–94] Influenza is associated with worse outcomes in people with cardiovascular and pulmonary diseases, diabetes, renal disease, obesity, asthma and late pregnancy, all conditions associated with chronic low-grade inflammation.

Statins are 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) inhibitors that act to reduce cholesterol and are commonly prescribed for cardiovascular indications. Statins also exert pleiotropic anti-inflammatory and immunomodulatory effects.[92,93] A large retrospective observational study of patients hospitalised with acute coronary syndromes (ACS) showed that inpatient statins reduced hospital mortality by 38–42% when compared with ACS patients who were not given statins.[95] Retrospective cohort studies of hospitalised patients with bacterial sepsis show that statins appear to reduce mortality.[92] More importantly, several observational studies have shown that outpatient statin treatment is associated with reductions in pneumonia hospitalisation and death.[96–101] However, not all observational studies have shown that outpatient statins offer significant protection.[102–104] All epidemiological studies, regardless of outcome, have limitations, including inadequate sample size,[102] misclassification of pneumonia, imprecise ascertainment of statin use and confounding variables (e.g. severity of underlying conditions and functional status[103]) and potential interactions of statins with other medications. One study abstract has reported the results of a retrospective cohort study in the USA that evaluated nearly 4000 adults hospitalised with laboratory-confirmed seasonal influenza.[105] In the 26% of patients receiving inpatient statin treatment, a 66% relative reduction in hospital mortality was reported.

Other potential immunomodulatory agents that should be considered for treating severe influenza include PPARα agonists (fibrates) and PPARγ agonists (glitazones).[92,93] Fibrates and glitazones have anti-inflammatory and immunomodulatory properties, in part mediated through increases in PPARα and PPARγ activity.[93,106] Activation of PPARα downregulates IL-6, iNOS and Cox-2, whilst PPARγ downregulates TNF-α and MCP-1. Both have shown beneficial effects in experiments in influenza virus–infected mice.[81,92,93] Even though these agents have no known antiviral activity, no increase in influenza virus replication has been reported thus far. Epidemiological data on the effects of these drugs in patients with pneumonia and influenza would be of great interest.

Adequately powered, randomised, controlled trials of statins and other immunomodulatory agents in influenza are needed. One study has been organised by the ARDS Clinical Trial network (Statin Trial for Influenza Patients: STIP; ClinicalTrials.gov identifier: NCT00970606) and another by the International Forum of Acute Care Trialists (InFACT).[107] However, recruitment of patients during the H1N1 pandemic was delayed, and very few were enrolled. This highlights the need for preparing the clinical research infrastructure to respond to a new pandemic virus and to conduct trials of immunomodulatory treatment of severe seasonal influenza and other acute respiratory infections.

Novel approaches to therapeutic interventions for influenza

When inflammation is good – inducible innate epithelial resistance

Dr Michael Tuvim (University of Texas, MD Anderson Cancer Center) described data illustrating how induction of innate immune responses in airway epithelial cells can protect against influenza infection in animal models. Previously the group has shown that an aerosolised *H. influenzae* bacterial lysate can completely protect mice from a lethal challenge with *Streptococcus pneumoniae* if administered 24 hours beforehand.[108] Bacterial lysate administration also prevented mortality after challenge with a range of other bacterial or fungal pathogens.[109] Lysate administered 24 hours before challenge with an H3N2 influenza virus increased mouse survival from 0% to 90%, reduced weight loss and decreased lung viral titres.[110] The protective effect of the lysate therapy was dependent upon the time of administration and decreased if given more than 24 hours before or 1 day after influenza challenge. Further studies of lysate treatment at later timepoints are needed to assess possible therapeutic value.

For prophylaxis, such an intervention would probably need to be taken on a sustained basis. In mice, multiple treatments were able to induce the same levels of protection as that observed after one treatment, i.e. repeated treatment did not cause tachyphylaxis.[110] The protective effect was site specific, as aerosolised bacterial lysate did not protect against influenza virus administered through i.p. or i.v. routes.[108] Unlike killed bacterial lysates, administration of live bacteria 1 day after influenza challenge promoted death in mice 3–4 days later.

The mechanism of protection induced by the lysate treatment in the bacterial challenge model was found to be associated with, but not dependent upon, the recruitment of leucocytes, predominantly neutrophils to the lung.[108] Lysate-treated mice exhibit enhanced bacterial killing that is correlated with an increase in antimicrobial peptides in the BAL (e.g. lysozyme and surfactant apoprotein D),[108] as well as upregulated gene expression of NFkB and a number of inflammatory cytokines including type I and II IFNs, IL-6 and TNF-α[109] However, experiments using IL-6 and TNF-α knockout mice and mAbs to these cytokines showed that protection was independent of them.[109] In influenza challenge studies, protection was also associated with (although not proven dependent upon) a significant rise in bronchial lavage IL-6 and TNF-α levels after lysate administration.[110] However, after influenza challenge, lavage cytokine levels are much lower compared to infected mice that were not given prior lysate treatment. Serum cytokines were very slightly raised after lysate treatment but quickly dropped again after 24 hours.

Bacterial lysate is a relatively crude mixture of multiple components. Using knockout mice, epithelial resistance to pathogens was found to require the TLR signalling component MyD88 but not TRIF. MyD88 is part of a cell signalling pathway component shared by a number of different TLR types. To elucidate which TLRs are involved in the protective functions of the bacterial lysate, TLR agonists, alone and in combinations, were used to pre-treat influenza-infected mice. Only a synergistic combination of TLR ligands was able to recapitulate the protective effect of the crude bacterial lysate. Specifically, a combination of TLR2 and TLR9 ligands (Pam2CSK4 and ODN synthetic ligands, respectively) induced protection to both bacterial and influenza virus challenge. All three synthetic type-C TLR9 ligands (ODN 2395, 10101 and M362) tested in conjunction with the TLR2 ligand in the influenza mouse model showed comparable protection (up to 80% survival rates).

In conclusion, bacterial lysate administration is able to induce protection against influenza through epithelial activation. TLR agonists can be delivered topically to simulate the effects seen with a crude bacterial lysate. The inflammation elicited is seen largely confined to the lung rather than systemic, but careful pre-clinical safety studies are necessary before such an intervention can be taken to humans. Other studies of interest will be testing combinations of antivirals with TLR agonists and determining effects of lysate therapy on secondary bacterial infections in virally infected animals.

The role of CC10 in respiratory infections

Dr Aprile Pilon (Clarassance Inc., USA) described the role of the mucosal protein CC10 (Clara cell 10-kDa protein) in respiratory infections. CC10 is produced by many tissues in the body, but the highest expression of CC10 is found in the respiratory tract, where it is mainly produced by the non-ciliated Clara cells of the epithelium and is the most abundant single protein normally found in respiratory mucosa.[111,112] CC10 appears to have multiple functions. It acts on Clara cells to facilitate cell trafficking and protein transport and probably upon other cell types, including vascular epithelial cells, neutrophils, lymphocytes and dendritic cells in a similar manner, but there is no consensus on its mechanism of action on other cell types. CC10 appears to act as an 'all is well' signal for epithelial cells. CC10 deficiency leads to severe inflammation and airway dysfunction and is associated with diseases such as pneumonia, ARDS, COPD, asthma and pulmonary fibrosis. CC10 maintains the airway epithelia and overall lung function through autocrine stimulation of Clara cell renewal and preservation of pulmonary structural integrity. CC10 can suppress NF-κB signalling and proinflammatory cytokine production which helps reduce vascular permeability, as well as accelerate viral clearance,[113] possibly by interfering with intracellular viral transport.[114] One audience member described recent work that

found that during inflammation, part of the CC10 promoter is downregulated. IFN-γ is able to upregulate the CC10 promoter, and TNF-α stabilises CC10 expression. CC10 is also upregulated in the respiratory tract by corticosteroids.

A recombinant CC10 (rhCC10) has completed phase I/II clinical trials in pre-term infants with respiratory distress syndrome (RDS).[115] One randomised, double-blind, placebo-controlled trial of 22 neonates found that a single intratracheal administration of 0, 1.5 or 5 mg/kg rhCC10 was safe and efficacious with a significantly decrease pulmonary inflammation indices in the short term. Total protein levels in tracheal aspirates showed a dose-dependent decrease 2–3 days after rhCC10 treatment, indicating reduced vascular permeability, and reduced numbers of infiltrating neutrophils and total cell counts were also seen from 1 day after treatment. During the 6-month follow-up, none of the eleven treated infants needed hospitalisation owing to a respiratory infection, compared to three of six untreated infants.

RSV-infected $CC10^{-/-}$ mice developed increased lung epithelial hypertrophy and had a greater lung cellular infiltrate and delayed viral clearance as compared to wild-type RSV-infected mice.[113] *In vitro* RSV infections of the epithelial-like HEp2 cell line also showed that the addition of rhCC10 significantly reduced viral titres. Further, pre-clinical studies to assess its potential applicability by different routes of administrations (e.g. topical, intravenous) and against different influenza strains would be of interest. Cotton rats infected with A/PR8 influenza virus and treated intraperitoneally with CC10 demonstrated a reduction in lung viral titres. Furthermore, rhCC10 suppresses airway constriction and inflammation in isolated rat lungs perfused with bacterial endotoxin (LPS). Rats given rhCC10 also produced less pulmonary TNF-α, IL-1β and IL-6 than rats given LPS alone.[116] Whether rhCC10 might prevent secondary bacterial infections warrants study in relevant animal models. Further clinical studies of topically delivered administered rhCC10 are warranted, and development of a parenteral formulation would be of interest for study in patients with severe illness.

Poly-ICLC TLR agonist interventional strategies: protection from respiratory threats through broad-spectrum activation of mucosal and adaptive immunity

Dr Andres Salazar (Oncovir Inc.) presented work on TLR agonists as interventional strategies for respiratory pathogens. Oncovir has developed two dsRNA therapeutic viral mimics that are TLR3 agonists: poly-ICLC, a synthetic dsRNA composed of polyriboinosinic-polyribocytidylic acid (poly-IC) stabilised with l-Lysine and carboxymethylcellulose, and a liposome-encapsulated poly-ICLC called LE Poly-ICLC. The poly-IC molecules activate TLR3 on epithelial cells and myeloid cells initiating innate immune signalling and cytokine production, thereby inducing a broad-spectrum antiviral state.[117] Poly-ICLC has been found to induce protection against a range of respiratory and mucosal pathogens in animal models, including SARS-CoV, vaccinia, anthrax, herpes and ebola virus.

In mice, intranasal poly-ICLC or LE Poly-ICLC induced protection against subsequent H5N1 virus and seasonal H1N1 and H3N2 virus infections.[118] Protection correlated with increased expression of cytokines including IFN-β, IFN-γ, TNF-α, as well as increased TLR3 mRNA.[118] Partial protection could still be induced when LE poly-ICLC was administered up to 3 weeks before challenge, whereas poly-ICLC could be given up to 7 days before challenge.[118] Poly-ICLC was also shown to be protective in a cotton rat model of influenza in which treated rats demonstrated a dose-dependent decrease in viral titres.[119] Interestingly, histology showed that poly-IC treatment caused more lung inflammation than observed in controls.

In phase I and II clinical trials with poly-ICLC (Hiltonol®, Oncovir Inc., Washington, DC, USA), intranasal dosing was well tolerated with no serious side effects. Future plans include further testing in appropriate animal models to confirm efficacy, followed by further clinical trials to confirm safety and assess efficacy. Using Hiltonol® as a vaccine adjuvant for intranasal live attenuated vaccines is also a consideration. Nasally delivered poly-ICLC and LE poly-ICLC may offer potentially effective prophylaxis or early treatment for influenza and other respiratory viruses.

Mediating hypercytokinaemia in influenza infections

Dr James Larrick (StormBio Inc., New Jersey, USA) described his company's focus on the treatment of immunologically mediated diseases, including influenza, where treatment aims to reduce the so-called cytokine storm. Drugs currently in development include superoxide dismutase mimetics, OX40 antibody constructs (to block OX40–OX40L interactions) and anti-TNF-α therapies. Superoxide dismutase mimetics function to limit the expression of ROS that contribute to lung immunopathology. Initial animal experiments with these compounds have shown only modest effects, however, and therefore it is uncertain whether they will be taken forward into clinical trials. In studies with a propriety OX40 antibody construct, pegylation was required to increase its plasma half-life, although this formulation also reduced cellular binding by 40%. Future work may include testing alternative anti-OX40 antibodies or using OX40:Ig fusion proteins.

Excess production of TNF-α has been associated with lung and multi-organ injury, such that neutralisation of TNF-α would be anticipated to reduce lung damage without compromising viral clearance by immune cells.

However, no observational studies or clinical data have been reported to date with regard to influenza and anti-TNF-α therapy. Direct experimental data are needed in relevant models of influenza-associated ALI. One point with regard to intervening in acute influenza is that the TNF-α response may be initiated so early on in infection that the timing of administration may be difficult to get right in a clinical trial.

General discussion and future directions

The goal of immunomodulatory interventions for the management of sepsis and severe influenza is to reduce adverse inflammatory effects whilst maintaining adequate immune responses. Dr James Larrick commented on the slow progress in the development of immunomodulatory agents for sepsis. Over the last 25 years, the number of novel drugs that have entered clinical trials is much lower than expected, and only one (activated Protein C) has been approved to date. However, there is very limited data on the use of Protein C for the treatment of influenza-associated ALI.[120] Influenza infection of mice causes a prothrombotic state concurrent with reduced ability to produce activated protein C.[121] However, a recent study showed that activated Protein C administered to mice 24 hours post-lethal H1N1 influenza infection caused a reduction in viral titres but did not appear to affect lung inflammation or survival rates.[122] Epidemiologic data regarding influenza outcomes amongst the many people being treated with anti-TNF-α or activated Protein C for other conditions would be of interest.

The critical importance of timing likely applies to many immunomodulatory interventions. Early cytokine gene dysregulation has been shown to occur within the first few hours after infection with H5N1 and 1918 influenza.[123] Major hurdles exist for obtaining new drugs into clinical trials including the registration process and overall expense of development. Increases in translational research and new research technologies (e.g. genome sequencing, microarray analysis) over recent years have yielded a substantial increase in the identification of new drug targets and potential therapeutic products.

Regulatory issues

Further discussion identified other regulatory and developmental issues for some of the immunomodulators. One specific question is how would and could a non-specific immunomodulator be approved without thorough mode of action mechanistic details. In the instance of pandemic influenza or other high-impact emerging infectious disease, streamlining of the regulatory approval processes needs to be explored, perhaps similar to the mock-up licensure that exists for pandemic influenza vaccines in Europe. An emergency use authorisation (EUA) procedure exists in the United States and was used for intravenous peramivir for treating hospitalised patients with pandemic H1N1 illness.

Long timelines are required to obtain drugs from discovery to market, and patent expiration times may discourage smaller companies from pursuing a particular molecule. It may be possible to reduce these regulatory hurdles if novel therapies for influenza are identified amongst drugs already licensed for the treatment of other diseases, so that established safety records already exist. This applies to several of the immunomodulatory agents proposed for influenza treatment (e.g. Cox-2 inhibitors, statins, fibrates, glitazones, macrolides, mesalazine). However, drugs identified and pursued in this way are of little interest to large pharmaceutical companies, and funding must be sought from elsewhere, normally from governmental or charitable funding bodies.

Therapeutic options

An additional therapeutic option is passive immunotherapy via convalescent blood products or immunoglobulin (Ig) which has been proposed for treating cases of severe influenza. Convalescent plasma and hyperimmune sera have recently been studied in the non-randomized treatment studies of patients with severe pandemic H1N1 illness (2009) in Hong Kong with encouraging results.[124,125] Convalescent blood products appeared to be beneficial during the 1918 pandemic, and passive immunotherapy has been used with apparent success in individual cases of H5N1 infection[126] and with clear benefit in murine models of H5N1 disease.[127,128] Such interventions may serve to clear virus by cross-reactive antibodies and also act as immunomodulatory factors by mechanisms that are yet to be fully elucidated. Further rigorously controlled clinical trials are needed to prove beneficial effects and, secondly, to elucidate the mechanisms of action.

IFN therapy is another possible means of treating influenza. Indeed, earlier observations on fatal human cases of influenza pneumonia reported the absence of interferon in lung tissues.[129] A more recent study in patients with severe pandemic H1N1 illness also found much lower plasma IFN-α levels compared to those with mild illness.[130] These broad-spectrum treatments may offer advantages over traditional approaches including avoiding the generation of resistance mutations. Novel immunomodulatory therapeutics of proven value in influenza or ARDS is currently lacking, although there is now a growing number of publications examining the impact of various immunomodulators in experimental systems, mainly in the mouse. Research is needed to further test a broader array of novel therapeutics, extend observations to other relevant animal models and to understand underlying modes of action. It will be important to determine and take into account the

differential host responses that are activated by different virus strains, e.g., H5N1 versus seasonal influenza. An alternative option would also be to target the host cellular interactome of the virus.[22,49–52] For example, blocking the Raf/MEK/ERK kinase and NF-κB pathways may also reduce disease,[53] and TLR3 knockout mice show improved survival upon infection with H3N2 seasonal influenza.[131]

In summary, the presentations during this workshop highlighted both the substantial progress that has been made in understanding the mechanisms of pulmonary injury in influenza and other respiratory virus infections and the complex, dynamic nature of the events at play. Inhibition of viral replication with efficient antivirals is likely essential but not sufficient to achieve optimal patient outcomes in severe human influenza. Effective immunomodulatory interventions, particularly combined with antivirals during periods of active viral replication, are needed for such patients. However, the functional redundancy of the pathways and rapid changes in responses over time involved create challenges for intervention. Considerable heterogeneity exists in innate immune responses related to both host and viral factors. Key questions regarding the particular targets for intervention, their appropriate level of inhibition, the timing (both initiation and cessation) of intervention within the course of infection and the appropriate patient populations remain to be resolved. Downregulation of overly exuberant innate immune responses also has the potential to upregulate viral replication and increases the risk of secondary bacterial infections. It is also clear that deficient innate immune responses exist in certain situations (e.g. inadequate interferon production or signalling) that might be amenable to targeted upregulation or exogenous supplementation.

The multiplicity of potential targets related to viral–host interactions and innate immune responses underscore the challenges in deciding which interventions to take to the clinic for rigorous testing. In turn, this uncertainty emphasises the importance of developing animal models predictive of disease pathogenesis in humans, understanding the limitations of these models, integrating findings from different models and identifying markers or surrogates that might predict benefit. Many of the presentations during this workshop addressed studies in murine models, especially mouse strains with defined genetic defects. The extension of findings in such models to other species is a key point for further study. Clinical testing of candidate agents needs to rigorously assess clinical, virologic and immune measures, in part because of the safety issues raised by modulating immune responses. Placebo-controlled studies of selected immunomodulators in uncomplicated influenza are now possible. These would provide useful information regarding possible effects on virology and immune responses but would be of uncertain value in predicting safety and efficacy in more severe disease states like influenza viral pneumonia. Consequently, controlled studies of particular immunomodulators as additions to antiviral therapy in hospitalised patients with severe illness will be necessary.

Appendix

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