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Use of *ex vivo* and *in vitro* cultures of the human respiratory tract to study the tropism and host responses of highly pathogenic avian influenza A (H5N1) and other influenza viruses

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The tropism of influenza viruses for the human respiratory tract is a key determinant of host-range, and consequently, of pathogenesis and transmission. Much of the available data on the tropism of influenza viruses in the human respiratory tract has been derived from autopsy studies of fatal influenza. While this provides important insights into the cells and tissues infected by different respiratory viruses, these data are of necessity restricted to late-stages of severe influenza disease, often following mechanical ventilation. Of the available animals models, only ferrets (and possibly guinea pigs in relation to transmission) are regarded as relevant surrogates for human influenza infection. Nevertheless, ferrets are not humans, and studies of the human respiratory tract remains of paramount importance.

The human respiratory tract is sub-divided into the upper respiratory tract including the nasopharynx and larynx, the conducting airways (trachea and bronchi) and the lower respiratory tract which comprises of the lung parenchyma. Each of these anatomical regions has different types of epithelial cells and influenza viruses differ in their tropism for different cell types at these sites.

The tropism of an influenza virus for the upper, conducting and lower airways has consequences for transmission and pathogenesis. Viruses with tropism for the alveolar epithelium may well cause severe viral pneumonia but may be less transmissible from person-to-person, while those that infect the upper airways are likely to be more transmissible but may cause less severe disease. Thus, experimental models that reflect these anatomical and physiological differences are relevant.

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Available experimental models used for assessment of virus tropism and replication competence in humans include a) *ex vivo* cultures of human respiratory tissues removed at biopsy or surgical excision which are excess to diagnostic requirements, b) primary cells from the human respiratory tract, used with or without differentiation in air-liquid interface cultures, or c) continuous cell lines derived from the human respiratory tract (e.g. A549) or cell lines derived from other animal tissues (e.g. Mardin-Darby Canine Kidney (MDCK)).

Experimental use of *ex vivo* cultures of human respiratory tissues removed at biopsy or surgical excision was first adopted in 1960's (Higgins et al., 1969; Roome et al., 1971; Tyrrell and Blamire, 1967; Tyrrell and Hoorn, 1965) as attempts to provide substrates for the isolation and growth of respiratory viruses. Subsequently, for convenience and reproducibility, semi-continuous and transformed continuous cells from various species have been used for the purposes of diagnostic virology. Primary and semi-continuous cell cultures were derived from aborted human embryos (e.g. human embryo lung fibroblast cultures) and from non-human primates (e.g. primary and secondary Rhesus monkey kidney epithelial cell cultures). Continuous transformed cell cultures used for influenza virus studies include MDCK cells and A549 cells derived from a type II pneumocyte lung carcinoma (Giard et al., 1973). These continuous cell lines require the addition of exogenous trypsin for cleavage of the precursor hemagglutinin molecule (HA₀) to the HA1 and HA2 components of the mature virion.

While these different cell substrates may be useful for virus isolation, diagnostics and vaccine production, they are not equally useful for providing insights on the tropism of influenza viruses in the human respiratory tract. In this review, we summarize available data on influenza virus receptor distribution, the profiles of virus binding and on virus infection and replication in relevant experimental models and compare these with what is known for the human respiratory tract, to identify physiologically relevant experimental systems for investigating the tropism of influenza viruses for the human respiratory tract.

Distribution of influenza virus receptors

Sialic acids linked to proteins or lipids on the cell surface are binding receptors for influenza viruses. Studies in the 1980s using desialylated and resialylated red blood cells demonstrated that human influenza viruses preferentially bind 2-6 Gal-linked sialic acids while avian influenza viruses preferentially attach to sialic acids with an 2-3 linkage (Rogers and Paulson, 1983). We used the lectin *Sambucus nigra* agglutinin (SNA) which binds 2-6 Gal, *Maackia amurensis* agglutinin (MAA) I which has a preference for

2-3Gal 1-4GlcNAc and MAAII which has a preference to bind 2-3Gal 1-4GI-3GalNAc to compare the glycan expression in *ex vivo* cultures of the human nasopharynx, bronchus and lung alveolar tissues with corresponding tissues from patients at autopsy who died of non-respiratory causes (Table 1). These findings are also compared with the glycan expression profiles in undifferentiated and differentiated primary human cell cultures (Chan et al., 2010b; Yu et al., 2011).

Overall, there is good concordance between the expression of the sialic acid receptors in the *in vivo* tissues and *ex vivo* organ cultures as well as the well-differentiated primary cell culture models but the receptor distribution in some undifferentiated primary human cell cultures and continuous cell lines do not correspond well with what is found in corresponding human tissues.

In humans, an almost complete differentiation of nasopharyngeal epithelium is found in a 12-13 week fetus *in utero* with two distinct cellular types, ciliated and microvillus-provided cells (Gulisano et al., 1992). The nasopharyngeal epithelium has five characteristic cellular types found on the surface, including the tall cylindrical ciliated cells; cylindrical cells

covered only with microvilli, secretory cells, flat epithelial cells, cuboidal cells with microvilli on the surface and many vesicles in the cytoplasm (Karchev and Kabakchiev, 1984). The cell surface of the nasopharyngeal epithelial cells is decorated with glycans with different terminal residues including sialic acids (Chew et al., 1991). Lectin binding studies of *ex vivo* nasopharyngeal, tracheo-bronchial and lung organ cultures accurately reflect the receptor profile observed in normal human tissues (Nicholls et al., 2007a; Nicholls et al., 2007c).

In endemic areas, exposure of humans to H5N1 infected poultry is very common but H5N1 disease remains exceedingly rare. It has been hypothesized those who get H5N1 disease may have differences in the distribution of avian influenza binding Sia₂₋₃ receptors in the respiratory tract. In a clinical study, the differences of sialic acid receptors distributed in different human tissues, including extrapulmonary organs, in H5N1-infected and non-infected patients was compared using lectin histochemistry (Yao et al., 2008). This study used biotinylated MAAII and SNA lectins to identify the Sia₂₋₃ and Sia₂₋₆ receptors present. They found no significant differences in Sia₂₋₃ and Sia₂₋₆ receptor distribution between the H5N1 infected and patients dying of non-pulmonary diseases in the trachea, brain, liver, kidney, heart and intestine. Unfortunately, severe distortion of the architecture in the nasal mucosa, pharynx, bronchi, bronchioles and lung parenchyma of H5N1-infected patients precluded comparison of the receptor distribution in these tissues from the control samples. The data from the non-infected human tissues indicated that Sia₂₋₃ glycans were scarce in the epithelial cells of the upper respiratory tract and in the bronchus. However, it should be noted that only MAAII, which detects the O-linked Sia₂₋₃ receptor was used. MAAI detects the N-linked Sia₂₋₃ receptor and would have provided additional relevant information on the distribution of N-linked Sia₂₋₃ receptors, but was not used in this study.

A number of physiological and pathological factors can affect the expression of sialic acids on the human respiratory tract, e.g. age, inflammation, etc. The upper and lower respiratory tissues of children have a higher abundance of N-linked Sia₂₋₃ receptors than adults (Nicholls et al., 2007a). Furthermore, inflammatory conditions can affect the expression of sialic acid receptors and therefore the epithelial susceptibility to influenza infection. A study using normal nasal mucosa and nasal polyp tissues explant cultures indicated that nasal polyps expressed higher levels of Sia₂₋₃ and Sia₂₋₆, as well as a more efficient influenza virus replication kinetics for both seasonal and H5N1 avian viruses (Suptawiwat et al., 2010). The upregulation of sialyltransferase (the enzymes responsible for adding sialic acid to galactose) expression detected in the nasal polyp could be a result of the inflammatory condition leading to the increased abundance of the expression of the corresponding sialic acid receptor. The increased availability of the receptor, together with the change in the inflammatory condition, including the up-regulation of various cytokines and transcriptional factors, e.g. NF- κ B, could be one reason for the NF- κ B dependence of influenza virus replication (Nimmerjahn et al., 2004).

The extracellular mucus secreted by respiratory epithelial cells (goblet cells and submucosal glands) can also profoundly affect the susceptibility of the respiratory epithelium to influenza viruses. Tests on the effect of mucin on influenza virus infection in hamsters was carried out by Wheeler and Nungester in 1942 (Wheeler and Nungester, 1942). As mucin in the respiratory tract contains an abundance of Sia₂₋₃Gal (Baum and Paulson, 1990), it may preferentially entrap avian influenza viruses whose HA expresses an affinity for these Sia₂₋₃ receptors (Couceiro et al., 1993). The activity of the virus neuraminidase in cleaving such linkages is likely to be important in allowing the virus access to the respiratory epithelium. Thorough washing of the epithelial surface to remove such mucus in differentiated respiratory epithelial cultures *in vitro* does alter its susceptibility to specific

viruses (Wong et al., 2012). It is important to note that such removal of mucus does not take place under physiological conditions and thus the presence of mucus in studies of virus tropism may need be taken into account. The pH of the human respiratory epithelium is also likely to affect the permissiveness of the cells to influenza viruses. Neuraminidase activity is enhanced at lower pH. Influenza viruses from different species differ in the optimal pH that triggers HA activation for fusion; human seasonal influenza requiring a lower pH than avian highly pathogenic H5N1. This in turn affects pH stability of different viruses (Krenn et al., 2011).

Binding of labeled influenza viruses to respiratory epithelium

The binding of inactivated labeled influenza viruses to respiratory epithelial tissues provides an alternative approach to investigate virus tropism (Imai et al., 2012; van Riel et al., 2006; van Riel et al., 2007). Formalin fixed paraffin embedded tissues of the human respiratory tract were used in these studies. It is known that sialic acids are not affected by the fixation process, thus the results appear physiologically relevant. H5N1 virus binds to type II cells within the lung while seasonal influenza virus predominantly binds type I cells (van Riel et al., 2007). Supporting this finding, it was recently reported that H5N1 virus infection and budding of new virus particles in ex vivo human lung cultures occurs solely within type II, rather than type I cells (Weinheimer et al., 2012). Studies of the human nasal septum, conchae, nasopharynx, paranasal sinuses and larynx revealed that the highly pathogenic H5N1 virus rarely attaches to the ciliated and goblets cells at these anatomical sites (van Riel et al., 2010).

Binding of whole influenza virus or influenza virus HA on glycan-arrays for determining virus binding preference for sialic acids

Glycan arrays were developed in 2004 (Blixt et al., 2004) and are a powerful tool which allows a more comprehensive analysis of virus binding preference to various glycans. Typically, virus or virus HA is added onto a glass slide coated with hundreds of glycans with different structures, degrees of sialylation and topologies. This allows an assessment of the influenza virus or virus HA binding affinity to a spectrum of glycans to be done in one experiment. Currently, four published glycan arrays are available and used in influenza virus-receptor binding studies with each of them containing a different collections of glycans (Childs et al., 2009; Liao et al., 2010; Stevens et al., 2006a), ranging from few dozens up to more than six hundred found in the array that is developed and updated by the Consortium for Functional Glycomics (<http://www.functionalglycomics.org/>). Using such technology, it has been possible to define the effect of a few amino acid changes in the HA which change the binding affinity of a H5 virus from a Sia₂₋₃ to a dual Sia₂₋₃ and Sia₂₋₆ binding profile HA (Stevens et al., 2008; Stevens et al., 2006b). However, until recently, the glycans that are present in the human respiratory tract were not well defined, and thus it was not clear which of the glycans found in these arrays were physiologically relevant for humans and also whether all the glycans found on the respiratory epithelium were represented in the available arrays. Recent studies have used mass-spectroscopy to comprehensively characterize the glycans present in the human respiratory tract. These studies reveal that of approximately 50 glycans present in the human respiratory tract, around one third are not represented in any of the glycan arrays currently available (Trevenan et al., 2013). Therefore, the conclusions based on glycan array studies on virus receptor-binding profiles so far should be treated with some cautions and the arrays need to be updated to include those glycans physiologically relevant to the human respiratory tract.

Infection and replication of influenza

Human *ex vivo* cultures

As expected from studies of influenza virus receptor distribution in human respiratory tract tissues, seasonal human influenza viruses as well as the pandemic H1N1 (2009) viruses efficiently infect and replicate in *ex vivo* cultures of the nasopharynx and upper airways. Influenza A H5N1 viruses can also infect and replicate in *ex vivo* cultures of the nasopharynx (Nicholls et al., 2007c) including the tonsils and adenoid although they apparently lack Sia₂₋₃ receptors, and also in the alveolar epithelia (Figure 2B) and alveolar macrophages in lung (Nicholls et al., 2007c; Shinya et al., 2006; van Riel et al., 2006). As expected, there is little H5N1 infection of the conducting airways, including the epithelia of bronchi (Figure 2C) and bronchioles. It is also noted that H5N1 viruses appear to replicate to high titer in the upper respiratory tract of ferrets but without transmission (Imai et al., 2012). The virus has also been reported to infect the ferret brain via spread along the olfactory nerves supplying the cribriform plate which implies competence of this virus to replicate in the ferret nasopharynx, which also apparently lacks the avian Sia₂₋₃ receptors as detected by lectin binding. The *ex vivo* cultures thus highlight some discordance with accepted dogma which is an area that deserves further research.

Although swine influenza viruses have a predilection for human-like Sia₂₋₆ receptors, interestingly, not all swine influenza viruses replicate in human *ex vivo* cultures of nasopharynx and bronchi (Chan et al., 2011b). Replication in human *ex vivo* cultures of nasopharynx and bronchi appears to identify swine influenza viruses that have the capacity to transmit by airborne droplets in the ferret model, and therefore those that pose a greater threat to transmit in humans (Yen et al., 2011). This suggests that replication of influenza viruses in human *ex vivo* cultures of the upper airways can be one factor used in risk-assessing animal viruses for pandemic threat.

Primary human respiratory epithelial cell cultures *in vitro*

While *ex vivo* cultures are superior in mimicking the physiological microenvironment of the respiratory tract, the complexity of the organ culture system makes it difficult to evaluate the role of individual cell types and these cultures can be maintained with good viability for a limited time (ranging from 4 to 10 days) (Booth et al., 2004; McBride et al., 2000). More importantly, the availability of human *ex vivo* organ cultures are limited and donor-to-donor variation cannot be well controlled. Therefore, *in vitro* cultures based on primary cells isolated from its original anatomical site, with or without differentiation in air-liquid interfaces *in vitro*, provide an important alternative and that can provide physiological relevance (as compared with immortalized transformed cell lines) and greater availability and reproducibility for more detailed experimentation. Available data on influenza virus tropism and infection in primary human respiratory epithelial cultures is summarized below.

Primary human nasal and nasopharyngeal epithelial cultures *in vitro*—Because of its anatomical location in the upper airways, nasal and nasopharyngeal epithelial cells are usually those first exposed to a respiratory pathogen such as influenza virus. Therefore, data generated from these cells are important in understanding the tropism and pathogenesis of influenza viruses in humans. Matrosovich and colleagues have developed well-differentiated nasal epithelial cell cultures *in vitro*, but have not systematically documented influenza virus tropism in these cells (Matrosovich et al., 2004). Highly pathogenic influenza H5N1 viruses and seasonal H1N1 virus replicated efficiently in undifferentiated nasopharyngeal epithelial cell *in vitro* cultures at both 33°C and 37°C (Nicholls et al., 2007b), correlating with observations in *ex vivo* cultures of the human nasopharynx. However, the use of undifferentiated nasopharyngeal cells needs to be treated with caution as the Sia receptor

distribution of undifferentiated nasopharyngeal epithelial cells do not closely mimic that seen in the human nasopharynx or in *ex vivo* nasopharyngeal cultures. The undifferentiated nasopharyngeal cells express both O-linked and N-linked 2-3 and Sia 2-6 receptors whereas human nasopharyngeal epithelium and *ex vivo* nasopharyngeal culture only express N-linked Sia 2-3 and Sia 2-6 receptors as determined by lectin binding studies.

Primary human differentiated and undifferentiated tracheo-bronchial epithelial cultures in vitro—

Tracheobronchial epithelial (TBE) cells or the normal bronchial epithelial (NHBE) cells are cells derived from the conducting airways. These primary cells have been used extensively to study respiratory disease, including influenza virus infections, because *in vitro* cultures can be differentiated in air-liquid interface cultures to generate epithelia that contain ciliated epithelial cells and mucus secreting goblet cells together with the secretion of human airway trypsin like proteases that mimic the conditions found in the human conducting airways. For example, as within human airways, influenza viruses replicate in these cultures without the need for adding exogenous trypsin to these cultures for the cleavage of the HA0 to HA1 and HA2. A comparison of the virus infection and replication kinetics as well as innate immune responses of human seasonal influenza H1N1 and avian H5N1 viruses in undifferentiated and well differentiated NHBE cells has been reported (Chan et al., 2010b). It was found that both differentiated and undifferentiated NHBE cells were permissive to H5N1 virus infection but productive H5N1 replication was observed only in non-differentiated NHBE. While H1N1 can replicate efficiently both differentiated and undifferentiated *in vitro* cultures, the well-differentiated NHBE cells correlate better with the observations from *ex vivo* bronchial organ cultures. These findings also correspond to the observation that while O-linked Sia 2-3 receptors may not be as prominent as N-linked Sia 2-3 receptors in human bronchial tissues, *ex vivo* cultures and well differentiated NHBE cells, they are well expressed in the undifferentiated NHBE cells, explaining the (non-physiological) permissiveness of these cells to the avian H5N1 virus which may dependent on binding to Sia 2-3 receptors (Chan et al., 2011c; Nicholls et al., 2007a)

In studies with well-differentiated tracheobronchial epithelial cell cultures reported by Matrosovich's group, H5N1 virus preferentially infects ciliated respiratory epithelial cells rather than non-ciliated cells and this was thought to be related to the binding preference of the virus HA protein to the 2-3-linked sialic acids, which was mainly expressed on ciliated cells (Matrosovich et al., 2004). In the studies reported by others (Chan et al., 2009; Ibricevic et al., 2006), differentiated epithelial cells express functional Sia 2-6 in both ciliated and goblet cells. In a more recent study using a low pathogenic H5N1 which primarily bound sia 2-3 moieties, removal of Sia 2-3 moieties in fully differentiated NHBE cells by specific sialidase failed to abrogate infection (Oshansky et al., 2011). These authors concluded that, at least with the low pathogenic H5N1 viruses, infection is not constrained by the need for sialic acid 2-3 residues. These findings highlight the need a better evaluation of the binding profiles of these viruses as well as a better understanding of the virus receptors on respiratory cells that are needed for virus replication. It is also important to note that virus attachment, though necessary, is not always predictive for efficient infection of the cells (Rimmelzwaan et al., 2007).

Ilyushina et al in 2008 attempted to evaluate the viral load at one-day post infection of 31 recombinant viruses with the A/Vietnam/1203/04 (H5N1) backbone. These viruses possessed mutations of the HA which altered binding affinity or specificity for Sia receptors. Comparison of the efficiency of infection and virus replication kinetics of these viruses would have potentially provided useful information on the contribution of these mutations on human bronchial epithelial cell tropism. However, as the purpose of their study was to evaluate the effect of changes in HA that affect neuraminidase-inhibitor susceptibility of

H5N1 viruses, only the fold-change of viral load with or without the neuraminidase-inhibitor treatments was shown. Thus we do not have the information needed to assess the effect of these H5-HA protein mutations on respiratory epithelial tropism, except for the conclusion that “human-like” HA specificity does not affect the neuraminidase-inhibitor susceptibility of the virus (Ilyushina et al., 2008). In a follow up study, they showed that mutant H5N1 viruses with decreased NA activity are better adapted to human airway epithelium (Ilyushina et al., 2012). This study also highlighted the possibility of using serial passage of virus in differentiated NHBE cells as a model to study the virus mutations relevant to the process of human adaptation. Likewise, influenza virus tropism in NHBE is being used as a model to assess whether a virus has potential to cause human infection (Suksatu et al., 2009). In this study, the two of the three H5N1 viruses isolated from cloacal swabs of open-bill storks replicated and caused cytopathic effect in NHBE cells. It has thus been suggested that open-bill stork-derived H5N1 viruses may not require additional mutations to cross the species barrier to infect humans.

Primary human alveolar epithelial cell cultures in vitro—The tropism of viruses for type I and type II alveolar epithelial cells is important for understanding virus pathogenesis in the lung. Although type I pneumocytes are numerically less than 50% of the total number of type II pneumocytes in the lung, they contribute to 90% of the alveolar surface area because of their flattened morphology. The type I pneumocyte is thin and flattened to allow gaseous exchange to take place and does not undergo further cell division. Type II pneumocytes are more cuboidal and account for less than 10 % of the alveolar surface area (Crapo et al., 1982). They act as the defenders and regenerators of the alveoli rather than contributing to gaseous exchange. They are responsible for the production of surfactant proteins (Crouch et al., 2000) which functions to lower the surface tension between air and alveolar fluid and thus to prevent alveolar collapse at end of the expiration. In addition, surfactant proteins contribute to antiviral defense (Hillaire et al., 2012). Pulmonary surfactant consists of lipids (90–95%) and four surfactant proteins (SP-A, SP-B, SP-C, and SP-D, 5–10%). Type II alveolar pneumocytes are progenitor cells which can proliferate and differentiate into type I pneumocytes following lung injury (Adamson and Bowden, 1974). They also play an important role in controlling the lung fluid transport between the apical and basolateral surface by means of the sodium channel to maintain the normal function of the lung (Mason, 2006).

The alveolar epithelium is the main site for the replication of highly pathogenic avian H5N1 viruses as shown by the demonstration of viral antigens in alveolar epithelial cells as well as in alveolar macrophages in autopsy lungs (Liem et al., 2008). Although viral antigen could be found in other extrapulmonary organs, major pathological changes, high viral load and frequent virus isolation are most reproducibly seen in the respiratory tract and these patients die primarily of their lung pathology and acute respiratory distress syndrome (Abdel-Ghafar et al., 2008; Suptawiwat et al., 2010; Uiprasertkul et al., 2005; Zhang et al., 2009).

There are only a few studies on influenza virus tropism in type I and II pneumocytes because the maintenance of type II cells on plastic surfaces *in vitro* is very difficult as they differentiate rapidly into a type I-like phenotype (Daum et al., 2012; Fang et al., 2006; Wang et al., 2007; Yu et al., 2011). The replication kinetics of human seasonal H1N1 and avian highly pathogenic H5N1 viruses were compared in primary human alveolar epithelial cells (Chan et al., 2005), demonstrating that both viruses infected these cells with similar efficiency (93-100% following infections with multiplicity of infection = 2) as evaluated by nucleoprotein staining and produced similar viral yield at 48hpi. However, given the rapid differentiation of type II pneumocytes to type I cells *in vitro*, the exact phenotype (type I vs type II) of these alveolar epithelial cells could not be defined with certainty. Later studies investigated virus infection and productive replication of influenza viruses in polarized type

I-like epithelial cells *in vitro*. These studies showed that both seasonal H1N1 and highly pathogenic H5N1 virus can efficiently infect type I cells from their apical and basolateral surfaces, but release of the virus is restricted to the apical side of the cells (Chan et al., 2009). In a comparison of H5N1 virus infection and one-step growth kinetics in cultures of differentiated primary human alveolar type I-like cells and alveolar type II cells grown on matrix gel, it was observed that influenza viruses have a higher efficiency of infection in type-I like cells (> 95%) than in type-II cells (26% $\pm$ 4%). The replication kinetics of H5N1 virus peaked earlier at 48 hpi with a 2.5 log increase in viral titer in type I pneumocytes compared to type II-like cells where replication peaks at 72 hpi but with a higher (4.5 log) increase in viral titres (Yu et al., 2011). This highlights the biological differences of type I-like cells and type II cells for both H5N1 and seasonal H1N1 influenza virus replication.

Continuous transformed cell lines

Continuous cell lines derived from the human respiratory tract have been used in some studies of influenza virus tropism and pathogenesis. However, continuous cell lines are often not representative of respiratory cells found *in vivo*. A549 cells were derived from a type II pneumocyte lung tumor (Giard et al., 1973) and are commonly used to represent normal human lung epithelium in studies of virus tropism and host responses. Although A549 cells express some characteristics of type II pneumocyte, including the synthesis of phospholipids, cytoplasmic lamellar bodies and apical microvilli, they do not mimic the true phenotype of its primary counterpart. For example they lack the expression of surfactant proteins A, B, Pro-surfactant C and furthermore, the produced surfactant C is expressed as a mutated form. More importantly, while low pathogenic influenza viruses can replicate in primary human pneumocytes without the addition of exogenous trypsin because these cells produce endogenous human airway proteases, exogenous trypsin must be added to support multiple rounds of virus replication in A549 cells (Das and Boggaram, 2007; Swain et al., 2008). When the sialic acid distribution in A549 cells is compared with those in primary type II-like pneumocyte (Table 1), A549 cells have far more Sia 2-6 than 2-3, which is the reverse of what is observed in type II pneumocytes *in vivo* and *in vitro*. Thus, A549 cells are not ideal surrogate for the study of influenza viral cell tropism.

Calu-3 or BEAS-2B cells are alternative continuous cell lines that have been used for studies of influenza viruses and compared with primary human bronchial epithelial cells (Stewart et al., 2012). Calu-3 cells are derived from a human lung adenocarcinoma and give reproducible transepithelial electrical resistance on polarization. However, ciliated cells are comparatively less abundant (or absent) when compared to normal bronchial epithelium. Furthermore, the high abundance of O-linked Sia 2-3 (Zeng et al., 2007) which is detected by MAAII binding, differs from the human bronchus where MAAII binding is absent. BEAS-2B cells are another continuous human bronchial epithelial cell line obtained from non-cancerous individuals that was transformed using adenovirus. However, these cells cannot differentiate into ciliated or goblet cells and cannot develop to form tight junctions. Moreover, there is so far no report on the sialic acid distribution in this cell line. It is less commonly used than Calu-3 for studies on influenza.

H5N1 tropism in non-epithelial cell types found in the respiratory tract

In addition to alveolar epithelial cells, the lung microenvironment also contains resident immune cells, including alveolar macrophages, dendritic cells, T cells, NK cells and lung stem cells. Some of these cell types have been found to stain positive for influenza virus antigen by immune-histochemistry in fatal cases of human H5N1 disease (Uiprasertkul et al., 2005). It is therefore relevant to investigate these cells with regard to their susceptibility and response towards H5N1 infection. Data from *in vitro* cultures of primary human cells are summarized below.

Alveolar Macrophage and peripheral blood monocyte derived macrophage

Extensive infiltration of macrophages into the alveolar space is reported in H5N1 disease (Peiris et al., 2004). Alveolar macrophages are consistently found to be positive for virus antigen in autopsy tissues of H5N1 infected patients (Gu et al., 2007). In *ex vivo* lung cultures, alveolar macrophages are positively stained with viral antigen after H5N1 virus infection *ex vivo* (Nicholls et al., 2007c; Weinheimer et al., 2012). Viral antigen within the macrophages in the lung could reflect productive or abortive infection, or may represent phagocytosis of other virus-infected cells by macrophages. Therefore it is important to examine the H5N1 virus tropism in this cell type.

In a comparison of the susceptibility of human alveolar macrophages and peripheral blood monocyte derived macrophage to H5N1 virus infection (at a multiplicity of infection of 2), over 95% of cells were infected in both cell types, as demonstrated by expression of viral protein at 16 hours post infection. Productive virus replication was seen in alveolar macrophages (2 log increase in viral yield) and in peripheral blood derived macrophages (1 log increase in viral yield). Seasonal H1N1 influenza virus led to a similar efficiency of infection (as demonstrated by virus antigen expression) in both cell types and the replication kinetics was comparable to that of H5N1 virus. However, H1N1 virus failed to replicate productively in alveolar macrophages (Yu et al., 2011). Others also found differences between alveolar and peripheral blood derived macrophages and reported that H5N1 virus replicates less efficiently in alveolar, as compared with peripheral blood monocyte derived macrophages (van Riel et al., 2011).

Endothelial cell tropism, host responses and the importance of endothelial integrity

The loss of endothelial barrier function and the development of microvascular leakage has been found to be a key determinant in the pathogenesis of bacterial sepsis. A recent study demonstrated that the endothelial barrier breakdown may occur in other viral infections such as H5N1 avian influenza virus (Steinberg et al., 2012). In the human lung microenvironment, the microvascular endothelial cells are essentially an immense capillary plexus. The capillary endothelium is composed of the flattened cytoplasmic extension of the endothelial cells, which by their contiguous arrangement forms a thin vascular tube. The cytoplasm of endothelial cells, like the squamous epithelial cells lining alveoli, rarely contain organelles. The microvascular capillaries run through the lung and are overlain by the thin layer of alveolar type I epithelial cells. In addition to its important but passive role in gas and liquid exchange, the capillary endothelium is also a major site of liquid and solute exchange in the lungs which is essential for the vital function of the gas exchanges.

Infectious H5N1 virus can be found in the blood and cerebrospinal fluid in birds (Muramoto et al., 2006) and several mammalian species and there is evidence of disseminated infection with a fatal outcome in some humans (Buchy et al., 2007a; de Jong et al., 2005; de Jong et al., 2006; Gu et al., 2007; Uiprasertkul et al., 2007). These observations suggest that the spectrum of H5N1 influenza A virus is wider than previously thought (de Jong et al., 2005; de Jong et al., 2006; Gu et al., 2007). However, the mechanism of dissemination of the H5N1 influenza virus is still unclear. Although autopsy data in influenza H5N1 virus infected patients is limited, diffuse intravascular coagulation (Claas et al., 1998; Shu et al., 2006; Wiwanitkit, 2008), diffuse alveolar damage with hyaline membrane formation, patchy interstitial lymphoplasmacytic infiltrates, bronchiolitis with squamous metaplasia and pulmonary congestion with varying degrees of hemorrhage have been identified (Buchy et al., 2007a; Gu et al., 2007; Uiprasertkul et al., 2007; Zhou et al., 2006a). The microvascular endothelium has a critical role in determining the permeability of the alveolar capillary barrier and hence controlling the influx of protein-rich oedema fluid into the airspaces which

occurs in acute lung injury. Inflammatory mediators are thought to play a critical role in the pathogenesis of this disorder (Minamino and Komuro, 2006).

Influenza virus antigen has been detected in lung endothelial cells of patients with pandemic H1N1 2009 virus infection (Shieh et al., 2010). Although viral antigen has not so far been detected in endothelial cells in patients with fatal H5N1 disease, given evidence of extra-pulmonary dissemination, it is likely that virus does infect lung microvascular endothelium.

The distribution of sialic acid receptors on endothelial cell of human tissues has been studied using staining with lectins, MAA II and SNA. Endothelial cells of all human organs express both avian influenza (Sia 2-3) and human influenza (Sia 2-6) receptors (Yao et al., 2008). It is therefore plausible that H5N1 virus may infect endothelial cells and that this may facilitate the dissemination of highly pathogenic avian H5N1 viruses to multiple organs (Gu et al., 2007). Although receptors for human adapted seasonal influenza viruses are also ubiquitous in the endothelial cells, the lack of a multi-basic cleavage site of the hemagglutinin in these viruses (in contrast to highly pathogenic avian viruses) restricts their capacity for replication in sites beyond the respiratory and gastrointestinal tracts because these other organs lack the human-airway-like proteases needed for the cleavage and activation of these hemagglutinin molecules. The presence of avian influenza receptor is thus not the only factor determining the capability of avian influenza A virus to infect cells.

In a study of polarized microvascular endothelial cells infected *in vitro*, H5N1 but not seasonal H1N1 virus efficiently infected these cells (Chan et al., 2009; Ocana-Macchi et al., 2009). H5N1 virus infected these cells from both an apical and basolateral side of the cell and new progeny virus was also released from both the apical and basolateral aspect of the cells. This provides a mechanistic explanation for how H5N1 may disseminate beyond the respiratory tract and also how virus may invade the lung from the systemic circulation, for example after infection via the gastro-intestinal route. There is anecdotal evidence of humans being infected with H5N1 virus though consumption of infected uncooked food. There is also evidence that inoculation of ferrets with H5N1 virus via the intragastric route can lead to virus infection that disseminates to the lung and other organs inoculation of ferrets (Shinya et al., 2011). Others have suggested that the higher expression of the Sia 2-3-linked receptors on pulmonary microvascular endothelial cells could explain the higher permissiveness of these cells to H5N1 infection and they found that the deletion of the H5 hemagglutinin multibasic cleavage site does not affect virus infectivity but results in decreased virus replication in endothelial cells (Zeng et al., 2012).

Neutrophils

During acute influenza virus infection, the infected alveolar epithelium secretes inflammatory mediators that leads to the recruitment of inflammatory cells including neutrophils and macrophages to the lung (Wareing et al., 2007). To date, there is little data on the significance of neutrophils in human influenza because neutrophil infiltration occurs during the early phase of inflammation (Tate et al., 2009; White et al., 2007) at a time when clinical biopsy or autopsy material is not readily available. One autopsy study of H5N1 disease in a pregnant woman found evidence of viral antigen in neutrophils in the placental blood and hypothesized that neutrophils may be a vehicle for virus dissemination (Zhao et al., 2008). Bring short-lived cells, there have not been studies so far to systematically investigate whether neutrophils can get infected by H5N1 virus *in vitro*.

We infected human neutrophils with seasonal H1N1 or highly pathogenic H5N1 virus *in vitro*. In six independent experiments using neutrophils from five different donors, we found a significant increase in influenza A matrix gene viral load in H5N1 infected cells whereas that for seasonal H1N1 virus was marginal (Figure 1) (Chan et al – unpublished data). These

findings suggest that H5N1 virus infects and replicates in human neutrophils. The findings also indicate a difference between H5N1 and seasonal H1N1 virus in this regard.

Dendritic cell and lymphocytes

Dendritic cells are professional antigen presenting cells which acquire virus antigen at the site of infection. This is followed by maturation of these cells with the expression of co-receptors and their migration to the draining lymph nodes. This may potentially also provide a route for dissemination of H5N1 infection. H5N1 virus can infect and replicate in monocyte-derived and blood myeloid dendritic cells whereas blood plasmacytoid dendritic cells are resistant to infection (Thitithanyanont et al., 2007). DC-SIGN on the surface of dendritic cells was responsible for the capture and transfer of H5N1 pseudotyped and recombinant particles to target cells (Thitithanyanont et al., 2007).

There has so far not been systematic study reporting on experimental infection of lymphocytes with H5N1 virus *in vitro* although some have reported H5N1 virus antigen in autopsy tissues (Zhang et al., 2009).

H5N1 infection of extrapulmonary cells

Astrocytes

Neuronal cells and glial cells of the brain have been found to express H5N1 virus antigen in autopsy tissues of H5N1 cases (Gu et al., 2007) (Zhang et al 2009). Differentiated human astrocytes and neuronal cell can be infected with both seasonal H1N1 and H5N1 virus *in vitro* but only astrocytes supported productive replication (Ng et al., 2010).

Placenta and fetus

H5N1 viral antigen was detected in the Hofbauer cells and cytotrophoblasts of the placenta from a pregnant woman who died from H5N1 infection. In the fetus, viral RNA and antigen was found in the lungs, circulating mononuclear cells, and macrophages of the liver. This appears to be the first evidence of transplacental transmission of H5N1 from mother to fetus (Gu et al., 2007).

Digestive tract

The digestive tract has been suspected to be a potential site for replication of H5N1 virus, given the high H5N1 viral load in rectal swabs (Buchy et al., 2007b) and the isolation of virus from faeces in a patient with H5N1 encephalitis (de Jong et al., 2005). However, detection of virus in the gastrointestinal tract may reflect swallowed respiratory secretions containing virus and is not conclusive. H5N1 virus antigen has been detected in the small and large intestinal epithelial cells at autopsy (Zhang et al., 2009). Sia₂₋₆-Gal was absent from epithelial surface of small and large intestines and was found only in lamina propria cells. In contrast, Sia_{2,3}-Gal was found on large intestinal epithelial cells and in lamina propria cells. Virus-binding studies showed that neither seasonal influenza A (H1N1) nor A (H3N2) virus bound to small and large intestinal epithelial surface, but they did bind to a subset of CD45⁺ leukocytes (Chan et al., 2011a)

Conjunctival epithelium

The conjunctival sac is connected to the nasopharynx via the lacrimal duct. The human conjunctival epithelium contains Sia₂₋₃ sialic acid receptors but not Sia₂₋₆ receptors. *Ex vivo* cultures of conjunctival epithelium culture also possess similar sialic acid expression profiles (Chan et al., 2010a; Olofsson et al., 2005; Terraciano et al., 1999). Therefore, it is considered as an alternative entry route for avian influenza virus infection

(Olofsson et al., 2005). Human seasonal H1N1 (pre-2009) and H3N2 influenza viruses fail to infect or replicate in *ex vivo* cultures of the conjunctival epithelium while avian H5N1 and H7N7 viruses do. Conjunctivitis has been repeatedly associated with human H7N7 infections in the Netherlands and elsewhere (Fouchier et al., 2004). H5N1 and H7N7 viruses can infect and replicate well in *ex vivo* conjunctival cultures but seasonal H1N1 and H3N2 viruses do not efficiently infect the conjunctiva. Surprisingly, pandemic H1N1 virus can efficiently infect and replicate in the conjunctival *ex vivo* cultures (Chan et al., 2010a), given its receptor binding profile being similar to that of seasonal influenza viruses (Maines et al., 2009) although one study using glycan array has reported dual binding of the pandemic H1N1 virus to both Sia₂₋₃ and Sia₂₋₆ receptors (Childs et al., 2009). The conjunctival tropism of pandemic H1N1 virus was further supported by a recent clinical report of bilateral acute conjunctivitis in patients with pandemic H1N1 infection (Mansour et al., 2012).

As with conjunctival epithelium, H5N1 but not seasonal influenza strains replicated to high titers in human retinal pigment epithelial cells (Michaelis et al., 2009). Ferret infection studies have shown that ocular inoculation of H5N1 virus can lead to lethal infection with systemic spread without involvement of the lower respiratory tract (Belser et al., 2012).

Investigation of Host Responses using primary human cells

Primary human cells and *ex vivo* cultures also provide insights into host responses to virus infection. The mechanisms underlying the severity of human disease, primarily as a result of a fulminant primary viral pneumonia caused by H5N1 virus remain unclear. Although early use of antivirals have a significant impact on reducing mortality associated with human disease, even oseltamivir treatment within 2 days of symptom onset is still associated with a case fatality ratio of >15% (Chan et al., 2012), a worrying scenario if H5N1 viruses with current virulence characteristics were ever to become pandemic. While the replication competence of the virus, lack of protective host immune responses and the tropism for the alveolar epithelium contribute to this unusually severe lung pathology, there is accumulating evidence that dysregulated host responses also contribute to the pathogenesis of this disease (Peiris et al., 2010). Data from studies of patients with H5N1 disease and experimental animal models are compatible with this hypothesis (Baskin et al., 2009; de Jong et al., 2006; Perrone et al., 2008). However, studies of H5N1 or seasonal influenza infected humans or animals *in vivo* does not allow differentiation of the contribution of increased viral replication efficiency from intrinsic differences between these viruses in eliciting aberrant host responses. By comparing host responses elicited by H5N1 and other seasonal or pandemic viruses in experiments using primary human cells *in vitro*, where the dose of infecting virus can be controlled, the intrinsic differences between these viruses can be investigated.

Apoptosis and cell death

Avian influenza H5N1 virus was shown to induce apoptosis in many cell types including macrophages and dendritic cells. In human monocyte-derived macrophages, TNF-related apoptosis-inducing ligand (TRAIL) was shown to be involved in the apoptosis-associated mechanisms in H5N1 virus infection through the activation of caspase-10. H5N1 virus triggered apoptosis, including the activation of caspases and PARP, instead of necrosis (Ekchariyawat et al., 2012; Zhou et al., 2006b). Viruses differed in the speed with which they led to apoptosis (Geiler et al., 2011; Mok et al., 2007). It is notable that apoptosis, by reducing the opportunity for productive virus replication and release of inflammatory mediators from the infected cells, can be a host defense mechanism in response to virus infection. iNOS has been found to be involved in the induction of target cell apoptosis. Kruppel-like factor 6 (KLF6) is a critical transcription factor essential for iNOS gene

expression following influenza virus infection. KLF6 gene silencing in human lung epithelial cells resulted in the loss of NO production, iNOS promoter-specific luciferase activity and the expression of iNOS. A significant reduction in influenza virus-mediated apoptosis was observed in KLF6-silenced cells, cells treated with iNOS inhibitor, and primary murine macrophages derived from iNOS knockout mice (Mgbemena et al., 2012).

Influenza H5N1 virus, but not seasonal H1N1 virus, has been shown to be involved in autophagic cell death, an alternative programmed cell death pathway in alveolar epithelial cells upon infection by the H5N1 virus (Sun et al., 2012). In addition, autophagy has been demonstrated to play a role in influenza induced cytokine induction. H9N2 influenza A viruses of the G1-lineage (which possess 6 internal gene segments in common with the H5N1 viruses that caused the outbreak in Hong Kong in 1997) was a more potent inducer of autophagy, in association with cytokine (CXCL10 and interferon-alpha) hyper-induction when compared with seasonal H1N1 and pandemic influenza H1N1 viruses in human blood macrophages (Law et al., 2010).

Innate immune responses

A major difference between the low pathogenic seasonal influenza and lethal influenza viruses such as H5N1 or the 1918 H1N1 virus is an increased release of inflammatory cytokines and chemokines that occurs in H5N1 or 1918 H1N1 virus infected macrophages or epithelial cells (Chan et al., 2005; Cheung et al., 2002; Kobasa et al., 2007; Perrone et al., 2008; Tumpey et al., 2000; Wong and Yuen, 2006; Yu et al., 2011). Comparative transcriptomic analysis of H5N1 and seasonal H1N1 infected human macrophages showed that both viruses elicit similar host response pathways but that H5N1 virus was a more potent inducer of these pathways including those of interferon induction and signaling, of tumor-necrosis factor alpha induction and signaling, and differentially activated apoptosis pathways (Lee et al., 2009). H5N1 virus strains may differ in their potency in activating innate immune responses (Sakabe et al., 2011). The host innate immune responses may also be affected by the differentiation status of the respiratory epithelial (NHBE) cells, in part because of the reduced permissiveness of these cells to avian H5N1 virus infection (Chan et al., 2009). Similarly, alveolar macrophages express much less pro-inflammatory cytokines in response to H5N1 virus infection compared to peripheral blood monocyte derived macrophages (van Riel et al., 2011; Yu et al., 2011). Since there is a massive increase of macrophages in the lung of H5N1 virus infected patients, it is important to understand whether such infiltrating macrophages have a cytokine phenotype more akin to alveolar macrophages or to peripheral blood monocyte derived macrophages. These provide good illustrations of the need to choose physiologically relevant cell subsets for such investigations.

Primary human peripheral blood monocyte derived macrophages have been used to study the signaling mechanisms associated with H5N1-mediated cytokine hyper-induction including the role of RIG-I, IFN regulatory factor 3 (IRF3), NF- κ B nuclear translocation and p38 kinase activation in the induction of IFN-beta, IFN-lambda1 and MCP-1, and their partial contribution to the induction of TNF-alpha (Hui et al., 2011; Hui et al., 2009). Human peripheral blood monocyte derived macrophage infected with influenza H3N2 or H5N1, but not with H1N1 viruses was associated with pronounced pro-inflammatory cytokine production and activation of relevant mitogen-activated protein kinase pathways as indicated by phosphorylation of p38, JNK and ERK 1/2 (Geiler et al., 2011). Soluble mediators secreted from influenza H5N1-infected human monocyte derived macrophages up-regulated RIG-I, MDA5, and TLR3 in adjacent un-infected cells thereby potentially sensitizing these cells by paracrine effects acting via IFNAR1/JAK but not IFN- γ receptor signaling (Hui et al., 2011). When compared with virus free supernatants of H1N1 virus infected cells, H5N1 virus infected cell supernatants were more potent at inducing these

effects in adjacent cells and in enhancing the cytokine responses to PolyIC and to both the seasonal H1N1 and H5N1 virus infection in a RIG-I-dependent manner. Thus, sensitizing neighboring cells by up-regulation of RIG-I contributed to the amplified cytokine cascades during H5N1 infection (Hui et al., 2011; Lee et al., 2008). Some of these paracrine mediator cascades were dependent on COX-2 activation (Lee et al., 2008). In dendritic cells and macrophages, influenza virus RNA interacting with TLR7 provides the first signal and the virus M2 ion channel protein provides the second signal for inflammasome activation by influenza viruses (Ichinohe et al., 2010).

The proteomic responses in the cytoplasm of H5N1 or H1N1 virus infected macrophages was evaluated using mass spectrometry-based quantitative proteomic profiling and showed significant perturbation of the host proteome upon viral infection as early as one hour after infection. This early host response distinguished H5N1 infection from H1N1 infection, the latter inducing less of a response. The most pronounced effect was observed on the translational machinery, suggesting that H5N1 might gain advantage in replication by using the cell protein synthesis machinery early in the infection (Cheung et al., 2012).

Interferon (IFN) is the first line of defense against influenza virus infections. Influenza viruses have developed various mechanisms to counteract this response, in order to gain a replication advantage in the host cells. The non-structural protein 1 (NS1) protein of influenza contributes to evading the host interferon responses (Geiss et al., 2002). H5N1 virus was showed to block the IFN signaling by reducing the IFN-inducible tyrosine phosphorylation of STAT1, STAT2 and STAT3 and inhibit the nuclear translocation of phospho-STAT2 and the formation of IFN-inducible STAT1:1-, STAT1:3- and STAT3:3-DNA complexes. Besides, NS1 induced the up-regulation of the JAK/STAT inhibitors, SOCS1 and SOCS3, suggested that NS1 can directly interfere with IFN signaling to enhance viral replication (Jia et al., 2010). Influenza A viruses not only suppress IFN-beta gene induction but also inhibit type I IFN signaling through a mechanism involving induction of the suppressor of cytokine signaling-3 (SOCS-3) protein (Pauli et al., 2008).

Recombinant viruses generated by reverse genetic methods that contain different combinations of genes from high-cytokine (A/Vietnam/1203/04; H5N1) and low-cytokine (A/WSN/33; H1N1) phenotype viruses suggested that the H5N1 polymerase gene segments, and to a lesser extent the NS gene segment, contribute to cytokine hyper-induction phenotype in primary human macrophages. Thus, a putative H5 pandemic virus that may arise through genetic reassortment between H5N1 and one of the current seasonal influenza viruses may have a markedly altered cytokine phenotype (Mok et al., 2009).

Many studies have been carried out to determine the functional contribution of inflammatory mediators released from the respiratory epithelium and macrophages to inflammation (summarized above). Consequences of the infection of endothelial cells have been less intensively studied. The most frequently used endothelial cell model in influenza A virus research is the umbilical vein endothelial cell (Ishiguro et al., 2004; Sumikoshi et al., 2008; Taub et al., 1993; Visseren et al., 1999). It is known to produce IL-6 (Ishiguro et al., 2004; Visseren et al., 1999), IP-10 and MIG (Ishiguro et al., 2004) in response to influenza A virus infection. There have been varying reports on the permissiveness of endothelial cells to influenza viruses (Colden-Stanfield et al., 1993; Friedman et al., 1981; Ishiguro et al., 2004; Sumikoshi et al., 2008; Visseren et al., 2000). The response of endothelial cell, in particular, the lung microvascular endothelial cells, to the highly pathogenic H5N1 influenza A virus subtypes remains unknown.

There is increasing evidence of heterogeneity (Craig et al., 1998) among endothelial cells from different tissues from *in vivo* (Swerlick et al., 1992) and *in vitro* (Haraldsen et al.,

1996; Nilsen et al., 1998). Endothelial cells from different sites may respond differently to inflammatory stimuli and have variable susceptibility to injury (Murphy et al., 1998). Data extrapolated from non-pulmonary or macro-vascular endothelium (Beekhuizen and van Furth, 1994), e.g. human umbilical vein endothelial cells (HUVEC), might not adequately represent cells from the lung. It is therefore important to use appropriate endothelial cell models that exhibits characteristics with direct relevance to respiratory diseases processes.

Conclusion

Primary human cells, differentiated cultures of respiratory epithelium and *ex vivo* cultures contribute to understanding influenza virus tropism for the human respiratory tract and the host responses to influenza virus infection. Taken together with data from clinical studies on infected humans and relevant animal models, these data can provide insights into virus transmission and pathogenesis and may provide understanding that leads to novel therapeutic interventions.

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1. Sialic acid expression in ex vivo organ cultures correlates with in vivo tissues. (83 characters)
2. Ex vivo cultures can be used to study innate immune response to flu infection (81 characters)
3. Ex vivo cultures can be used to study virus tropism. (54 characters)
4. Ex vivo cultures are useful for risk assessing animal viruses. (64 characters)

Figure 1.

Influenza matrix gene copy number detected after 1, 6, 21 hpi and normalized with β -actin gene expression. Human neutrophil was isolated from fresh blood and infected in a MOI of 2.

Figure 2.
H5N1 virus infected ex vivo cultures of human A) nasopharyngeal, B) bronchial and C) alveolar epithelium with red arrows indicating the infected cells stained positive with influenza viral antigen in reddish brown.

Table 1

Summary table comparing the expression of sialic acid receptor of ex vivo organ culture models, in vitro primary epithelial cell cultures and continuous cell lines of human respiratory tract to the nasopharynx, bronchus and lung of humans, using lectin binding method

		Lectin	MAAI	MAAII	SNA
Anatomical site	Culture Model	Differentiation Stage	2-3 N-linked	2-3 O-linked	2-6
Nasopharynx	In vivo		++	–	++
	Ex vivo		++	–	++
	In vitro	Undifferentiated	++	+	–
Bronchus	In vivo		++	–	++
	Ex vivo		++	–	++
	In vitro	Undifferentiated	++	++	+
		Well-differentiated	++	–	++
	Cell line	Calu-3 polarized	O-linked 2-3 2-6 (Zeng et al., 2007)		
Lung	In vivo		+	++	+
	Ex vivo		+	++	+
	In vitro	Type-I like	++	++	+
		Type-II like	++	+	+
	Cell line	A549 (Type II-like), NCI-H661 cells	2-3 << 2-6 (Guo et al., 2009; Sieben et al., 2012)		

Table 2

Attachment of labeled influenza virions to human respiratory epithelia for comparison with distribution of influenza receptors by lectin binding (see table 1)

Virus Attachment					
	HPAV H5N1	Seasonal H1N1	Seasonal H3N2	H7N7 (2003)	H1N1pdm (2009)
Conjunctiva	No Data	No Data	No Data	No Data	No Data
Nasal/ Nasopharynx	No (scant binding to nasopharynx and paranasal sinuses, ciliated cells)	Yes (Ciliated > goblet cells)	Yes (Ciliated > goblet cells)	No Data	Yes (Ciliated > goblet cells)
Trachea	No	Yes	Yes	No Data	Yes
Bronchus	Minimal	Yes	Yes	No Data	No Data
Bronchiole	Yes	No	Minimal	Yes	No Data
Lung	Yes (Type II)	Yes	Yes	Yes	No Data
References	(Chutinimitkul et al., 2010; van Riel et al., 2006; van Riel et al., 2007)	(van Riel et al., 2010; van Riel et al., 2007)	(Chutinimitkul et al., 2010; van Riel et al., 2007)	(de Wit et al., 2010)	(van Riel et al., 2010)

Table 3

Infection and replication of influenza viruses of different subtypes in human respiratory and conjunctival epithelia

Virus Infection and Replication					
	H5N1	H1N1	H3N2	H7N7	H1N1pdm
Conjunctiva	Yes	No	No	Yes	Yes
Nasal/ Nasopharynx	Yes	Yes	Yes	No Data	Yes
Bronchus	Limited infection without productive replication	Yes	Yes	No Data	Yes
Lung	Yes (type II pneumocytes)	Yes	Yes/ No (strain dependent)	Yes	Yes
Reference	(Nicholls et al., 2007c; Weinheimer et al., 2012)	(Chan et al., 2010a; Nicholls et al., 2007c)	(Chan et al., 2010a; Chan et al., 2011c; Nicholls et al., 2007c)	Chan et al., unpublished data.	(Chan et al., 2010a; Chan et al., 2011b)