IMMUNE RESPONSE TO H5N1 VIRUS INFECTION IN HUMANS

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ABSTRACT
Background: The menace of H5N1infection in humans till date is a worldwide problem. H5N1 influenza viruses are prevalent in poultry in Asia and have recently spread to other continents like Africa and Europe. H5N1 influenza virus infections in the human population are rare due to their inefficient direct human-to-human transmission. H5N1 infection in human is followed by strong inflammatory response which is usually characterized by elevated levels of cytokines and chemokines in serum, believed to be important in the severe pathogenesis that develops in a high proportion of patients. Of particular concern are the elevated levels of tumor necrosis factor-alpha, a protein associated with tissue destruction at sites of infection. H5N1 virus causes severe influenza disease in humans, manifested by acute respiratory distress syndrome, multi-organ failure, and high mortality rates of 60%.
Aim: To understand the human immune responses to H5N1 so that effective control of severe influenza disease caused by H5N1 and rationally design of new immunotherapies and vaccines can be achieved.
Conclusion: This review discussed the human immune responses to H5N1 viral infection, with emphasis on H5N1 virulence factors and their impact on the modulation of the innate immunity in humans.
Keywords: H5N1; Cytokine; Immunity; Infection; Humans.

INTRODUCTION

H5N1 virus, a subtype of Influenza A virus, which is a negative-sense RNA virus belonging to the family "*Orthomyxoviridae*" and genus "Influenza virus", continues to zoonotically transmit to humans causing severe disease and poses a pandemic threat (Peiris *et al.,* 2007). H5N1 has eight gene segments that encode 11 proteins: hemagglutinin (HA), neuraminidase (NA), matrix proteins M2 and M1, nonstructural (NS) proteins NS1 and NS2 (nuclear export of vRNPs), the nucleocapsid, and the four polymerases, the PB1 (polymerase basic 1), PB1-F2, PB2, and PA (polymerase acidic) proteins (Chen *et al.,* 2001)

The highly pathogenic H5N1 virus attracted widespread attention in 1997, when a H5N1 outbreak in Hong Kong resulted in 18 human infections and 6 deaths (Shortridge *et al.,* 1998). Subsequent outbreaks of the H5N1 subtype occurred in China, Indonesia, Malaysia, Vietnam, and Thailand poultry industries in the early 2004, resulting in 13 human cases with 9 deaths in Vietnam, and 27 cases with 20 deaths in Thailand (Ungchusak *et al.,* 2005). Since then, multiple reassortant H5 HPAIV strains have spread across Asia and into Europe, the Middle East and Africa, giving rise to multiple subtypes (H5N2, H5N5, H5N6, and H5N8). Humans in close contact with AIV-infected poultry, and on rare occasion with other AIV-infected humans can become infected (Li *et al.,* 2008). Since 2003, dispersion of H5N1 viruses has led to epizootics in about 60 countries on three continents, and has caused 403 human cases and 254 deaths, as of 27 January 2009. Thus, the fatality rate is >50% for H5N1 infections (WHO, 2008). Although, several studies addressed the calculations of more realistic rates of H5N1 infections, taking into account mild asymptomatic cases and low transmissibility, these are still estimated to be >30% (Li *et al.,* 2008).

Countries that had the highest number of documented cases of H5N1 include Egypt, Indonesia, Vietnam, Cambodia, and China, where the total numbers ranged from 52 to 344 (WHO/GIP, 2015). The overall prevalence of H5 viruses increased in China since 2010 (Su *et al.,* 2015).

Patients infected with H5N1 influenza virus developed mainly severe respiratory disease, accompanied by fever, cough, shortness of breath, and pneumonia, that frequently progressed to acute respiratory distress syndrome (ARDS) and multiorgan failure (Yuen *et al.,* 1998; Hui, 2008; Yu *et al.,* 2008). In fatal cases, the median time from onset to death was 9 to 10 days (Abdel-Ghafar*et al.,* 2008). As of August 2012, more than 600 confirmed cases of infection by H5N1 viruses have been reported to the World Health Organization (WHO), with a lethal outcome in 59% of those documented cases (WHO, 2012). However, there is evidence that indicates that this lethality rate might be overestimated due to reduced sensitivity of the WHO confirmation criteria, no official H5N1 confirmation by health providers in rural areas, and subclinical or mild infections (Wang *et al.,* 2012). Luckily, avian strains of IAV are not efficient at infecting humans (Beare and Webster, 1991) and direct transmission from human to human has been reported only in close family clusters, with very limited spread of the virus (Yang *et al.,* 2007).

The Effect of H5N1 Viruelent Factors

The haemaglutinin (HA) of H5N1 plays an essential role in initiation of infection, since it interacts with sialic acid (N-acetylneuraminic acid) receptors (SA) on the cell surface (Weis *et al.,* 1988), which allows for subsequent endocytosis of the viral particle. It also mediates fusion of the endosomal and viral membrane (Wiley and Skehel, 1987). The main host species barrier that limits transmission of avian IAV to humans consists of the different receptor specificity of avian and human viruses (Shinya *et al.,* 2006; van Riel *et al.,* 2006; Nicholls *et al.*, 2007). SA are found in a variety of glycosidic linkages, principally as α-2,3- or α-2,6-linkages to galactose or *N*-acetylgalactosamine residues in *N*- and *O*-linked glycans, although α-2,8-linked SA oligomers can be found as well(Rutishauser*et al.,* 1988). It is known that HA from human isolates usually binds SAα2,6, which are abundant in the human upper respiratory tract, while the one from avian isolates usually has affinity for SAα2,3 (Rogers and Paulson, 1983). Therefore, the low abundance of SAα2,3 in the human respiratory tract is one of the limiting factors for the lack of transmission of avian influenza viruses in humans (Herfst*et al.,* 2012; Imai *et al.,* 2012). Although SAα2,6 are the most abundant in the respiratory system in humans, some cell types have been shown to present SAα2,3 glycoconjugates (therefore being susceptible to infection by H5N1 viruses) mainly in the lower respiratory tract, including type II pneumocytes and alveolar macrophages (Shinya *et al.,* 2006; van Riel *et al.,* 2006; Nicholls *et al.,* 2007). Interaction of the HA with cell receptors is also believed to contribute to the induction of cytokine production (Ramos and Fernandez-Sesma, 2012).

One of the most important virulence determinants of the HPAIV is the presence of a multibasic cleavage site (MBCS) as shown in avian and mammalian models (Horimoto*et al.,* 1994; Suguitan*et al.,* 2012). This polybasic amino acid sequence in the cleavage site allows post-translational processing of the precursor HA0 to HA1 and HA2, which is necessary for fusion activity and virus entry, by furin and other subtilisin-like proprotein-processing endoproteases that are ubiquitously expressed in tissues (Stieneke-Grober*et al.,* 1992; Horimoto*et al.,* 1994).

PB1-F2 is a small protein encoded in the +1 reading frame of PB1 (Chen *et al.,* 2001) that localizes to the mitochondria and has been shown to induce apoptosis in infected cells, specifically in immune cells, by altering the mitochondrial membrane potential (Chen *et al.,* 2001; Gibbs *et al.,* 2003; Zamarin*et al.,* 2005). It is important to note that the PB1-F2 protein is not expressed by all the IAV.

In many swine and human isolates, protein translation is interrupted because stop codons are introduced in the reading frame (Chen *et al.,* 2001; Zell *et al.,* 2007). On the contrary, PB1-F2 is highly conserved in H5N1 avian influenza viruses (Zell *et al.,* 2003) and the residue 66S is present in some of the HPAIV (Conenello*et al.,* 2007). Therefore, the expression of PB1-F2 and the presence of the amino acid 66S are considered virulence markers, which is critical for surveillance purposes. Zamarin*et al*. (2005) unraveled the mechanism by which this small protein induces apoptosis. They showed that PB1-F2 interacts with the adenine nucleotide translocator 3 (ANT3) and the membrane voltage-dependent anion channel 1 (VDAC1) of the mitochondrial permeability transition pore complex (PTPC). They also found that PB1-F2 also renders the cells more sensitive to pro-apoptotic stimuli, like TNF-α. On the other hand, other group showed that PB1-F2 can also oligomerize to form pores in the mitochondrial membrane (Brunse*t al.,* 2007). In addition, another virulence mechanism for PB1-F2 has been recently identified and characterized, namely the ability of PB1-F2 to inhibit type I IFN production both in cell culture and *in vivo* (Conenello*et al.,* 2007; Varga*et al.,* 2011). The C-terminal domain of PB1-F2 interacts with the mitochondrial antiviral signaling (MAVS) protein, decreasing the mitochondrial membrane potential and resulting in the inhibition of RIG-I-mediated of IFN-α/β production (Varga*et al.,* 2012).

Another element from IAV known to be involved in the deregulation of the antiviral immune response is the polymerase complex. One of the polymerase components, the PB2 bearing a lysine in position 627, which is predominant in human IAV and in some HPAIV strains, is considered a virulence determinant in mammals for H5N1 IAV, since viruses bearing this residue are more virulent and pathogenic in mice than those with a glutamic acid, encoded in PB2 from low pathogenic avian IAV strains (Hatta*et al.,* 2001). Interestingly, 627K has been also demonstrated to be associated with strong production of TNF-α, IFN-β and IP-10 in human primary macrophages and type 1–like pneumocytes infected with HPAIV H5N1, and therefore is thought to contribute to the elevation of cytokines in serum in H5N1 infected patients (Mok*et al.,* 2009).

Nevertheless, components of the polymerase complex are also involved in the evasion of the innate immunity. Each component of the viral polymerase complex, PA, PB1 and PB2, has been shown to interact with MAVS. However, PB2 has the strongest MAVS-induced IFN-α/β production inhibitory ability (Iwai *et al.,* 2010). IAV polymerase also contributes to the host protein shut-off by two independent mechanisms. Kuo and Krug (2009) showed that PA is able to stabilize the NS1-CPSF30 complex of an H5N1 NS1 lacking the residues F103/M106, which allows the inhibition of the post-transcriptional processing of cellular pre-mRNA. On the other hand, it is known that, in order to hijack the host transcriptional system, IAV polymerase associates with the host RNA polymerase II (Pol II). It has been described that this interaction results in the degradation and inhibition of Pol II, which seems to contribute to the inhibition of the cellular gene expression and therefore, of the antiviral response (Vreede*et al.,* 2010a) (Vreede and Fodor, 2010b).

In the infected cell, NS1 is localized in the nucleus and NS2 in the cytoplasm. NS1 is a multifunctional protein that plays an important role as a post-transcriptional regulator in the influenza virus life cycle: it binds virion RNA (Hatada*et al.,* 1997, Marion *et al.*, 1997a; Marion *et al.*, 1997b) poly(A)-containing RNA (Qiu and Krug, 1994), U6 snRNA (Qiu, 1995), and some viral and cellular proteins. Some strains of H5N1 subtype are highly virulent because NS1 protein inhibits antiviral interferon α/β production (Lin, 2007). NS1 plays a crucial role in protecting the influenza virus against the cellular cytokine response (Hatada*et al.,* 1999; Seo, 2002).

The NS2 protein was previously thought to be presented only in infected cells, until it was shown to exist in the viral particle (Richardson and Akkina, 1991). It is also referred to as nuclear export protein (NEP) because it plays an important role in mediating the export of viral RNP from nucleus to the cytoplasm through export signal and via interaction with Crm1 protein (O'Neill, 1998, Neumann, 2000). The NS2 protein is involved in viral assembly by interacting with the M1 protein (Schmitt and Lamb, 2005). Recently, it has been proposed that NS2/NEP protein regulates influenza virus transcription and replication that is independent of its viral RNP export function (Robb, 2009).

H5N1 and "Cytokine storm".

Inflammation acts as a double-edged sword during viral infections. While inflammation can promote the recruitment of immune cells, uncontrolled and exacerbated inflammation, as observed in many AIV human cases, is associated with systemic edema and extensive tissue damage. Humans infected by H5N1 frequently present high serum levels of pro-inflammatory cytokines and chemokines (To *et al.,* 2001; De Jong*et al.,* 2006; Thitithanyanont*et al.,* 2010), which is believed to contribute to the disease pathogenesis. Acute respiratory distress syndrome (ARSD), which has been compared to the severe acute respiratory syndrome virus due to Coronavirus (SARS-CoV) and associated with exaggerated inflammatory responses (Ng *et al.,* 2006), has been frequently observed in patients infected by H5N1 IAV with severe degrees of disease (Yuen *et al.,* 1998). It is well established that human infection with avian viruses, like H5 subtypes, can lead to "cytokine storms." Analyses of serum and plasma of hospitalized patients infected with H5N1 revealed hypercytokinemia of pro-inflammatory mediators (MCP-1, MIF, IL-6, IL-8, IP-10) and angiogenic/growth factors (HGF, SCF) (De Jong*et al.,* 2006; Wang *et al.,* 2014; Guo*et al*., 2015), and can be linked to fatal disease outcomes (Guo*et al.,* 2015). Similar findings were observed in macaques, ferrets, and mice challenged with HPAIV H5N1. Extensive tissue damage and hypercytokinemia are consistently present in H5N1-inoculated animals (Tumpey*et al.,* 2000; Zitzow*et al.,* 2002; Govorkova*et al.,* 2005; Baskin *et al.,* 2009). Interestingly, anti-inflammatory molecules, including IL-10, were detected in serum of H5N1-infected patients (De Jong*et al.,* 2006). Furthermore, limited production of anti-viral type I IFN, which in part serves to limit viral replication, was observed in respiratory epithelial cells infected with H5 subtype in vitro (Belser*et al.,* 2011; Zeng*et al.,* 2007). This indicates that there may be an attempt by the immune system to modulate inflammation, although this could be too little too late. Murine studies demonstrated that blocking pro-inflammatory cytokines, such as TNF, IL-6, and CCL2, did not improve survival of mice challenged with HPAIV (Salomon *et al.,* 2007), suggesting that multiple mechanisms, in addition to inflammation, are likely to exacerbate pathology. Mechanistically, blocking pro-inflammatory mediators in humans has not yet been preformed for AIVs.

INNATE IMMUNE RESPONSE DURING H5N1 INFECTION

The innate immune response, composed of both cellular and soluble mediators, forms the first line of defense during viral infections. The protective role of innate immunity is clearly demonstrated during HPAI infection of birds whereby, ducks exhibit asymptomatic or mild disease, while chickens become ill (Kuchipudi*et al.,* 2014). Following H5N1 infection, primary lung cells isolated from chickens showed highly elevated immune and pro-inflammatory responses compared to duck cells, which appeared to be mediated via inhibition of STAT-3 (Kuchipudi*et al.,* 2014). In humans, immunohistological analyses of lung tissues in five fatal cases of H5N1 showed an influx of neutrophils into the alveolar spaces, while TNF and IL-6 were detected in monocytes/macrophages in situ (Nakajima *et al.,* 2013). The role of monocyte-derived macrophages (MDMs) and alveolar macrophages in protection versus pathology in avian influenza remains controversial.

Robust pro-inflammatory responses, including TNF and IP-10 were detected in culture supernatants in H5N1-infected human MDMs in vitro (Cheung *et al.,* 2002; Sakabe*et al.,* 2011; Friesenhagen*et al.,* 2012). This was dependent on the viral isolate and independent of viral replicative capacity in MDMs (Cheung *et al.,* 2002; Sakabe*et al.,* 2011; Friesenhagen*et al.,* 2012). Conversely, alveolar macrophages isolated from human lungs showed limited cytokine secretion after HPAIV infection (van Riel *et al.,* 2011). The latter study demonstrated that the permissiveness to infection was lower in alveolar macrophages than in MDMs (van Riel *et al.,* 2011). These data indicate differential roles of tissue-resident macrophages versus circulating and differentiated monocytes during avian influenza infections (Chan *et al.,* 2013). During the steady state, tissue macrophages have been shown to provide homeostatic functions, including resolution of inflammation via removal of apoptotic debris and cells (Davies *et al.,* 2013). Conversely, MDMs have an inflammatory effector profile, suggesting that they have the potential to contribute to inflammation-induced pathology (Davies *et al.,* 2013; Italiani and Boraschi, 2014). Together, these studies highlight the importance of understanding how microenvironments drive differential levels of inflammation during avian influenza infection.

The innate immune responses to IAV have also been studied in endothelial cells by using primary human *ex vivo* models as well. The study by Zeng*et al*. (2012) showed that primary human lung micro-vascular endothelial cells (HMVEC) support viral replication of H5N1 viruses and infection results in strong up-regulation of pro-inflammatory genes. Accordingly, other studies using Human Umbilical Vein Endothelial Cells (HUVEC) showed that H5N1 viruses induced strong inflammatory responses, mostly mediated by activation of the NF-κβ (nuclear factor kappa-light-chain-enhancer) pathway (Schmolke*et al*., 2009; Viemann*et al*., 2011; Zeng*et al*., 2012), supporting a possible role for endothelial cells in the hyper-induction of cytokines in infected patients.

Natural Killer (NK) cells also represent important players in the innate immunity to viral infection. Upon activation and recruitment to the site of infection, NK cells produce cytokines and destroy infected cells (Brandstadter and Yang, 2011). There is evidence that influenza virus HA can induce activation of NK cells, by interacting with the receptors NKp46 and NKp44 (Arnon*et al.,* 2001; Mandelboim*et al.,* 2001; Ho *et al.,* 2008), which is thought to be a mechanism for killing infected cells. This interaction seems to be dependent on the sialylation of the receptor, and have been demonstrated for H5 and H1 (that bind through SAα2,3 and SAα2,6, respectively). However, NKp46 interactions with H5, as opposed to the ones with a swine origin H1 2009, are insufficient to activate the NKp46-mediated killing (Achdout*et al*., 2010), suggesting that H5 pathogeneicity might be increased by this lack of ability to activate NK cells. On the other hand, another study showed that infection with pseudotyped particles expressing influenza virus HA induced higher levels of human NK cell activation as shown by up-regulation of the activation marker CD69 (Du *et al*., 2010), and in this case H5N1 and H1N1 1918 expressing pseudo-particles induced higher levels of activation that the ones expressing an H1N1 2009 HA, which suggests different outcomes depending of the viral HA. Due to the variety of sialylatedglycans that HA from different IAV strains interact with (Stevens *et al.,* 2006a; Stevens *et al*., 2006b), it would be possible that those interactions have diverse outcomes depending on the IAV subtype/origin, resulting in different levels of pathogenesis.

Conclusion

H5N1 viruses induce severe disease in humans, probably as a consequence of the induction of an exacerbated innate immune response that leads to “cytokine storm”.

The virulent components of H5N1 have been involved in both induction and inhibition of the innate immune response, and for that reason the contribution of these different elements at different stages of the infection in human could lead to failure of the innate immune response to control virus spread and subsequent tissue damaging pro-inflammatory burst.

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