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Immune responses to infection with H5N1 influenza virus

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Abstract

Influenza A H5N1 viruses remain a substantial threat to global public health. In particular, the expanding genetic diversity of H5N1 viruses and the associated risk for human adaptation underscore the importance of better understanding host immune responses that may protect against disease or infection. Although much emphasis has been placed on investigating early virus–host interactions and the induction of innate immune responses, little is known of the consequent adaptive immune response to H5N1 virus infection. In this review, we describe the H5N1 virus-specific and cross-reactive antibody and T cell responses in humans and animal models. Data from limited studies suggest that although initially robust, there is substantial waning of the serum antibody responses in survivors of H5N1 virus infection. Characterization of monoclonal antibodies generated from memory B cells of survivors of H5N1 virus infection has provided an understanding of the fine specificity of the human antibody response to H5N1 virus infection and identified strategies for immunotherapy. Human T cell responses induced by infection with seasonal influenza viruses are directed to relatively conserved internal proteins and cross-react with the H5N1 subtype. A role for T cell-based heterosubtypic immunity against H5N1 viruses is suggested in animal studies. Further studies on adaptive immune responses to H5N1 virus infection in both humans and animals are needed to inform the design of optimal immunological treatment and prevention modalities.

Keywords

Influenza A H5N1 virus; Infection; Immune response

1. Introduction

Since the first documented case of human infection with HPAI H5N1 influenza virus in Hong Kong in 1997, HPAI H5N1 viruses have diversified genetically and spread to over 60 countries on 3 continents, resulting in over 600 documented human cases of H5N1 infection with high mortality. These viruses remain a substantial economic burden for global agriculture and a considerable threat to public health. To date, over 20 distinct clades and subclades of H5N1 have been identified in domestic poultry and wild birds. The criteria

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used to classify viruses into clades and subclades has been described (WHO OIE FAO, 2012). Human infections with H5N1 viruses belonging to four first order clades (clades 0, 1, 2 and 7) and multiple subclades (1.1, 2.1.2, 2.1.3.2, 2.2, 2.2.1, 2.2.1.1, 2.3.4, 2.3.2.1, 2.3.4.2) have been reported to date. Direct or close contact with sick or dead poultry and visiting a live poultry market are the major risk factors for illness due to H5N1 virus infection (Kandun et al., 2008; Mounts et al., 1999; Van Kerkhove et al., 2011; Zhou et al., 2009). Detection of H5N1 cases has depended largely on recognition of hospitalized suspect cases (Abdel-Ghafar et al., 2008; Uyeki, 2009). More rarely, human H5N1 cases with milder clinical illness have been reported in sentinel surveillance settings (Brooks et al., 2009). Although limited human-to-human transmission has been documented on several occasions (Kandun et al., 2008; Ungchusak et al., 2005; Wang et al., 2008a), H5N1 viruses currently circulating among birds lack the ability for sustained transmission and spread among humans. Nevertheless, the recent demonstration that experimental reassortment with A(H1N1)pdm09 virus and/or acquisition of mutations can confer respiratory droplet transmissibility of H5N1 viruses in a ferret model, support the pandemic potential of H5N1 viruses (Herfst et al., 2012; Imai et al., 2012).

Most H5N1 human infections occur in children and young adults; the median age of cases reported is 19 years (WHO, 2012). In hospitalized cases, high pharyngeal virus replication, low peripheral T cell counts and high plasma levels of inflammatory cytokines and chemokines were associated with fatal disease outcome (de Jong et al., 2006). Extrapulmonary spread of the virus to the blood, central nervous system and gastro-intestinal tract has been documented in critically ill patients with fatal outcome (de Jong et al., 2005, 2006). Overall, H5N1 pathogenesis is characterized by high and prolonged viral shedding and hypercytokinemia, a phenomenon associated with lung injury.

The characteristics of the early host innate response to H5N1 virus infection, relative to infection with seasonal influenza viruses have been well studied in multiple animal models and in vitro and ex vivo cell culture systems and will be dealt with in detail in other articles in this issue (articles by Peiris, Katze, Belser and Tumpey). In contrast, there is only very limited information about the adaptive immune response to H5N1 infection in humans and relatively little information from animal models which have largely focused on the pathogenesis of fatal H5N1 disease. Here we review the available information on the adaptive immune response to H5N1 virus infection in humans and laboratory animal models.

2. Antibody responses in H5N1 virus infected persons

2.1. Methods of antibody detection

The development of strain-specific serum antibody responses to the hemagglutinin (HA) protein is regarded not only as a serologic marker of infection with influenza viruses but also is the primary immune correlate of protection against antigenically closely related influenza viruses. HA-specific antibodies that recognize epitopes on the globular head of HA can neutralize virus infectivity by inhibiting attachment of the virus to sialic acid containing glycan receptors and entry into host cells. The ability to detect anti-H5 HA specific antibodies and/or antibodies that are cross-reactive with H5 HA depends on the

method used. The hemagglutination-inhibition (HAI) assay is a convenient method to detect and quantify antibodies that bind to the globular head of HA and inhibit virus binding to receptors. In this way, the HAI is a surrogate method for the detection of neutralizing antibodies (Katz et al., 2011; WHO, 2011). In the HAI assay, HA-specific antibodies in sera inhibit the virus binding to and subsequent agglutination of red blood cells which with few exceptions are derived from avian species. On the other hand, virus neutralization assays directly detect functional antibodies that inhibit virus entry or otherwise block virus replication. The 2-day microneutralization assay detects only antibody directed against the HA, whereas virus neutralization assays with longer incubation periods and plaque reduction neutralization assays which both involve multiple cycles of virus replication may detect antibodies to the other surface glycoprotein of influenza A viruses, the neuraminidase (NA), which is known to block virus egress from infected cells and therefore cell-to-cell virus spread (Hassantoufighi et al., 2010). In general direct binding assays such as enzyme-linked immunosorbent assay (ELISA) lack specificity for detection of subtype- and strain-specific anti-HA responses, particularly in sera from adults (Burlington et al., 1983; Rowe et al., 1999). An early challenge in detecting human antibody responses to H5N1 virus infection was the poor sensitivity of traditional HI assays using avian erythrocytes (Rowe et al., 1999). Other studies had previously reported the inability to detect HA-specific antibodies to avian influenza viruses in experimental infections of mammalian species using the traditional HI assay, even in cases where infection was confirmed by virus isolation (Beare and Webster, 1991; Hinshaw et al., 1981). Using a virus microneutralization (MN) assay, Rowe et al., demonstrated the presence of neutralizing antibodies in convalescent sera from virologically confirmed H5N1 cases identified during the 1997 Hong Kong outbreak (Rowe et al., 1999). Direct comparison of the MN assay and the HI assay using avian red blood cells demonstrated that the MN assay was more sensitive in detecting antibodies in human sera. Subsequently, the use of horse red blood cells was shown to substantially improve the sensitivity of the HAI for the detection of anti-H5 HA antibodies in human post-infection and post-vaccination sera (Stephenson et al., 2003, 2004). Whereas turkey and chicken RBCs possess surface sialic acid receptors possessing both α2,3 and α2,6– galactose linkages, horse RBCs predominantly express sialic acid receptors containing α2,3– galactose linkages, which are preferentially recognized by H5N1 and most other avian influenza viruses (Ito et al., 1997). Goose RBCs have been proposed as an alternate to horse RBCs for improved HAI assay sensitivity for detection of anti-H5 antibodies (Louisirirotchanakul et al., 2007). However, HAI assays using horse RBCs or those from other species require further evaluation for their relative sensitivity and specificity for detection of antibodies in human sera against different genetic clades of H5N1 viruses.

To overcome the need for live virus in neutralization assays and heightened biocontainment requirements, neutralization assays using retroviral vectors pseudotyped with H5 HA have been developed (Nefkens et al., 2007; Temperton et al., 2007; Wang et al., 2008c). Pseudotype neutralization titers typically cover a broader range than traditional virus neutralization and HI assays and have been used to detect broadly cross-reactive HA stem region antibodies which are poorly or not detected by MN and HI assays (Corti et al., 2010; Ding et al., 2011; Sui et al., 2009; Thomson et al., 2012). Finally, it should be recognized that substantial inter-laboratory variability exists in technical aspects and the determination and calculation of titer endpoints for serological assays for detection of H5N1 antibody

responses and therefore only within laboratory (and optimally within assay) comparisons among titers are valid. An international antibody standard that reduces inter-laboratory variability has been developed for clade 1 H5N1; additional antibody standards for clade 2 viruses are needed (Stephenson et al., 2009).

2.2. Kinetics of the serum antibody responses in H5N1 virus-infected persons

Using the MN assay, the kinetics of the primary antibody response to the 1997 clade 0 H5N1 virus was examined in 16 H5N1 confirmed cases (8 children <14 years and 8 adults aged 19–60 years). With two exceptions, serum neutralizing antibodies to A/Hong Kong/156/97 virus were detected in convalescent sera from 15 of 16 cases collected 14 or more days post symptom onset (pso). One 60 year old case had detectable neutralizing antibody on day 6 pso (titer of 80) while another adult with an underlying chronic illness had no neutralizing antibody detected in sera collected on day 23 pso. Neutralizing antibody titers in sera from either H5N1 virus-infected children or adults collected between 14 and 26 days pso ranged from 80 to 2560 and were at least 640 by 3 weeks pso (Katz et al., 1999), based on and the earlier convention of identifying the initial serum antibody dilution in the assay as 1:20 (Rowe et al., 1999). Therefore, in this study, the kinetics of the antibody response to the avian H5N1 virus was generally similar to that seen for primary responses to human influenza A H3N2 and (H1N1)pdm09 pandemic viruses (Miller et al., 2010; Murphy et al., 1973; Veguilla et al., 2011). Although an H5 HA-specific ELISA was found to lack specificity for the detection of anti-HA antibody in adults, the ELISA was highly sensitive and specific for detection of H5-specific antibody in children. H5 HA-specific IgM and IgG were detected in sera collected 11 or more days pso in 7 of 8 and 8 of 8 children tested, respectively (Katz et al., 1999; Rowe et al., 1999).

There have been limited studies using appropriate methods to detect serum antibody response in persons virologically confirmed to be infected with H5N1 viruses that have circulated since 2003. Kitpathi et al. reported on antibody responses in 8 Thai patients, including four fatal cases infected with a clade 1.1 H5N1 viruses between 2004 and 2006. As observed for the 1997 clade 0 H5N1 antibody response, a four-fold or greater rise in serum neutralizing antibody generally was detected in sera collected 15 or more days pso, although one adult patient mounted a robust response (neutralizing antibody titer of 320 with initial serum dilution of 1:10) by day 5 pso; one other adult patient had no detectable response by day 27 pso. In the four survivors, neutralizing antibody was detected for 3 to 4 years after infection. Among these, one pediatric and one adult case, with neutralizing antibody titers of 640–1280 approximately 3 weeks after infection, demonstrated a ≥8-fold drop in titer by 5 and 18 months, respectively. Antibodies detected by HAI assays using either horse or goose RBC showed similar titers and trends. Similarly, Cheng et al. (2008) provided a single case report of an adult infected with a clade 2.3.4 virus in 2006 for whom a peak neutralizing antibody titer was detected about 4 weeks pso, with an 8-fold waning of the response approximately 7 months later. In a study of 11 H5N1 Cambodian patients with severe disease, peak MN titers generally were detected 2–4 weeks pso and then declined gradually by approximately 4-fold, but were still detected at titers of ≥80 in 4 surviving patients 2 years after infection (Buchy et al., 2010). The geometric mean MN titer from 4 patients at 4–8 weeks pso was 540. In contrast, persons with asymptomatic or

mild, unreported illness that were identified through sero-epidemiological surveys in H5N1 endemic areas, had a GM MN titer of 149 4–8 weeks after exposure; less than half of these individuals had detectable antibody 10–11 months after exposure (Buchy et al., 2010). These limited data suggest that virologically confirmed H5N1 virus infected persons who experienced severe disease had higher peak H5 antibody titers than those with mild or asymptomatic infections, and that with similar waning of the response, severely ill patients retained H5-specific antibody for at least 2 years.

2.3. Specificity of the antibody responses in H5N1 infected persons

The characterization of monoclonal antibodies (mAbs) generated from memory B cells of survivors of H5N1 virus infection has provided an understanding of the fine specificity and repertoire of the human antibody response to H5N1 virus infection. In addition, the ability of such mAbs to prevent or treat H5N1 disease in mice, suggests a strategy for alternate therapies for human H5N1 cases. Simmons et al. (2007) generated mAbs from Vietnamese survivors of H5N1 clade 1.1 virus infection through the generation and single-cell cloning of Epstein–Barr Virus transformed memory B cells. Four mAbs that neutralized A/Vietnam/1203/2004 virus in vitro were characterized; two mAbs neutralized only clade 1 virus while the other two cross-neutralized clade 1 and 2 viruses. While two mAbs also elicited cross-clade inhibition of pulmonary virus replication and inflammation and limited extrapulmonary virus spread, another was effective in vivo but did not neutralize virus in vitro, suggesting that its mechanism of action required additional soluble factors or cell-based in vivo factors for its effect as has been described previously (Gerhard, 2001). In one study, an anti-HA mouse mAb complement component C1q together with additional serum factors was shown to enhance in vitro neutralization activity (Feng et al., 2002). Two of the above-mentioned H5N1 mAbs were characterized further using a whole genome phage display library (GFPDL) in *Escherichia coli*, expressing fragments of 15–350 amino acids in length representing all known proteins of A/Vietnam/1203/2004 virus and were shown to recognize an HA1 peptide encompassing the first 223 amino acids of HA1; the mAb with broader in vivo reactivity demonstrated a 50-fold higher binding affinity for the HA1 peptide compared with another mAb which demonstrated only clade 1 specific protective efficacy in mice, suggesting that both specificity and avidity may contribute to effective in vivo neutralization of H5N1 virus (Khurana et al., 2009). Another mAb generated from memory B cells of a survivor of H5N1 clade 2.3.4 was also shown to exhibit broad cross-clade (clades 0–9 with the exception of clade 7.2) neutralizing activity as assessed by the pseudotype neutralization assay. This mAb exhibited prophylactic and therapeutic efficacy against clade 2.3.4 and clade 1 viruses in mice and recognized a conformational epitope at the tip of the globular head encompassing HA1 residues 118, 121, 161, 164 and 167 (H5 numbering) which is highly conserved among most H5N1 clades (Hu et al., 2012). As an alternate strategy, Kashyap et al. (2008) developed a combinatorial antibody library from bone marrow derived memory B cells from Turkish survivors of H5N1 clade 2.2 virus infection. Over 300 unique antibodies were identified of which approximately 50% were directed against the H5N1 HA protein. Three of four HA-specific mAbs selected for further characterization showed broad neutralization activity against clade 1 and 2 H5N1 viruses as well as viruses of the H1 subtype. One antibody not only neutralized H5N1 viruses but also former seasonal and influenza A(H1N1)pdm09 virus, and exhibited both prophylactic

and therapeutic activity in protecting mice from a lethal A(H1N1)pdm09 virus infection in mice (Kashyap et al., 2010). Using comparative sequence alignment, the epitope recognized by such subtype cross-reactive mAb was presumptively identified as a highly conserved pocket in HA2 formed by non-contiguous amino acid residues 19–56 and encompassing the fusion peptide, similar to the previously identified target of broadly neutralizing influenza subtype cross-reactive antibodies in non-H5N1 immune human donors (Ekiert et al., 2009; Sui et al., 2009). Two baculovirus-expressed human mAbs generated by screening a Fab antibody phage library derived from a patient recovered from a clade 2.3 H5N1 virus infection recognized distinct epitopes within HA1 (Sun et al., 2009). One recombinant mAb neutralized clade 0, clade 1 and clade 2 H5N1 viruses recognized a linear epitope comprising residues 116–123 of HA1, while the other mAb neutralized only clade 2 viruses and recognized an overlapping but non-contiguous conformational epitope that also included residues Lys152 and Asn155 of HA1. Both antibodies when administered prophylactically protected mice from lethal clade 2.3 H5N1 virus infection. Whole-genome-fragment phage display libraries (GFPDL) technology has been used to investigate the specificity of antibodies in polyclonal sera pooled from five Vietnamese survivors of clade 1 H5N1 virus infection. Pooled polyclonal sera reacted with large HA1 peptides encompassing antigenic sites A–E as defined for human H3N2 viruses (Wiley et al., 1981) as well as peptides encompassing the C-terminal of HA1 and the HA2 fusion peptide region. Furthermore, this study identified antibodies in convalescent sera that recognized the NA in the proximity of the enzyme active site, the ectodomain of M2 (M2e) as well as the PB1-F2 protein. Epitopes within PA, NP and M1 were also identified but were also recognized by sera from a control group with no known H5N1 virus exposure and therefore likely represented cross-reactive antibody responses induced by exposure to human influenza A virus subtypes. Antibodies against NA have been shown to reduce seasonal influenza virus replication and disease severity in humans, whereas anti-M2e antibodies contribute to viral clearance and enhanced survival against various subtypes including sublethal challenge with H5N1 viruses in animal models (Couch et al., 1974; Murphy et al., 1972; Tompkins et al., 2007; Wang et al., 2008b). Interestingly, mAbs derived from IgG^+ memory B cells from non-H5N1 immune human donors that recognized a conserved conformational epitope within the M2e, demonstrated the ability to protect mice from lethal challenge with a clade 1 H5N1 virus (Grandea et al., 2010). A further understanding of the possible contribution of antibody recognizing M2, NA or PB1-F2 in amelioration or immunopathology of human H5N1 virus infection is needed.

2.4. H5N1 subtype cross-reactive antibody responses

Evidence from limited seroprevalence studies using HAI or VN assays suggests that overall, there is little or no cross-reactive serum antibody against the H5 subtype among humans. Even in populations with occupational exposure to H5N1 virus infected birds, or persons residing in areas that experienced H5N1 outbreaks in poultry and human H5N1 cases, studies conducted since 2003 and using WHO testing recommendations and criteria for seropositivity reveal low seroprevalence of anti-H5 antibody with relatively few asymptomatic cases detected (Van Kerkhove et al., 2011). Early studies with the clade 0 H5N1 viruses suggested that cross-reactive MN antibody to H5N1 viruses were more frequently detected in adults ≥60 years and were rarely or not detected in younger persons (Rowe et al., 1999). A subsequent study assessing the immunogenicity of H5N1 clade 1

vaccine also found higher base-line (pre-vaccination) levels of HAI and VN H5 antibodies among persons >60 years compared with younger adults (18–60 years) (Leroux-Roels et al., 2009). Several studies have demonstrated an increase in serum cross-reactive antibodies against H5N1 viruses in a minority of persons following receipt of seasonal inactivated influenza vaccine using either VN assays with low input virus (Gioia et al., 2008) or a PN assay which more readily detects heterosubtypic antibodies, including those that bind to the stem region of the HA (Ding et al., 2011; Garcia et al., 2009). Indeed, Corti et al. (2010) isolated mAbs from four adults immunized with seasonal influenza vaccines that neutralized H5 pseudotype virus, the majority of which recognized a pre-fusion epitope in the stem region of H5 HA. Antibodies that cross-react with avian N1 have also been detected at low frequency in sera from a small group of human donors. Among 38 individuals tested, 31 had detectable NA-inhibition titers of ≥20 (range 20–320) against human N1 (A/New Caledonia/20/99: H1N1), whereas approximately a quarter of persons tested had low levels (range 20–80) of functional NA-inhibition antibody that recognized the avian N1 from H5N1 virus (Sandbulte et al., 2007). To what extent, if any, the variable and modest levels of heterosubtypic antibodies recognizing H5N1 HA or NA that arise in humans following seasonal influenza vaccination or infection contribute to amelioration of H5N1 disease is unknown. Such cross-reactive immunity may be more pronounced in older persons with repeated exposure to seasonal influenza viruses. If cross-reactive immunity offers some cross-protection against H5N1 viruses in older adults, this may be one reason for the relatively younger mean age of H5N1 cases (WHO, 2012). However other factors including possible age-related differences in exposure, healthcare seeking behavior and overall younger age structure of countries with endemic H5N1 virus circulation may also contribute. In any case, these studies high-light the need for additional studies to further assess the impact of cross-reactive antibody responses on H5N1 virus infection.

3. Antibody mediated immunity in animal models of H5N1 virus infection

Several mammalian models of H5N1 infection have been developed and used, primarily to better understand the virus–host interactions contributing to H5N1 virus pathogenesis and to assess the immunogenicity and protective efficacy of a wide spectrum of countermeasures against H5N1 viruses (for reviews see Belser et al. (2009) for pathogenesis studies; Bodewes et al. (2010) for vaccine strategies). Since the mouse and ferret are the most commonly used H5N1 virus animal models, they will be the focus of this section. The use of these models generally focuses on the recapitulation of severe human H5N1 disease, and typically involves high dose challenge of animals with lethal outcomes, usually before the development of the antibody response. Furthermore, highly lethal H5N1 viruses typically disseminate to multiple extra-pulmonary organs in mice and ferrets and infection of the central nervous system is associated with lethal outcome. Depletion of circulating lymphocytes is an early feature of H5N1 virus lethal infection in mice and ferrets (Maines et al., 2005; Tumpey et al., 2000). Reduced numbers of CD4+ and CD8+ T cells in lungs and mediastinal lymph nodes, and significantly greater levels of apoptotic leukocytes, particularly in germinal centers in the spleen were a feature of lethal H5N1 virus infection in BALB/c mice (Tumpey et al., 2000). Thus, in lethal infections, development of adaptive immune response may be substantially compromised. Therefore, opportunities to study

antibody responses following H5N1 virus infection in animals generally are limited to low dose sub-lethal challenge or the use of H5N1 viruses which do not cause lethal disease.

BALB/c mice infected with a high dose (10^6 50% egg infectious doses (EID_{50}) of a non-lethal clade 0 H5N1 virus (A/Hong Kong/486/97) generally achieved serum HAI titers of ≥80 about 14 days post-infection (Katz et al., 2000). By reducing the infecting dose, a range of serum HAI titers (≤20 to ≥80) were achieved in surviving animals. Subsequent challenge of mice with a related but highly lethal clade 0 H5N1 virus demonstrated that pre-challenge serum HAI titers of ≥40 were associated with protection from death in ≥90% of animals (Katz et al., 2000). Similarly, serum neutralizing antibody titers of 40–160 were detected in mice surviving intranasal infection with lower doses (10^3 EID_{50}) of clade 2.1 and 2.3 viruses (Lipatov et al., 2009). Infection of mice with a non-lethal 2003 clade 1 virus (A/Hong Kong/213/2003) also demonstrated robust serum IgG1 and IgG2a as well as nasal wash IgG and IgA anti-H5 HA antibodies (Lu et al., 2006). Antigenic sites have been identified for H3 (sites A–E) and H1 (sites Sa, Sb, Ca and Cb) subtypes based on genetic analyses of antigenic drift variants and the localization of point mutations within antigenic regions in escape mutant viruses selected with mAbs (Caton et al., 1982; Wiley et al., 1981). In the same way, epitopes on the H5 HA recognized by antibodies were characterized by selecting escape mutants with a panel of mAbs generated from mice infected and then inoculated subcutaneously with clade 1 H5N1 virus. The substitutions identified in escape mutants mapped to antigenic sites corresponding to site B as identified in H3 viruses (overlapping with site Sa of H1 viruses) or site A. Substitutions selected in escape mutants from a third group of mAbs appeared to overlap both site A and B (Kaverin et al., 2007).

Intranasal delivery of a single high dose (50 mg/kg) of mouse polymeric H5-HA specific IgA mAb administered up to 72 h prior to H5N1 virus infection, protected 100% of animals from death and inhibited virus replication in the trachea, lungs and brains, demonstrating the potential protective effect of mucosal IgA responses against H5N1 viruses (Ye et al., 2010). The IgA mAb also exhibited broad HAI and neutralizing reactivity against multiple clades and sub-clades of H5N1 viruses. Passive transfer of murine N1 NA-specific IgG mAb which demonstrated NA inhibition activity against both clade 1 and 2 viruses, provided partial protection (50%) of mice from a lethal clade 1 H5N1virus infection, reducing lung viral titers by approximately 10-fold (Shoji et al., 2011).

The importance of B cell immunity to protection from H5N1 virus infection was demonstrated by comparing the outcome of lethal H5N1 virus infection in C57Bl/6 mice deficient in antibodies (μMT mice). Immunocompetent and μMT mice infected with a low pathogenic H5N2 were subsequently challenged with a genetically closely related HPAI H5N1 virus. Immunocompetent mice which had robust serum HAI antibody titers at day 14 post infection and detectable antibodies at the time of challenge approximately 7 weeks later, were completely protected from lethal disease. In contrast, μMT mice succumbed to lethal infection with similar kinetics to naïve animals (Droebner et al., 2008). B cell responses may also contribute to the primary response to H5N1 virus infection. Mice deficient in IL-17 infected with H5N1 virus exhibited a decreased frequency and number of B220+ B cells in the lungs on day 5 post-infection and displayed greater pulmonary immunopathology and succumbed to lethal challenge more rapidly than immunocompetent

controls (Wang et al., 2011). Other studies suggest a role for pre-existing natural (IgM) responses in the early antiviral host responses and a role for neutralizing IgG antibodies in clearing virus infection in mice (Baumgarth et al., 2000; Palladino et al., 1995).

Ferrets are now generally accepted to be the most relevant laboratory animal model for the study of influenza virus pathogenesis and transmission. However, there remains limited data on the kinetics of the antibody and B cell response to infection with H5N1 viruses, in part due to the high lethality of many clade 1 and 2 viruses and the lower post-infection HAI titers achieved compared with primary infection of ferrets with seasonal H1 and H3 viruses, when using avian RBC for detection. The use of horse RBC to detect HAI antibodies may enhance the titers detected 14 or more days after infection with H5N1 viruses such that they approach the high HAI titers detected with avian RBC following infection of ferrets with human seasonal influenza viruses. For example, ferrets infected with clade 0 or A/Hong Kong/213/2003 (clade 1) that survived infection achieved high HAI titers (1280) using horse RBC (Maines et al., 2006). The HAI antibody responses in animals that survive a clade 1 virus infection appeared to be more variable, perhaps being both strain and dose dependent (Jackson et al., 2009; Maines et al., 2006; Middleton et al., 2009). Treatment of H5N1virus-infected ferrets with antiviral drug Oseltamivir, promoted survival of ferrets and enabled evaluation of serum antibody responses. Ferrets infected with a low dose of clade 1 virus 3 weeks earlier exhibited low homologous titers (20–40) using a chicken RBC HAI assay, but titers were 4–8-fold higher when measured using a related H5N1 virus, A/Hong Kong/213/2003 (Govorkova et al., 2007). This virus has a unique substitution in the globular head of HA (S223N) that alters receptor binding properties such that when used as an antigen in HAI tests it yields substantially higher titers when avian RBC are used (Hoffmann et al., 2005). Using a VN assay, Lipatov evaluated serum neutralizing antibody titers in ferrets infected intranasally with 10^6 EID_{50} of virus. Animals infected with clade 2.1, 2.2 and 2.3 viruses 15 days earlier had titers ranging from 80 to 2560. Interestingly, ferrets fed meat of chickens infected with clade 2.3 virus also seroconverted achieving neutralizing antibody titers of 160–320. These animals exhibited mild respiratory disease and had virus recovered from nasal washes. These animals were infected presumably through pharyngeal contact with high concentrations of virus in the infected meat (Lipatov et al., 2009). Taken together these results indicate that titers in ferrets surviving H5N1 virus may be generally robust depending on the dose and strain of infecting virus, as long as the appropriate methods are used for detection. There are no studies exploring B cell responses in ferrets infected with wildtype H5N1 viruses. However, some information on the kinetics of the IgM and IgG response can be gleaned from the study of ferrets infected intranasally with live attenuated cold-adapted (ca) vaccine viruses. H5N1 virus-specific IgM antibody secreting cells (ASC) were measured in lymphocyte populations isolated from paratracheal lymph nodes on days 5 and 10 post-infection and were of greater magnitude following infection with ca A/Hong Kong/213/2003 virus vaccine compared with ca A/Vietnam/1203/2004 virus vaccine (Cheng et al., 2009). IgG ASC were higher on day 10 compared with day 5 and, similar to IgM responses, they were of greater magnitude following infection of animals with the ca A/Hong Kong/213/2003 virus vaccine. The relative magnitude of the ASC responses correlated with the level of serum neutralizing antibodies. These results suggest that primary infection of ferrets with a live attenuated H5N1 virus results in

the induction of ASC responses in a similar timeframe to ASC responses observed in humans administered seasonal trivalent LAIV viruses (Sasaki et al., 2007). Furthermore, they demonstrate the ability to detect cellular responses in ferrets as an approach to expand the utility of this model.

4. Cell-mediated immune responses

Upon infection with influenza viruses, virus-specific CD4+ and CD8+ T cell responses are induced. CD4+ T cells comprise the T helper cell subset whereas the CD8+ T cells comprise cytotoxic T lymphocytes (CTL). T helper cells play an important role in the adaptive immune response and are essential for the differentiation and proliferation of antibody producing and memory B cells and the induction of CTL responses. CTL recognize and eliminate virus-infected cells and thus contribute to protective immunity. The role of cell mediated immune response in A/H5N1 influenza virus infection is poorly understood. However, since the majority of T cells induced by infection with seasonal A/H3N2 and A/H1N1 influenza viruses are directed to relatively conserved proteins like the M1 protein and the nucleoprotein, it was anticipated that these cells would cross-react with influenza A viruses of other subtypes, including those of the H5N1 subtype. Assessing the extent of cross-reactivity of human influenza virus specific T cells with H5N1 viruses has been the topic of various studies (Cusick et al., 2009; Jameson et al., 1999; Kreijtz et al., 2008; Lee et al., 2008; Roti et al., 2008). Using synthetic peptides representing T cell epitopes of human influenza A/H3N2 and A/H1N1 viruses and their variants derived from amino acid sequence of H5N1 viruses, it was shown that CD4+ and CD8+ T cells directed to seasonal influenza viruses could cross-react with H5N1 viruses and respond by lysing target cells and/or producing IFN-γ. These results were confirmed by using stimulator/target cells that were either transfected with plasmids expressing an influenza virus gene, infected with a viral vector driving the expression of influenza virus proteins or infected with influenza virus of various subtypes including H5N1. Of interest, indeed the majority of CD4+ and CD8+ T cells were directed to the NP and the M1 protein, but also T cells of both subsets were detected against the polymerase proteins PB1, PB2 and PA. However, the major membrane protein, the hemagglutinin (HA) and neuraminidase (NA) were targeted by CD4+ T cells only (Lee et al., 2008). This may be explained by the relative inefficient processing of these antigens by the endogenous route of antigen processing and presentation, which may be in favor of antigens that are more abundantly present in the cytosol and peptides derived from these proteins may outcompete those derived from HA and NA for presentation by MHC class I molecules. In HLA-DR1 transgenic mice, infection with a human A(H1N1) virus induced HA- and NA-specific CD4+ T cells that cross reacted with corresponding epitopes present in an H5N1 virus, confirming that cross-reactive CD4+ T cells also can be induced to HA and NA proteins (Richards et al., 2009; Roti et al., 2008).

Although the role of virus specific T cells in heterosubtypic immunity to influenza virus infection is relatively well documented (for reviews see Grebe et al. (2008), Hillaire et al. (2011a), Rimmelzwaan and McElhaney (2008)) evidence for protection against H5N1 viruses is sparse and stems predominantly from animal models. It was shown that mice that were primed by infection with an influenza virus of the H9N2 subtype were protected against challenge infection with highly pathogenic avian influenza virus

A/Hong Kong/156/97 (H5N1). Even in μMT mice that lack antibodies partial protection was observed indicating that cell mediated immune responses were the basis for the observed protection (O'Neill et al., 2000). Furthermore, after infection with a human A(H3N2) influenza virus, mice were protected from infection with the more recent H5N1 strain A/Indonesia/5/05 (Kreijtz et al., 2009). In the latter study, the protection correlated with anamnestic virus-specific CD8+ T cell responses detected after the challenge infection with the H5N1 strain. In chickens, heterosubtypic immunity to lethal infection with HPAI H5N1 virus induced by primary infection with a virus of the H9N2 subtype correlated with virus-specific T cell responses, in particular CD8+ T cell responses. By adoptive transfer and depletion studies it was demonstrated that indeed these cells were responsible for affording protection against infection with H5N1 strain. Of note, it was demonstrated recently that adoptive transfer of T cells obtained from donor mice that were infected with a human A(H3N2) virus afforded recipients protection against infection with A(H1N1)pdm09 virus (Hillaire et al., 2011b). Thus the presence of cross-reactive T cells induced by infection with seasonal H3N2 and H1N1 influenza virus may mitigate the severity of illness caused by infection with H5N1 viruses. This may also explain why there is disproportionate age distribution of H5N1 cases (Smallman-Raynor and Cliff, 2007). Younger subjects are more likely to develop severe disease, which may be explained by less exposure to seasonal influenza A viruses and subsequently insufficient induction of cross-reactive T cell responses, although other confounding factors cannot be excluded.

Of interest, recently T cells were detected in 3.2% of subjects at high risk for exposure to H5N1 viruses in Vietnam (but without clinical symptoms typical for H5N1 infection) that reacted with peptides derived from the HA of H5N1 influenza viruses and not with those derived from the HA of seasonal influenza H3N2 and H1N1 viruses (Powell et al., 2012). In patients that had recovered from (severe) infection this proportion was higher (16%) whereas none of control subjects displayed reactivity with H5 derived peptides. However, the presence of H5 reactive T cells correlated poorly with the presence of H5 specific antibodies measured with the horse erythrocytes HAI assay. This discrepancy is poorly understood, although it was suggested that differences in persistence of T cells and antibodies may partially account for it. Also in this high risk Vietnamese cohort highly cross-reactive CD4+ and CD8+ T cells were detected specific for peptides derived from relatively conserved internal and polymerase proteins.

The magnitude of the T cell response to H5N1 virus and the likelihood to detect virus-specific T cells after infection may also depend on the rate of virus replication and the size of the inoculum. It was shown in mice (Hatta et al., 2010) that inoculation with a high dose of a fast replicating virus prohibited the induction of strong virus-specific CD8+ T cell responses and immune control of the infection, whereas infection with a lower dose of a slower replicating virus allowed the induction of robust virus-specific CD8+ T cell responses. Alternatively, inhibition of virus replication by administration of neuraminidase inhibitor oseltamivir also resulted in the induction of strong virus specific CD8+ T cell responses, which further contributed to the control of the infection. The differential kinetics and magnitude of virus replication of these H5N1 viruses in mice is known to be associated with a single amino acid substitution in the PB2 gene (E627K) (Hatta et al., 2001). This substitution was also associated with early impaired T cell receptor signaling and T cell

activation in the lungs of H5N1 virus-infected mice (Fornek et al., 2009). Although a precise mechanism for this effect was not identified, as mentioned earlier, 1997 H5N1 viruses bearing the PB2 (E627K) are known to cause T cell depletion in mice (Tumpey et al., 2000).

5. Conclusions

Despite ongoing epizootics of H5N1 in multiple countries and widespread human contact with infected birds, documented transmission of H5N1 viruses to humans remains an infrequent event. Nevertheless, the high case fatality ratio among H5N1 virus-infected persons, and the continuing genetic evolution of H5N1 viruses with associated risk for human adaptation, underscore the public health importance of better understanding both virus and host factors that contribute to H5N1 virus disease, and in particular, host immune responses that may protect against disease or infection. This review has highlighted the fact that detailed knowledge of the adaptive immune response to H5N1 virus infection remains relatively sparse and many questions remain.

The fact that most H5N1 cases occurred in children and young adults has led some to speculate that older adults are less susceptible to H5N1 virus infection or disease because of pre-existing cross-reactive immunity acquired through exposure to seasonal influenza A viruses, although other reasons including age-related differences in poultry contact, health-care seeking behavior and overall population demographics cannot be excluded. While there is growing evidence that B and T cell responses elicited by human influenza A viruses cross-react to varying degrees with H5N1 viruses, the ability of such cross-reactive immune effectors to ameliorate influenza virus infection in humans is not known. The infrequency of human H5N1 virus infection together with the high case fatality ratio makes the study of protective immunity to H5N1 infection in humans unfeasible. However, additional emphasis on better understanding the capacity of subtype cross-reactive adaptive responses to protect against seasonal influenza A virus disease or infection is warranted. Human experimental challenge systems provide one opportunity to identify cross-reactive immune responses that correlate with resistance to seasonal influenza viruses, although this approach by necessity can only investigate the consequences for mild disease (Killingley et al., 2011; Wilkinson et al., 2012). Studies in animals examining the roles of cross-reactive antibody and T cell responses in both non-lethal and lethal disease may improve our understanding of their relative contribution to protection against H5N1 viruses. The development of a broader range of immunological reagents for the ferret, widely considered to be the optimal animal model for the study of human influenza disease, would facilitate such studies.

Limited studies summarized here suggest that although initially robust, there is substantial waning of the serum antibody responses in survivors of H5N1 virus infection. In contrast, following primary infection with seasonal influenza viruses, young children retained peak titers for over one year (Wright et al., 1977). Furthermore, the 2009 H1N1 pandemic demonstrated the overall longevity of serum antibody responses in humans who had been infected more than 60 years earlier with a related H1N1 virus, and presumably maintained, at least in part, due to successive boosting through subsequent influenza A virus infections (Hancock et al., 2009). A better understanding of the immune response to H5N1 virus infection in individuals with varying clinical presentations is needed. Ideally,

prospective, longitudinal studies among populations exposed to H5N1 virus infected birds could assess the frequency RT-PCR-confirmed infection and investigate both serological and cell-mediated responses over time.

The characterization of mAbs derived from survivors of H5N1 virus infection offers hope for immunotherapy as an alternate intervention strategy in hospitalized H5N1 virus-infected patients. In three separate severely ill cases, immunotherapy with convalescent serum or plasma was administered and all patients recovered however, the true effect of H5N1 convalescent sera was unclear since other therapies were coadministered (Uyeki, 2009). The identification of antibodies that recognize highly conserved peptides within HA2, M2e and PB1-F2 proteins of H5N1 viruses has formed the basis for the development of a rapid test strategy for the detection of H5N1 virus infections (Khurana et al., 2011). Further knowledge of the adaptive immune response to H5N1 virus infection in humans is needed to expand our abilities to detect, treat and ultimately prevent this rare, but highly lethal disease. In addition, this knowledge may also help mitigate the impact of larger outbreaks these viruses may cause in the future.

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