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Immune Responses to Avian Influenza Viruses

Marios Koutsakos,* Katherine Kedzierska,* and Kanta Subbarao*†

Avian influenza A viruses (IAVs) naturally infect different avian species, and aquatic birds are their natural reservoir. Sporadically, avian IAVs can be transmitted to humans, and some, such as H5N1 and H7N9 viruses, cause severe disease in humans. Antigenically novel avian influenza viruses that infect and cause disease in humans pose a potential pandemic threat if they are able to spread efficiently from person to person. The immune response of the host is crucial in determining disease pathogenesis and is the basis for the development of control strategies. In this review, we examine the innate and adaptive immune responses to avian influenza viruses and their role in disease and recovery. Furthermore, we discuss the progress in developing vaccines against avian IAVs and summarize obstacles in designing universal and pandemic influenza vaccines. The Journal of Immunology, 2019, 202: 382–391.

Influenza A viruses (IAVs) infect humans and several animal species, including pigs, horses, dogs, and marine mammals and birds, but waterfowl and shorebirds are their natural hosts. IAVs are classified into different subtypes based on the antigenicity of their major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). IAVs bearing 16 different HA and 9 different NA subtypes have been isolated in various combinations from waterfowl and shorebirds. These infections are usually asymptomatic, and the virus is shed from the gastrointestinal tract. Occasionally, avian IAVs spill over into other hosts, including other avian species, such as chickens or turkeys, or mammals, in which they can cause a range of consequences, from asymptomatic infection to severe disease and death.

The reason for concern about avian IAVs infecting humans is their pandemic potential. An influenza pandemic can ensue if three conditions are met: the population lacks pre-existing immunity to the novel virus, the virus causes disease, and the virus is able to spread efficiently from person to person through sustained chains of transmission, causing community-wide outbreaks. Although seasonal influenza epidemics are caused by influenza A and B viruses, influenza B viruses do not pose a pandemic threat because they lack a nonhuman reservoir in nature from which novel influenza B viruses can be introduced into humans. Influenza viruses are enveloped viruses with a segmented, negative-sense RNA genome organized in eight gene segments that each encode one or more proteins. Two mechanisms account for the enormous genetic diversity of IAVs: point mutations that occur because the error-prone RNA-dependent RNA polymerase lacks proofreading function, and genetic reassortment, through which a virus can acquire a novel genotype by deriving entire gene segments from two different parent viruses that coinfect a cell. Both of these phenomena occur continuously in nature. The 1957 H2N2 and 1968 H3N2 influenza pandemics were caused by reassortant viruses that derived three or two gene segments, respectively, from an avian IAV and remaining gene segments from a previously circulating human IAV (1, 2). Avian IAVs did not replicate well or cause clinical illness in human subjects who were experimentally infected with avian IAVs (3). Therefore, it was believed that direct human infection with avian IAVs was rare and avian IAVs would infect humans and cause disease only if they reassorted with a human IAV.

In 1997, 18 human infections with avian H5N1 IAV were identified in Hong Kong associated with a range of clinical illness from mild to lethal infections (4). Infected poultry were the source of the virus. The outbreak of human infections was controlled by actions of the agricultural authorities in Hong Kong that included restriction of imports and closure of the live bird markets (5). However, the precursor viruses continued to circulate in wild birds, and the viruses re-emerged in humans in 2004 and continue to cause sporadic human cases (6). The avian H5 IAVs have continued to evolve in nature, with mutations and ongoing reassortment resulting in new genotypes that are now referred to as H5Nx viruses isolated in many countries. To date, 860 human cases and 454 deaths have been reported in 16 countries due to H5 IAVs (7). In 2003 and 2004, human infections with H7N7 and H7N3 avian IAVs were reported in the Netherlands and Canada with genotypes.
few fatalities (8, 9). However, in 2013, a large outbreak of severe human infections caused by low pathogenicity H7N9 avian IAV was reported in China (10). Each year since then, outbreaks of H7N9 infection have been reported in “waves.” The fifth wave, in 2017, was particularly severe (11). In 2017, highly pathogenic (HP) H7N9 viruses emerged in poultry in China and caused at least 25 human infections, but it is not clear whether the clinical illness caused by HP H7N9 viruses was more severe than that caused by low pathogenicity H7N9 viruses (12). To date, 1567 human H7N9 infections and 615 deaths have been reported (13).

Is disease associated with avian IAVs more severe than seasonal influenza?

Human disease with some avian IAVs, such as H5N1 and H7N9, is associated with a much higher case fatality rate than seasonal influenza viruses and pandemic influenza viruses, including those that caused the 1918, 1957, 1968, and 2009 pandemics, although there is controversy about the precise case fatality rate because mild cases are not likely to be counted (14, 15). Some other avian IAV subtypes (e.g., H10N8 and H6N1) have caused fatal infections (16, 17), but the total experience with these subtypes is limited. Still others, including H7N7, H7N3, H7N2, H7N4, and H9N2 viruses, have caused human infections with rare fatalities (8, 9, 18–21).

Clinical findings in H5N1 infection. Most of the laboratory-confirmed cases of H5N1 infection were hospitalized patients with severe illness complicated by acute respiratory distress syndrome (ARDS) and multiorgan failure, although some milder illnesses have been reported (22, 23). Among H5N1-infected patients, high viral load, lymphopenia, unusually high serum levels of IFN-γ inducible protein-10 (IP-10), and high serum levels of inflammatory cytokines and chemokines were associated with a fatal outcome (24). Hemophagocytosis was reported in several cases (23).

Clinical findings in H7N9 infection. As was seen with H5N1 infections, H7N9 infection in humans causes rapidly progressive pneumonia, with leukopenia and lymphopenia and substantially increased serum cytokine and chemokine concentrations in fatal cases (25). In a case series of 111 patients with laboratory-confirmed avian H7N9 IAV infections, 97.3% presented with pneumonia, and lymphopenia was observed in 88.3%. A total of 61.3% had at least one underlying medical condition, and this was the only independent risk factor for the development of ARDS (26). In a subsequent case series of 256 patients, 65.6% were admitted to intensive care (27). Transcriptional profiling in eight patients, all of whom required mechanical ventilation or extracorporeal membrane oxygenation, revealed that cell cycle and neutrophil-related immunity were activated at the acute stage of infection, whereas T cell processes, including selection, differentiation, and stimulation, were activated during the recovery phase (28).

The innate immune system detects viral infections through the recognition of pathogen-associated molecular patterns by pattern recognition receptors. Influenza virus infection is recognized by at least three distinct classes of pattern recognition receptors: TLRs, nucleotide oligomerization domain (NOD)–like receptors and retinoic acid–induced gene I (RIG-I)–like receptors (reviewed in Refs. 29 and 30). The adaptive immune response to an IAV is initiated when the virus interacts with dendritic cells (DCs) that line the upper respiratory tract. The DCs migrate to regional lymph nodes, where the primary or secondary immune response develops. IAV proteins in the cytoplasmic and lysosomal processing pathways of the infected cell present peptides bound to MHC class I and II proteins that are recognized by CD8+ CTLs and CD4+ Th cells, respectively (reviewed in Ref. 31). Infection of DCs by IAVs does not normally result in cell death or productive viral infection, but HP H5N1 avian IAV can cause productive infection and can also kill certain human DC subsets (32, 33).

Adaptive immunity to avian IAVs

As is the case for seasonal IAVs, the viral HA and NA are the primary targets of the Ab response. The application of sophisticated new tools has revealed that there is some cross-reactivity of B and T cell responses to seasonal and avian IAVs (Fig. 1), but their role in ameliorating illness is not known (40).
The viral HA is a trimer with a large globular and immuno-dominant head that includes the receptor binding site and several antigenic sites. Mutations at antigenic sites on the HA head are driven by Ab pressure. The ability to detect Ab directed against the HA of avian IAVs depends on the method(s) used (reviewed in Ref. 40). Widely used assays for detection of anti-HA Ab against seasonal IAVs required modifications to increase their sensitivity to avian IAVs. Briefly, the use of horse RBCs instead of turkey RBCs for hemagglutination inhibition (HAI) assays, more widespread use of micro-neutralization assays, the use of pseudotyped retroviruses instead of infectious influenza viruses in neutralization assays, and the development of assays to detect Abs to the HA stalk or stem are some of the key changes that were made in the assays (reviewed in Ref. 40). The kinetics of the Ab response to the H5N1 virus are generally similar to primary responses to seasonal H3N2 and H1N1pdm09 viruses; a 4-fold or greater increase in serum neutralizing Ab titer was observed ≥15 d after symptom onset (41), and the titers are robust.

The Ab titer in severely infected patients was generally higher than in patients with mild or asymptomatic infection; titers declined significantly over the next 6–8 mo in both groups (42, 43), although Ab remains detectable for a longer period (up to 2 y) in people with severe disease (40, 42). The rapid decay of Abs to avian IAVs contrasts with the persistence of Abs to seasonal IAVs (44, 45), but the mechanism underlying this observation has not been elucidated.

Abs to the HA, with neutralizing or HAI activity, are protective in animal models, as is intranasal delivery of a high dose of a polymeric H5 HA–specific IgA Ab (46). Passive transfer of an anti-NA Ab protected mice from lethal challenge (47). Ferrets are the preferred model for studying influenza disease and transmission, but there are limited data on the B cell response to avian IAVs in ferrets because of a paucity of reagents and the virulence of wild-type H5N1 viruses in these animals. H5N1 virus–specific IgM and IgG Ab-secreting cells were detected by ELISPOT in the paratracheal lymph nodes of ferrets infected with an H5N1
live attenuated influenza vaccine (LAIV) on days 5 and 10 postinfection, and their magnitude correlated with serum neutralizing Ab responses (48). The kinetics of these responses were consistent with the human Ab-secreting cell response to seasonal LAIV (49).

\[ CD8^+ T \text{ cells provide broad cross-reactive immunity to avian influenza viruses} \]

CD8\(^+\) T cells play an important role in reducing disease severity and promoting recovery following infection with seasonal (50, 51), pandemic (52, 53), and avian (54, 55) influenza viruses. In contrast to pre-existing strain-specific neutralizing Abs directed mainly at the HA head, T cells recognize highly conserved viral peptides derived predominantly from internal and conserved viral proteins (56–60). As a consequence, pre-existing pools of cross-reactive memory T cells are particularly important for mediating immunity towards novel avian IAVs. Indeed, a thorough dissection of cellular immune responses in a cohort of patients hospitalized with severe H7N9 disease at the Shanghai Public Health Clinical Centre in China provided important insights into cellular immunity toward avian IAVs (54, 55). Longitudinal analyses of PBMCs obtained from H7N9-infected individuals clearly indicated that recovery from severe disease was mediated by several immune effectors, dominated by CD8\(^+\) T cells (54, 55). Specifically, individuals who recovered from avian H7N9 IAV infection exhibited robust IFN-\(\gamma\)CD8\(^+\) T cell responses, whereas individuals who succumbed to infection had few or no IFN-\(\gamma\)-producing cells and displayed prolonged activation of exhausted PD-1–expressing CD38\(^+\)HLA-DR\(^+\) CD8\(^+\) T cells. This was accompanied by significant differences in the transcriptomes of activated CD38\(^+\)HLA-DR\(^+\) CD8\(^+\) T cells between fatal and nonfatal cases. Single-cell RNA sequencing analysis demonstrated distinct gene expression profiles related to clinical outcome, with a clear segregation especially at the early (day 11/day 12) time points after disease onset. Most apparent differences were associated with the heat shock protein DNAJB1, IFN-induced transmembrane protein 3 (IFTM3), lactate dehydrogenase A (LDHA), replication protein A3 (RPA3), proteasome subunit \(\alpha\) type-5 (PSMA5), and programmed cell death protein 5 (PDCD5) genes during fatal H7N9 disease. Furthermore, recovery was associated with robust expansion of cross-reactive CD8\(^+\)TCR \(\alpha\)\(\beta\) clonotypes, whereas delayed and limited expansions of novel IAVs in humans.

T cells other than the CD8\(^+\) T cell populations have also been implicated in immunity to avian IAV infection (61). In particular, CD4\(^+\) T cells from healthy adults showed cross-reactivity toward H5N1 viruses (62). Because the presence of cross-reactive CD4\(^+\) T cells correlates with protection during infection with human IAVs (63), such cross-reactivity would likely be beneficial during avian IAV infection. Additionally, depletion or efflux of the innate mucosal-associated invariant T (MAIT) cell subset from the circulation has been observed in severe human infection with avian H7N9 IAV (64). Although the exact role of MAIT cells in avian IAV infection is unclear, activation of MAIT cells by viruses generally results in the upregulation of cytotoxic molecules like granzyme B and the release of antiviral cytokines (64); thus, they may play a role in promoting recovery. Taken together, T cells play a key role in recovery from newly emerged avian IAV infections.

\[ \text{Pathogenesis of severe disease} \]

Severe lymphopenia is a hallmark of severe infection with avian IAVs in humans and in mice (65–67). Two H5N1 viruses were selected for evaluation in a mouse model. One was recovered from a fatal human infection associated with lymphopenia; this virus caused lymphopenia and was lethal for mice. The other virus was isolated from a nonfatal human case without lymphopenia; this virus replicated in the lungs of mice but was not associated with lymphopenia or lethality (32). We found that lethal H5N1 infection enhanced Fas ligand (FasL) expression on plasmacytoid DCs (pDCs), resulting in apoptosis of influenza-specific CD8\(^+\) T cells via a Fas-FasL–mediated pathway; this was not seen in mice infected with the nonlethal H5N1 strain (32), pDCs, but not other DC subsets, preferentially accumulated in the lung draining lymph nodes of lethal H5N1 virus–infected mice, and the induction of FasL expression on pDCs correlated with high levels of IL-12p40 monomer/homodimer in the lung draining lymph nodes. Thus, data from the mouse model demonstrated that pDCs played a deleterious role in lethal H5N1 infection and contributed to lymphopenia (32). As in mice, H5N1 infection in macaques resulted in prolonged margination of circulating T lymphocytes. In contrast to the findings in mice, apoptosis of DCs in the lungs and draining lymph nodes was observed in macaques early during infection (39).

Microarray analysis revealed that IFN response genes were highly enriched in the lungs of ferrets infected with H5N1 viruses compared with ferrets infected with a seasonal influenza virus (68). CXCL10 is a potent chemoattractant for activated Th1 lymphocytes and NK cells and, along with type I and III IFNs, plays a role in the development of innate and adaptive immunity (68). CXCL10 gene expression was significantly upregulated and remained upregulated through day 4 postinfection when the animals were necropsied. Administration of an antagonist of CXCRC3 to H5N1-infected ferrets resulted in a reduction of disease severity and delayed mortality, indicating that unregulated host IFN responses are at least partially responsible for the severity of H5N1 infection (68).

TGF-\(\beta\) acts as a global regulator of immunity by controlling the initiation and resolution of inflammatory responses, and many pathogens evade host immune responses by regulating TGF-\(\beta\). Several IAV subtypes activate TGF-\(\beta\) in vitro and in vivo through their NA proteins, but H5N1 viruses failed to do so. The administration of exogenous TGF-\(\beta\) to H5N1-infected mice delayed mortality and reduced virus titers, and neutralization of TGF-\(\beta\) during infection increased morbidity. The authors speculate that the failure to activate TGF-\(\beta\) may contribute to exacerbated immunopathology (69).

Macaques infected with an H5N1 virus developed severe disease associated with severe bronchiolar and alveolar lesions, an excessive and sustained type I IFN response, and innate immune induction, but the animals recovered (39, 70). Global transcriptional changes were investigated in bronchi because the virus induced an apoptotic response in the lungs and bronchi. Expression of type I IFN–stimulated genes was upregulated as early as 12 h postinfection and remained elevated.
at 24 and 48 h postinfection, and CXCL10 and CXCL11 were among the most highly induced chemokine genes (70).

The fact that severe H5N1 infections in humans are associated with ARDS and similar pathologic findings are seen in ARDS associated with sepsis, gastric acid aspiration, and pulmonary infection caused by other pathogens prompted Imai and colleagues (71) to search for a conserved injury pathway for ARDS. They found that oxidative stress and TLR4 signaling were the key pathways that controlled the severity of lung injury induced by H5N1 virus and other lung pathogens, including severe acute respiratory syndrome coronavirus and anthrax. Rapid formation of oxidized phospholipids in the lungs of TLR4 mutant mice was associated with a rapid onset of acute lung injury. Furthermore, H5N1 infections in humans resulted in the local activation of the oxidative stress machinery and oxidized phospholipid formation in the lungs (71).

Vaccine-induced immunity against avian IAVs with pandemic potential

Inactivated subunit vaccines. The first attempts to develop vaccines against H5 and H7 IAVs used technology that was licensed and used to manufacture seasonal influenza vaccines, and the products were unadjuvanted, inactivated subunit vaccines (Fig. 2). However, it quickly became clear that H5 and H7 vaccines did not induce nearly as robust an Ab response as seasonal influenza vaccines (reviewed in Ref. 72). A 6-fold higher Ag dose (two doses of 90 µg of H5 HA) than for seasonal influenza vaccines (15 µg of HA) was required to elicit an HAI Ab titer of 1:40, which is considered a “protective” titer (73). The Ab was highly strain-specific and did not cross-react with H5N1 viruses from different clades as they evolved (Fig. 2). The Ab titer declined rapidly (within 6 mo) but was boosted with an additional (third) dose and lasted longer (74).

These studies drove investigations into the use of adjuvants to boost the Ab response (75). The addition of alum had little or no effect (76, 77), but oil-in-water adjuvants such as AS03, MF59, and AFO3 improved immunogenicity greatly and allowed dose sparing (78–80). An HA Ag dose as low as 3.75 µg was immunogenic when administered with an oil-in-water adjuvant, and the breadth of the Ab was enhanced, allowing cross-reactivity with H5N1 viruses from different genetic clades (reviewed in Ref. 72). Analysis of the binding specificity of the Ab using genome fragment phage display libraries and surface plasmon resonance demonstrated that MF59 enhanced the diversity of the Ab epitope repertoire and binding affinity (Fig. 2) (81, 82). Inactivated H7N7 and H7N9 subunit vaccines were also poorly immunogenic when tested without adjuvants in clinical trials, but the addition of oil-in-water adjuvants enhanced the immunogenicity of the H7N9 subunit vaccine significantly (83, 84). Inactivated subunit H5N1 vaccines with and without adjuvant have been licensed and stockpiled in some countries.

LAIV. Seasonal LAIV were developed in Russia and the United States and have been licensed in several countries. The underlying principle of both LAIV is that the highly attenuated vaccine viruses are administered intranasally and are restricted to replicating in the upper respiratory tract by temperature-sensitive and attenuating mutations in the internal protein genes of the vaccine donor virus(es). Each LAIV is a reassortant virus that derives its HA and NA gene segments from a wild-type influenza virus on a backbone of six internal gene segments from the vaccine donor virus. The replication of the LAIV, although limited, is sufficient to induce systemic and mucosal Ab and T cell responses that protect the host from subsequent infection (Fig. 2) (85–87). The potential advantages of generating LAIV for pandemic influenza viruses are that the vaccine would induce a systemic and mucosal humoral and cellular immune response and a greater breadth of protection against a range of IAVs, the vaccine would be safe because the temperature-sensitive mutations would prevent the vaccine virus from replicating at the core body temperature of the lungs, and the yield of LAIV in embryonated hen’s eggs would provide 10– to 100-fold more doses per egg than inactivated subunit vaccine. Under a collaborative research and development agreement with MedImmune, we generated candidate pandemic LAIV (pLAIV) against six different avian IAV subtypes (H2, H3, H5, H6, H7, H9) on the backbone of the United States LAIV (Ann Arbor/66/60 cold-adapted). Similar pLAIV were developed in Russia on the Russian LAIV backbone (88, 89). The candidate vaccines developed by the National Institutes of Health and MedImmune were evaluated in animal models, and several pLAIV were evaluated in phase 1 clinical trials (90–95). The pLAIV on the United States LAIV backbone were safe and well tolerated, but primary vaccination did not elicit an immune response that was detectable by standard serologic assays, including HAI, virus neutralization, or ELISA. Remarkably, however, when pLAIV recipients were given a booster dose of matched pandemic inactivated influenza vaccine (pIIV), a very robust and cross-reactive Ab response was detected as early as 7 d postboost (96), indicating that the pLAIV had established long-lasting immune memory that could be recalled with a dose of inactivated subunit vaccine (Fig. 2). We have confirmed this phenomenon with three different pLAIV–pIIV combinations with different intervals between pLAIV prime and pIIV boost (90, 93, 96). In addition to a high titer Ab response in 64–79% of vaccines, the breadth of the Ab response elicited by the pLAIV–pIIV prime–boost combination covers multiple clades of H5 and H7 viruses (90, 96). As discussed below, this feature is highly desirable in a pandemic influenza vaccine strategy. In the event that a novel influenza virus emerges and the population lacks pre-existing immunity, a pLAIV of the same subtype could be administered as the priming dose, and a booster dose of a matched pIIV could be administered when available. Although it is theoretically possible that natural exposure to the novel virus could recall immune memory established by pLAIV priming sufficiently well to confer protection, it is difficult to envision a scenario to prove it.

We were puzzled by the fact that we were unable to detect evidence of pLAIV priming before the pIIV boost and turned to a nonhuman primate model to explore this further. African green monkeys vaccinated with H5N1 pLAIV alone, pIIV alone, or prime–boost with pLAIV–pIIV replicated the serologic findings in humans. We were only able to detect an H5-specific HAI Ab response in the animals that received the H5N1 pLAIV prime followed by pIIV boost. Further exploration that included sampling peripheral blood and a variety of lymphoid organs for H5-specific B cells revealed that intranasally administered pLAIV induced a highly localized germinal center B cell response in the mediastinal lymph node that was rapidly

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FIGURE 2. Vaccine approaches against avian influenza viruses with pandemic potential. (A) Avian influenza HA proteins delivered as standard inactivated influenza vaccines (such as pIIV) are poorly immunogenic, but this can be overcome with higher doses of Ag or the addition of adjuvants. The latter also increase the breadth of reactivity of vaccine-induced Abs. pLAIV formulations do not elicit detectable Ab titers on their own, but robust serological responses can be detected following boosting with pIIV. (B) Novel universal influenza vaccine approaches include the use of engineered HA proteins that encompass past or predicted mutations at antigenic sites, targeting the highly conserved HA stem domain, targeting the NA protein, and targeting highly cross-reactive CD8+ T cells.

Recalled systemically and detected at numerous distant sites after administration of pIIV (97).

Similar observations have been reported with several other priming vaccines, including DNA and a recombinant adenovirus expressing the H5 HA. The Ab response to the primary immunization is weak or not detectable, but subsequent administration of a pIIV boost elicits a robust Ab response (98, 99). It would be interesting to compare the different prime–boost strategies in a head-to-head comparison and to explore whether DNA and adenovirus-vectorized vaccines also induce severely localized germinal center responses that are recalled systemically after pIIV boost.

Challenges in pandemic influenza vaccine development

There is an enormous genetic and antigenic diversity among avian IAVs, and the viruses are constantly evolving in nature through reassortment and mutation. Within the 16 HA and 9 NA subtypes there are many cocirculating lineages of viruses that can be carried great distances by migratory birds. The pandemic potential of the vast majority of avian IAVs is not known, although the World Health Organization (WHO) has developed a risk assessment matrix, the Tool for Influenza Pandemic Risk Assessment, to assess the pandemic likelihood and impact of an influenza virus. In general, avian IAVs that have caused documented human infections are assessed using the Tool for Influenza Pandemic Risk Assessment. In collaboration with the World Organization for Animal Health and Food and Agriculture Organization, the WHO monitors avian IAVs with pandemic potential and recommends the development of vaccines for those that pose the greatest threat.

The current WHO strategy is to generate a library of vaccine seed viruses against selected avian IAVs. This involves reassortment of the HA and NA gene segments of the selected avian IAVs onto the background of six internal protein genes of the vaccine donor virus (influenza A/Puerto Rico/8/34) that is used for seasonal influenza vaccines. If there are known virulence determinants in the HA (or NA) gene segments, such as the multibasic amino acid cleavage site in the HA of H5N1, H7N7, and some recent H7N9 viruses, they are removed by genetic engineering. Vaccines can be manufactured from these seed viruses using the same processes that are used with the licensed seasonal influenza vaccines, and several such vaccines have been manufactured and tested in clinical trials. As discussed above, standard unadjuvanted subunit influenza vaccines elicit highly strain-specific Ab responses, whereas the inclusion of an oil-in-water adjuvant could broaden and boost the Ab response.

What are some of the potential pitfalls of the current strategy and possible solutions? First, vaccine seed viruses are only produced against avian IAVs that are recognized to pose a pandemic threat. Yet, the reservoir of avian IAVs is known to include 16 HA and 9 NA subtypes with great genetic and antigenic diversity. In 2009, the H1N1 pandemic was caused by a virus that was not recognized to pose a pandemic threat. A vaccine that induces broad cross-protection against IAVs within a subtype or, ideally, a universal influenza vaccine that protects across all subtypes would solve the problem of not having recognized the pandemic potential of a virus until it emerges and spreads in humans. Second, currently licensed influenza vaccine technology induces strain-specific immunity. In addition to oil-in-water adjuvants, prime–boost strategies combining different platforms should be investigated further.

Broadly cross-protective and universal influenza vaccines

Several approaches have been explored to increase the breadth of the HA-specific Ab response. Our approach to selecting viruses to target for pLAIV development was to 1) select a subtype of interest, 2) generate postinfection ferret antisera against 10–14 temporally and geographically distinct viruses from different genetic clades, and 3) test the antisera against the viruses in a checkerboard fashion to identify the virus(es) that induced the highest titer Ab that also cross-reacted most broadly against the other viruses. We used this approach to select H2, H6, H5, and H7 viruses for pLAIV development (100–103) and evaluated their breadth of immunogenicity and efficacy in mice and/or ferrets that were challenged with homologous and heterologous IAVs (102). Two other approaches designed to generate a more broadly cross-reactive
Ab response are to engineer a computationally optimized, broadly reactive Ag (104), in which a consensus HA sequence is generated that incorporates all of the significant amino acid changes that have occurred in the past, or to generate an antigenically advanced HA that incorporates changes that are predicted to occur (105). The addition of NA may also be a good strategy to enhance the breadth of influenza vaccine-induced immunity (106, 107).

**Novel approaches.** A completely novel approach to achieve universal immunity to all IAVs requires a change in focus to targets other than the highly variable HA head. A highly conserved epitope in the stalk of the HA, which is also referred to as the HA stem, is the current lead target for a universal influenza vaccine. Two circumstances led to the recognition of a conserved epitope in the HA stem. The first was exposure of humans to a novel HA during the 2009 H1N1 pandemic. The 2009 H1N1 infection failed to recall memory B cells against the HA head because it was novel but recalled the rare B cells directed at the conserved HA stem (108). This HA stem response would not have been amplified by exposure to seasonal H1 HA because the HA head is immunodominant. Second, advances in technology made it possible to isolate and immortalize plasmablasts and memory B cells to produce human mAbs. Human mAbs directed against the HA stem epitope cross-reacted broadly in vitro with a range of HA subtypes and were highly effective in protecting experimental animals from lethal challenge with IAVs of many subtypes, including avian IAVs (109–111). Although the HA stem is subdominant, it is an attractive target for a universal influenza vaccine.

**T cell–based vaccines**

The ability of CD8+ T cells to provide protection against antigenically novel influenza viruses is based on their ability to recognize small, virus-derived peptides presented by MHC class I glycoprotein (HLA-I in humans) on the surface of infected cells. These peptides are most often derived from the internal proteins of the virus, which show considerably less antigenic diversity than the surface glycoproteins. Indeed, memory CD8+ T cells from healthy adults with no prior exposure to avian influenza viruses can cross-recognize epitopes derived from H5N1 (62, 112) and H7N9 avian IAVs (58, 59). A set of six universal CD8+ T cell epitopes has been identified (58) that could form the basis of a T cell–based vaccine to complement Ab-based approaches. These epitopes were termed “universal” because they represent highly conserved peptides, and they are composed of six highly prevalent HLA-I alleles (HLA-A2, HLA-A3, HLA-B8, HLA-B18, HLA-B27, and HLA-B57), providing broad population coverage. However, it is important to note that these HLA-I alleles would be underrepresented in some ethnic groups, including indigenous populations around the world, especially Indigenous Australians and Alaskans, because of ethnic differences in HLA allele distribution (58). Thus, further identification and characterization of universal epitopes is necessary to achieve complete population coverage.

As the currently licensed inactivated influenza vaccines do not boost CD8+ T cells or innate T cells (113), novel vaccination strategies that elicit cross-reactive memory T cells are highly desirable to provide universal immunity against current and emerging influenza viruses. Several approaches are under development, including replication-incompetent viruses that are able to infect cells but are limited to a single cycle of replication because one or more of the genes that are essential for viral replication have been deleted or silenced (114–118) and vectored vaccines that encode one or more internal protein genes of influenza (119). There is no doubt that T cell immunity is more cross-reactive than HA Ab-mediated immunity. However, the kinetics of IAV replication are rapid: peak viral replication occurs within 2 d of infection. Given the kinetics of recall of T cells, a vaccine based only on T cell responses will not prevent infection and may not blunt the peak of viral replication. However, a robust T cell response is very likely to result in earlier clearance of virus, and this could result in less severe disease and/or reduced duration of illness and hospitalization. Although a universal vaccine may take several years to develop, a combination of strategies to induce Ab and T cell immunity could provide both strain-specific immunity and cross-reactive immunity in the nearer term.

**Conclusions**

Much of our knowledge about the immune response to avian IAVs is a result of careful study of H5 and H7 human infections and the human immune response to investigational pandemic influenza (H5 and H7) vaccines. These data are supported by studies in animal models. Although there are many commonalities, there are also some notable differences between the immune responses to avian and seasonal human IAV infections. Avian influenza virus infections are associated with a cytokine storm and an exaggerated innate immune response. The Ab response to the HA is not as robust as with seasonal influenza viruses. Recovery from severe disease is mediated by several immune effectors, dominated by CD8+ T cells. Much progress has been made in the development of pandemic influenza vaccines, most notably the incorporation of oil-in-water adjuvants and a prime–boost strategy combining different platforms such as LAIV, DNA, or a vectored vaccine followed by a protein vaccine. These approaches increase the breadth of the Ab response from a highly strain-specific response observed with an unadjuvanted subunit vaccine. The intermediate and ultimate goals of vaccine development, which are the generation of vaccines that induce broadly cross-reactive immunity against all IAVs within a subtype or a universal influenza vaccine that provides immunity against all IAV subtypes, are within sight.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


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