Influenza-Specific Antibody-Dependent Cellular Cytotoxicity: Toward a Universal Influenza Vaccine

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Influenza virus has a large human and economic toll, with seasonal influenza resulting in ~300,000–500,000 deaths/y worldwide and more than an estimated $26.8–87.1 billion/y in healthcare costs in the United States alone (1, 2). Yearly vaccination is recommended to reduce the burden of influenza disease. The fraction of severe illness prevented for the 2012–2013 season was estimated by the U.S. Centers for Disease Control and Prevention as being a low 17.3% (confidence interval: 16.2–18.0%), with an estimated half of the U.S. population receiving the influenza vaccine (3). Trivalent influenza vaccine preparations require precise selection of circulating strains (typically an H1N1, H3N2, and type B influenza virus) based on surveillance data and have reduced protection when strains are mismatched (4). Further, seasonal vaccines provide little protection from avian-origin viruses, such as H5N1 and H7N9 (5). With growing demand for broader therapeutic agents (6) and the rising number of viral isolates resistant to current antiviral treatments (7, 8), a broadly protective universal vaccine would be an important development toward curtailing the impact of influenza virus on the human population.

Influenza A viruses (IAVs) are enveloped RNA viruses belonging to the family Orthomyxoviridae. The genome comprises eight ssRNA segments that encode for 11 distinct polypeptides, including 8 structural viral proteins and 3 nonstructural proteins (Fig. 1A). The entry and release of influenza virus from host cells is mediated by the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), respectively. Following receptor-mediated endocytosis, the reduced pH within endosomes triggers a conformational change in HA to facilitate fusion of viral and host cell membranes and release into the host cytosol. The transmembrane M2-ion channel protein is involved in acidification of the virion, which is essential for uncoating of the virus. The internal components of the influenza virus consist of matrix 1 protein (M1); the ribonucleoprotein complex, which consists of the viral RNA segments; the polymerase complex proteins polymerase basic 1 (PB1), polymerase basic 2, and polymerase acid; and nucleoprotein (NP). Nonstructural protein 1, nonstructural protein 2, and PB1 frame 2 are only found within infected cells and are responsible for overcoming host immune mechanisms (9) (Fig. 1A).

Many studies have focused on the induction of broadly neutralizing Abs (Nabs) or cross-reactive CTLs to define immune correlates of protection from influenza virus infection. CTLs targeting peptides from NP or M1 were shown to be effective in protecting from severe influenza infection (10–13) and correlated with the reduction of symptomatic influenza disease in humans (13, 14). Further, CTLs recognizing a broad range of influenza viruses are present in healthy human subjects (15, 16). Conversely, Nabs directed to HA or NA can inhibit virus entry or prevent virus release from host cells, respectively (17, 18). Influenza vaccine studies primarily measure hemagglutination inhibition (HI) titers, such Abs that mediate HI target epitopes surrounding the receptor-binding site of influenza HA (Fig. 1B) (19, 20). However, Abs that mediate HI tend to be highly specific for the infecting virus or vaccine strain and amenable to escape through point mutations or glycosylation of the receptor binding site of the globular head. In contrast, subsets of Nabs exist that do not mediate HI and bind the conserved helix-A
region sitting between the HA₁ and HA₂ regions (known as anti-stem Abs) (21–23). The stem region is highly conserved among influenza viruses, and anti-stem Abs tend to mediate their neutralization activity by either blocking viral fusion with, or preventing cleavage of, the HA protein (24, 25). Vaccine regimens that target the HA stem region have given promising results in mouse models but have yet to be translated into human clinical studies (26–28).

Abs induced toward influenza virus can mediate a number of nonneutralizing functions, including complement-mediated lysis (29, 30), phagocytosis (31), and Ab-dependent cellular cytotoxicity (ADCC) (32–36). In some instances, these nonneutralizing Abs mediate distinct antiviral activity; however, in other cases, these additional functions can increase the potency of Nabs. In this review, we describe previous studies on influenza-specific ADCC and new technical developments in the study of influenza-specific ADCC that may aid in future vaccine development.

Influenza-specific ADCC: past and present

ADCC uses effector arms of both the innate and adaptive immune responses to kill target cells (Fig. 2). ADCC is initiated by secreted IgG Abs (IgG1 or IgG3 subtypes in humans and IgG2a or IgG2b subtypes in mice) binding to Ags on the surface of target cells. Effector cells, such as NK cells (but also neutrophils and monocytes), which in humans express the activating FcγRIIIα (CD16) receptor (in mice the FcγRIV receptor has the highest affinity for IgG2a), are then able to bind to the Fc region of the surface-bound Ab (37, 38). Ligation of the CD16 receptor on the effector cell leads to phosphorylation of the C-terminal ITAM to activate Ca²⁺-dependent signaling pathway, resulting in the release of preformed granzyme B and perforin granules from endosomes (39). Both granzyme B and perforin cause DNA fragmentation and apoptosis of the target cell. Additionally, ligation of the CD16 receptors on effector cells can result in the secretion of several antiviral cytokines and chemokines, including IFN-γ, and TNF, and β-chemokines (e.g., MIP-1α and MIP-1β), which have important antiviral and immunopathological properties (40, 41).

Early studies performed during the late 1970s and the early 1980s established a role for ADCC against influenza virus. In 1977 and 1978, Greenberg et al. (32, 33) suggested that lymphocytes in peripheral blood were capable of mediating cytotoxicity toward influenza virus–infected cells in the presence of small quantities of Abs secreted from PBMCs. PBMCs isolated from healthy volunteers were found to mediate robust cytotoxicity toward either HeLa cells or BHK-21 cells infected with H3N2 (A/Hong Kong/2/68) virus and labeled with [³²Cr]. Greenberg et al. (42) later confirmed that the presence of HA-specific Abs secreted by PBMCs correlated with the ability of lymphocytes to mediate cytotoxicity against influenza virus–infected cells. This is consistent with our recent studies showing that cross-reactive ADCC-mediating Abs against a broad range of HA and NA proteins from IAV strains were present in serum from healthy volunteers (36).

Greenberg et al. (32, 42) also showed that vaccination with an inactivated influenza vaccine or infection with influenza virus resulted in increased cytotoxicity from PBMCs within 7 d. Hashimoto et al. (34) performed follow-up studies showing that ADCC-mediating Abs were found in serum from children recently vaccinated with either inactivated or live-attenuated vaccines or infected with influenza virus. They suggested that the cytotoxicity was likely the result of NK cell activity. Our recent studies show that infection of macaques with a seasonal H1N1 virus induces cross-reactive ADCC-mediating Abs toward a heterologous H1N1 virus. These ADCC Abs declined to lower, but still detectable levels, by 30 d postinfection. However, the ADCC Abs expanded significantly within 7 d postinfection with a heterologous H1N1 virus, highlighting the infection-mediated induction of memory B cells capable of producing ADCC Abs (43).
Recent studies by our group showed that healthy humans have broad cross-reactive ADCC-mediating Abs, likely as a result of multiple prior influenza infections. We found that young adults with no known prior exposure to (consistent with a lack of HI Abs that recognized) a 1968 H3N2 virus had robust ADCC-mediating Abs to this strain (36). In our studies, most influenza virus–specific ADCC-mediating Abs induced via vaccination or infection were shown to be broadly reactive to strains within a given subtype (32, 34, 44–46). However, we detected cross-reactive ADCC-mediating Abs toward avian H5N1 and H7N7 strains in some individuals in the absence of prior exposure (36). Presumably, these cross-reactive ADCC-mediating Abs target conserved regions of the influenza HA, including the stem region. This is consistent with our recent studies showing a reduction in titers of ADCC-mediating Abs when measuring responses against the HA1 protein compared with the whole H1 protein (S. Jegaskanda, K. Vandenberg, K.L. Laurie, L. Loh, M. Kramski, W.R. Winnall, K. Kedzierska, S. Rockman, and S.J. Kent. manuscript in preparation). Such ADCC-mediating Abs are likely expanded following heterologous influenza infection (43, 47, 48).

The levels of cross-reactive ADCC-mediating Abs in healthy humans make measuring the generation of new ADCC responses difficult. Abs and NK cells capable of mediating ADCC are detectable in cord blood samples, suggesting that ADCC-mediating Abs are present at low levels in all individuals throughout their lifetime (35). This may shape the newborn’s immune response to influenza virus, with the broader ADCC-mediating maternal Abs having a greater protective effect on the newborn (49, 50). We speculate that, in addition to the waning of Nabs, the loss of maternal ADCC-mediating Abs following cessation of breastfeeding may lead to increased influenza infections in infants. More detailed studies on the induction of cross-reactive Abs by multiple infections and the influence of ADCC-mediating Abs on the protection of infants are required to make such conclusions.

**In vivo protection from influenza infection by ADCC-mediating Abs**

The characterization of protective Ab responses to influenza virus in mouse models has typically focused on Nab function. However, recent studies suggest that the nonneutralizing functions provided by the Fc region of the Ab are important for the potency and the protective ability of HA-specific Abs (22, 51). A study by Corti et al. (22) showed that the broadly neutralizing human F6 Ab attributes most of its in vivo activity to its FcR-binding properties. Mice administered 3 mg/kg of the FcR mutant (termed Fl6-LALA) Fl6 Ab had a 60% reduction in survival compared with Fl6 Ab or Fl6 complement mutant (termed Fl6-KA) when challenged with a lethal dose of PR8 virus. However, the transfer of human IgG1 Abs into mice that express mouse FcR may be suboptimal. A recent study by DiLillo et al. (52) showed that administration of 4 mg/ml of a mouse IgG2a form of Fl6 Ab (which can mediate ADCC in mice) protected mice from lethal challenge, whereas 4 mg/ml of mouse IgG1 Fl6 Ab form (which does not mediate ADCC in mice) did not. The protection afforded by mouse IgG2a Fl6 Ab was abolished when administered to Fcer1g−/− mice (mice lacking the FeRγ-chain). Further, the

**FIGURE 2.** Schematic diagram of ADCC to influenza virus–infected cells. IgG Abs (IgG1/IgG3 in humans or IgG2a/IgG2b in mice) bind to viral Ags expressed on the surface of influenza virus–infected cells (left panel). Effector cells, such as NK cells (also neutrophils or monocytes), bind to the Ab Fc-region using their FcRIII receptor (in mice FcRIV, middle panel). Upon Ab ligation, cytotoxic granules are released, and antiviral cytokines are expressed (right panel). This results in apoptosis of the infected cell and a reduction in viral replication.
human IgG1 form of the FI6 Ab was directly shown to mediate ADCC toward influenza virus–infected target cells. This confirms the importance of ADCC for the protective activity of the FI6 broadly Nab in mice.

DiLillo et al. (52) provide further support for the in vivo use of influenza-specific ADCC, showing that, for HA stem Nabs, the subclass and FcR function are important for protection from lethal influenza infection. Mice administered the mouse IgG2a subclass of the anti-stem 6F12 broadly Nab showed reduced weight loss and increased survival following challenge with a lethal dose of PR8 virus compared with those administered the mouse 6F12 IgG1 subclass (does not mediate ADCC) or a 6F12 FcyRII-binding-deficient Ab (DA265). The protection provided by the IgG2a 6F12 Nab in mice was FcγR dependent, because administration of IgG2 6F12 to either Fcγ-chain–deficient mice (Fcer1g−/− mice; which lack FcγRI, FcγRII, FcγRIII, and FcγRIV) or FcγR-null mice (which lack FcγRI, FcγRIIb, FcγRIII, and FcγRIV) resulted in reduced survival following lethal challenge with PR8. The ability of stem Abs to mediate ADCC was shown by the activation of donor NK cells in the presence of human IgG1 6F12 and A549 cells infected with PR8 virus. In addition, DiLillo et al. (52) showed that the anti-stalk Abs 2G02, 2B06, and 1F02 protected mice from A(H1N1)pdm09 virus challenge in a FcγR-dependent manner. Not surprisingly, FcγR binding was critical for the potent protection provided by anti-stem Abs because the mutant form of 6F12, deficient in FcγR binding (DA265), afforded protection only at concentrations >16 mg/kg, whereas 4 mg/kg of unmodified IgG2a 6F12 provided complete protection from lethal PR8 challenge. Indeed, these findings may explain the ability of anti-stem Abs CR9114 and CR8043 to protect mice from lethal challenge with a type B virus or H7N7 virus, respectively, although these Abs did not mediate in vitro neutralization of these viruses (21, 53). However, the ability of Abs CR9114 and CR8043 to mediate ADCC toward these strains needs to be assessed.

The investigation of stem Abs’ versus globular head Abs’ ability to mediate ADCC is particularly interesting. DiLillo et al. (52) suggested that only anti-stem Abs can bind in the correct conformation to ligate FcγRs. In their study, the one strain-specific anti-HA head Ab (PY102) investigated was insufficient at mediating FcγR binding and ADCC activity; however, more Abs to the globular head of HA need to be tested before clear conclusions can be drawn. Further, a polyclonal anti-HA ADCC response may provide greater ligation of CD16, as seen for HIV ADCC (54). Clearly, a larger subset of anti-HA head Abs needs to be tested to determine their overall potential to mediate ADCC.

Our study and other studies characterized the role of NK cells in mediating ADCC against influenza virus–infected cells (34–37). However, other cells at the site of influenza infection, including neutrophils and alveolar macrophages, express CD16 receptors and could mediate ADCC activity. The relative importance of different cell subtypes as effectors of ADCC activity is unclear.

The influence of influenza ADCC-mediating Abs on the 2009 pandemic

In April 2009, a novel swine-origin H1N1 influenza virus entered the human population and caused the first pandemic of the 21st century (55–58). In contrast to seasonal influenza, which has the highest morbidity and mortality in the elderly, it was younger individuals (<60 y of age) who were most affected during the 2009 pandemic. Older individuals seemed to demonstrate a level of protection against severe A(H1N1)pdm09 infection (58, 59). According to Hancock et al. (59), a proportion of individuals (39/115, 34%) born before 1950 (>59 y of age) had detectable cross-reactive Nab titers against the A(H1N1)pdm09 virus. However, the presence of Nabs in some individuals may only partially explain the reduced incidence of severe (H1N1)pdm09 virus infection in individuals >60 y of age. We recently showed that of the individuals >45 y of age with undetectable HI titers to A(H1N1)pdm09, nearly all (28/31, 90%) had ADCC-mediating Abs to the A(H1N1)pdm09 virus prior to the 2009 pandemic (44). Numerous animal studies showed that infection with seasonal H1N1 viruses, particularly with H1N1 viruses circulating pre-1960, can provide a level of protection from severe A(H1N1)pdm09 virus infection (21, 60–63). In studies in which Nabs against A(H1N1)pdm09 could not be detected, the levels of nonneutralizing binding Abs correlated closely with protection from severe A(H1N1)pdm09 infection (12, 61). Indeed, our studies showed that previous infection of macaques with seasonal H1N1 virus induced cross-reactive ADCC-mediating Abs toward A(H1N1)pdm09 (43). The expansion of cross-reactive ADCC-mediating Abs targeting the stem region of the influenza virus HA fits well with epidemiological studies. Future passive-transfer studies and isolation of mAbs from donor samples prior to the 2009 pandemic are required to determine the targets and protective ability of polyclonal ADCC-mediating Abs induced by seasonal H1N1 infection.

ADCC-mediated activity toward internal proteins of influenza virus

Vaccination strategies that target internal proteins of influenza virus, namely NP and M1, have shown promise in animal models. Amino acid sequences of NP and M1 are highly conserved across different IAV subtypes and strains (i.e., ~90% sequence identity), making these proteins attractive vaccine targets. Until recently, only T cell–mediated immunity toward internal proteins was considered plausible for effective heterologous protection from influenza virus infection. However, protein- or vector-based vaccine constructs containing NP can induce nonneutralizing Abs (64–67). Passive transfer of nonneutralizing polyclonal Abs or mAbs toward NP was associated with protection from lethal influenza challenge in mice (64, 68), although the mechanism of Ab-mediated protection remains unclear. Of interest, studies showed that intracellular NP Ag is transiently expressed on the surface of influenza virus–infected cells (69, 70) and, therefore, could represent a target for ADCC. Bodewes et al. (71) investigated the in vitro Fc-mediated effector functions of anti-NP Abs, demonstrating that a human anti-NP Ab did not mediate neutralization or complement-dependent cytotoxicity or improve presentation by opsonization. Studies on HIV ADCC by our group showed that ADCC can be mediated to peptides derived from internal HIV proteins, including Pol and Vpu (72, 73). Our limited studies in macaques suggest that anti-NP Abs mediate ADCC activity, because anti-NP Abs could activate macrophage NK cells in vitro (46). Further studies are required to determine whether virus-infected cells expressing NP can be killed by ADCC, as well as the ability of anti-NP ADCC-mediating Abs to mediate protection in vivo. The targeting of conserved regions of NP or other internal proteins (M1, PB1) for the induction of ADCC-mediating Abs may be a very useful strategy for vaccine design (74, 75).
of other effector functions that also contribute to effective immunity. It is clear that ADCC-mediating Abs are found in most individuals, and such Abs are cross-reactive against a broad range of influenza subtypes. This represents a unique turning point in our field and provides a possible avenue to assist in the development of a universal influenza vaccine.

Disclosures
The authors have no financial conflicts of interest.

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