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Influenza and Antibody-Dependent Cellular Cytotoxicity

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Despite the availability of yearly vaccinations, influenza continues to cause seasonal, and pandemic rises in illness and death. An error prone replication mechanism results in antigenic drift and viral escape from immune pressure, and recombination results in antigenic shift that can rapidly move through populations that lack immunity to newly emergent strains. The development of a "universal" vaccine is a high priority and many strategies have been proposed, but our current understanding of influenza immunity is incomplete making the development of better influenza vaccines challenging. Influenza immunity has traditionally been measured by neutralization of virions and hemagglutination inhibition, but in recent years there has been a growing appreciation of other responses that can contribute to protection such as antibody-dependent cellular cytotoxicity (ADCC) that can kill influenza-infected cells. ADCC has been shown to provide cross-strain protection and to assist in viral clearance, making it an attractive target for "universal" vaccine designs. Here we provide a brief overview of the current state of influenza research that leverages "the other end of the antibody."

Keywords: ADCC—antibody dependent cellular cytotoxicity, influenza, antibodies, animal models, vaccine targets, mAb

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INTRODUCTION

Influenza causes 3–5 million cases of severe illness and 290,000–650,000 deaths annually (1). Influenza is caused by orthomyxoviruses that have a segmented, negative-strand RNA genome that encodes its own RNA-dependent RNA polymerase that results in ∼1 error per replicated genome (2–4). Accumulating errors cause small changes over time that allow viral escape (5, 6), a process called antigenic drift. Reassorting of the segmented influenza genome can produce novel influenza strains to which there is no preexisting immunity in the human population, a process called antigenic shift, and it is thought this mechanism produced the 1918, 1957, and 1968 pandemics (7).

The primary means of combatting both seasonal and pandemic influenza are quarantine and isolation (8), strict hygiene (9), and vaccination (10–12). Influenza vaccines were developed starting in the 1940s (13–16), and seasonal vaccines now include multiple antigens either as inactivated or live-attenuated products (17–20). Selection of representative influenza strains requires the ongoing worldwide analysis of circulating influenza (21), leading to potential mismatch with low vaccine efficacy (22). Public health leaders have called for the development of "universal" influenza vaccines (23), but at this time it is not clear what kinds of immunity such a vaccine should elicit.

Vaccines can prevent symptomatic disease, reduce disease duration, and reduce viral shedding, and infectivity to other persons. To accomplish these, a vaccine can elicit responses that inhibit

influenza virions and/or enhance the clearance of influenza-infected cells. The influenza virion has three virally-encoded surface antigens (**Figure 1A**): a trimeric glycoprotein hemagglutinin (HA) that binds to sialic acid on cell surface receptors promoting virion endocytosis followed by fusion of viral and host cell membranes, a tetrameric neuraminidase (NA) that cleaves sialic acid to release virions from infected cells, and enhance passage through respiratory mucins, and a proton channel (M2 matrix) that helps the release of the viral genome after acidification in endocytic vacuoles. These proteins are present in most split-virus vaccine products (24), but the exact contribution of each of these, or of other influenza proteins that are not surface expressed, to vaccine efficacy is unclear.

ADCC ACTIVITY AGAINST INFLUENZA

Antibody specificity is mediated by binding of Fab (fragment antigen binding) domains to their antigenic target, while the other end of the antibody is a constant region, the Fc (fragment crystallizable) domain, that provides a link between antibody recognition of infected cells and effector cells. NK cells, monocytes/macrophages, and neutrophils all have Fc-receptors (FcRs) on their surface, and the combination of both activating and inhibitory signals direct the immune response (25). In humans, NK cells are generally thought of as the primary effector for ADCC, but ADCC of influenza-infected cells has been demonstrated using neutrophils, monocytes, lymphocytes, and cord blood cells (26–28).

An increasing body of literature has demonstrated the possible protective role of non-neutralizing antibodies for pathogens as diverse as HIV-1 (29, 30), herpes simplex virus (31–33), Ebola (34, 35), and influenza (36–38). Influenza-infected cells can be recognized by antibodies that bind to proteins that play important roles within the viral life cycle. As noted above, HA initiates the viral life cycle by binding sialic acid and mediating viral entry into cells via fusion after receptor-mediated endocytosis (2). During this process, M2 matrix channels promote pH equilibration, a critical step in the release of the viral genome from the endosome (39). After influenza genome replication and protein synthesis, viral components are transferred to the plasma membrane where viral budding occurs. NA removes sialic acid from glycoproteins allowing the release of newly assembled virions from the surface of infected cells and preventing aggregation (2). HA has been the focus of most vaccine designs because HA evolves more rapidly than other antigens (40), but more conserved antigens like NA, M2, and nucleoprotein (NP) have also been considered attractive targets for vaccine designs. Antibodies against these other antigens do not appear to directly prevent infection but can target infected cells (19), and antibodies against all of these proteins have mediated killing of infected cells *in vitro* (**Figure 1B**).

Antibody-dependent cellular cytotoxicity (ADCC) against influenza was described by Greenberg et al. (26) and ADCC activity can be mediated by multiple cell types (27, 28, 41) and arises after infection or vaccination (41–43). Most early work suggested that ADCC was primarily directed against HA (44), although the contribution of other antigens to ADCC could not be ruled out (43). Study of influenza-specific monoclonal antibodies (mAbs) have shown that antibodies directed against the globular head often lack breadth while those directed against the more conserved HA stem can recognize multiple strains and subtypes of HA (4). Both specificities of HA antibodies have mediated ADCC (**Figure 1B**) but antibodies with neutralization and hemagglutination inhibition (HAI) activity tend to be directed against the HA head domain (**Figure 1A**).

ADCC-mediating antibodies also target other proteins on the virus surface (**Figure 1B**). Murine mAbs against influenza B NA mediated ADCC and provided protection in mouse challenge (45), but whether this activity contributes significantly to human protection is not known. NA antibody titers have correlated with protection against influenza infection in humans (46–50), but NA responses have not been studied at the same level of molecular detail as HA responses (51). NA antibodies are thought to act by decorating infected cells thus making them targets for complement fixation and ADCC, and by inhibiting enzymatic activity and preventing virions from escaping respiratory mucins (19). NA is present in most vaccines derived from influenza virions (24), but few vaccine candidates focus on NA antigens and their relative contribution to protection is not well-quantified. There is growing interest in NA as a component vaccine-mediated protection, and the recent formation of the NAction! working group (51) is focused on filling key knowledge gaps.

M2 is a highly conserved antigen necessary for the virus life cycle (39) and a number of studies showed antibodies to M2 mediated ADCC and protected in mouse studies (36, 52, 53). M2 is present at low copy number on virions and may be occluded by the larger HA and NA proteins (19) (**Figure 1A**), and while vaccine candidates based on M2 have advanced to human clinical trials (54, 55), it is not yet known whether these approaches will provide broad cross-protection when tested in human efficacy trials.

Curiously, a protein that might not be expected on the surface of either virions or virus-infected cells has been shown to mediate ADCC *in vitro*. Influenza nucleoprotein (NP) is involved in replication and packaging of virus RNA segments into virions (56), but studies have shown that NP is surface expressed on infected cells (57, 58). Humans given seasonal vaccine demonstrated H7N9 cross-reactive ADCC activity that correlated with binding to NP (59). Antibodies against both NP and the internal RNA-binding and structural matrix 1 (M1) protein did not mediate ADCC against target cells expressing those specific antigens, but immune complexes of those proteins primed natural killer (NK) cells to secrete cytokines (60) (**Figure 1C**); the authors suggested such activity could contribute to an anti-viral environment. Whether these kinds of activities play an important role in cross-protection against human infection is not yet known.

ADCC IN MOUSE MODELS

The receptors and cell types important for human immune response may not have the same effect or use the same pathways

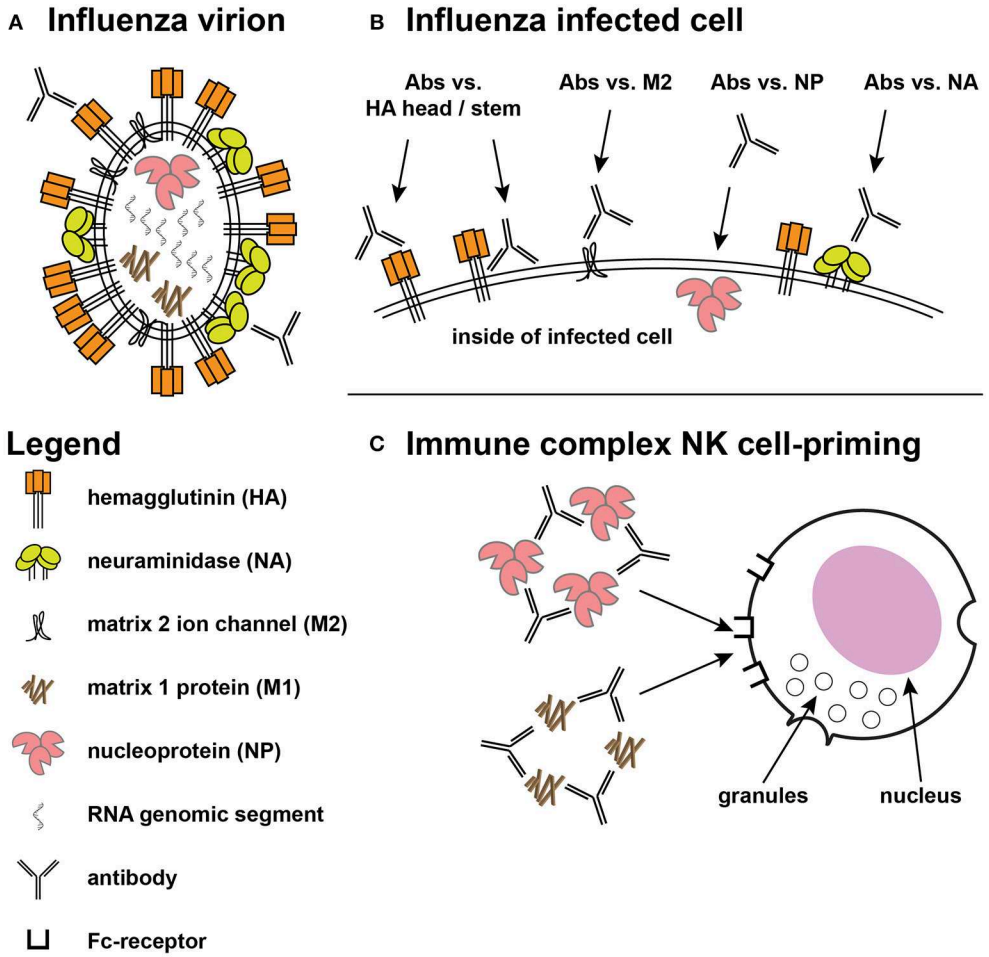

FIGURE 1 | Targets for protective influenza responses. **(A)** Influenza virion showing the tight packing of viral hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2) ion channels on the surface. Inside the virion are the 8 genomic segments, nucleoprotein (NP), and the structural matrix 1 (M1) protein. **(B)** The surface of an infected cell shows the same antigens spread out on the surface, providing access for ADCC-mediating antibodies directed at the HA head and stem, along with greater access to the M2 ion channel. Antibodies against NP have also shown ADCC activity. **(C)** Immune complexes of NP and M1 have been shown to prime NK cells to secrete cytokines.

in animal models. In fact, there are numerous differences among antibody isotypes and subclasses (61) in animal models typically used for immune studies, as well as in FcR sequences and cellular distributions (62), and such differences may result in species-specific mechanisms of ADCC activity against influenza.

With that caveat, mouse models have provided suggestive evidence for the kinds of immunity that might be protective in humans. DiLillo et al. administered human mAbs to transgenic mice that expressed human FcRs, and demonstrated that for both HA stem-directed antibodies (37) and HA head-directed antibodies (63) that Fc-FcR interactions were required for protection against lethal challenge. Interestingly, transgenic mice receiving neutralizing anti-HA and anti-NA antibodies were only protected against challenge if they were matched for Fc-Fc?R binding while strain specific anti-HA and anti-NA antibodies protected regardless of Fc-FcR matching (63). Similar studies of antibodies against M2 have also shown Fc-FcR dependence, with IgG1 M2-directed antibodies requiring matching FcRs to protect against lethal challenge (52, 53).

Wild-type mice have also demonstrated ADCC-mediating antibody protection. For example, murine antibodies generated against H7N9 influenza were protective, but ADCC-mediating antibodies required efficient Fc-FcR interaction to protect (64). Vaccination against H5N1 that elicited ADCC activity protected against lethal H5N8 challenge in mice (65), and protection against H7 influenza challenge also correlated with ADCC activity (66). Passive transfer of HA stem-specific human sera demonstrated protection against lethal challenge in a manner highly correlated with ADCC activity (67). Protection is not limited to HA-directed antibodies, as antibodies against influenza B NA also mediate ADCC and protect against lethal virus challenge (45).

However, not all mouse studies have suggested a protective effect of ADCC. Evaluation of candidate vaccines that targeted

specific epitopes on the HA head domain were capable of eliciting potent ADCC responses *in vitro*, but mice given these immunogens were more sensitive to lethal challenge (68, 69), and examination of lung tissue suggested damage caused by the immune response. Whether this kind of damage could occur in humans is not known.

OTHER NON-PRIMATE MODELS

Swine are susceptible to influenza and they are thought to be a key species in development of new strains by antigenic shift (70). Because of the major economic impact of swine influenza disease, vaccination is common and new vaccine candidates are tested in swine (71). Experimental infection models have been used to test swine for protection against challenge following vaccination (72) and as a model of enhanced influenza disease (73). Studies in this latter model have suggested that a lack of neutralizing activity against the challenge strain combined with ADCC-mediating antibodies can produce enhanced disease in swine (73, 74), suggesting that caution may be appropriate in the development of vaccines that do not elicit traditional correlates of influenza protection (75). Furthermore, differences between swine and human FcRs make direct measures of ADCC in swine challenging, and have confounded passive infusion studies of human antibodies (76). The swine model is important for improving our understanding of influenza pathogenesis, but it remains to be seen whether success or failure in the swine model will directly translate to human trials.

The ferret model may also provide insights into influenza immunity, although direct measures of ADCC activity in ferrets is also challenging. For example, immunization of ferrets to elicit HA stem-directed antibodies showed that ADCC in a reporter assay correlated with protection, though the assay used human FcRs due to the lack of ferret-specific reagents (77). Passive transfer of immune sera directed against the HA stem (78), infusion of ADCC-mediating mAbs (79), and immunization to induce ADCC-mediating antibodies (65) have all protected ferrets against heterologous influenza challenges. Unfortunately, the lack of ferret-specific reagents limits the depth of investigation possible at this time, and as with swine, it is not clear whether these studies will translate directly to human trials.

NON-HUMAN PRIMATE STUDIES

Non-human primate models have not traditionally been used for influenza research due to high cost and the low level of symptoms following experimental infection (80), but recently a number of studies have evaluated the protective capacity of ADCC in non-human primates. For example, protection was observed in rhesus macaques infected with a pre-pandemic H1N1 A/Kawasaki/173/2001 who were subsequently challenged with H1N1 A/California/04/2009; in this study ADCC activity correlated with control of the second H1N1 infection (81). Protection was also observed in a study of H5 immunization of rhesus macaques challenged with pandemic H1N1, and ADCC activity correlated with reduced viral shedding after infection (82). In cynomolgus macaques, vaccines that elicit ADCC-mediating antibodies have been shown to decrease shedding of influenza (83), and passive infusion of a human ADCC-mediating mAb protected against infection (84). However, given the differences between human and non-human primate Fc-FcR biology (85), it is not clear if non-human primate studies will be predictive of human study outcomes.

HUMAN STUDIES

Human studies have focused on examining people after natural infection, vaccination, and/or the isolation of human monoclonal antibodies. Natural infection studies have shown that influenza can imprint the immune system in a manner that provides protection against heterologous influenza exposure. For example, healthy adults and children in the US were found to have ADCC-mediating antibodies against H5N1 and H7N9 strains (86), despite the fact that those strains have not circulated in the US. Intravenous immune globulin, a product derived from plasma donation, contained ADCC-mediating antibodies that cross-reacted with multiple influenza strains; e.g., immune globulin collected from donors prior to the emergence of the 2009 pandemic strain was active against the newly emergent strain (87). During the last influenza pandemic a number of studies suggested that older adults were less likely to be infected by the newly emergent strain, and older persons were more likely to have ADCC-mediating antibodies against the 2009 H1N1 pandemic strain (88), suggesting that these responses may have contributed to the relative protection observed. However, such antibodies were not well correlated with protection in children (89, 90), indicating that *in vitro* activity alone may not provide an accurate correlate of efficacy.

ADCC-mediating antibodies that arise during severe infection may also correlate with outcomes. A study of seasonal and H7N9 human infections demonstrated that persons who did not survive infection were more likely to have low ADCC activity (91). It is not known whether ADCC-mediating antibodies being present prior to infection would have been protective.

Vaccine studies have examined the ability of different constructs to elicit cross-reactive ADCC-mediating antibodies. For example, seasonal vaccination of older adults indicated that they had strong boosting of ADCC activity, including activity against H5 and H7 strains (92). Similarly, a study of H7 vaccination found ADCC activity against many group 2 influenza strains (93), and H5 vaccination induced ADCC responses to multiple strains (94).

Testing of many of human-derived antibodies showed protection in mouse studies as described above and have helped define vaccine design targets. For example, antibodies against the vestigial esterase domain of H3 HA (95) or influenza B (96) mediate ADCC and block other viral activities, but whether such antibodies can be easily elicited by vaccination and protect is not known. Others have examined why HA head-directed antibodies are less efficient at mediating ADCC (97) or why antibodies that bind at or near the sialic acid receptor binding site of HA lack ADCC activity (98, 99). Data suggested that ADCC

activity against influenza-infected cells requires that sialic acid on NK cells must also bind HA. Several studies have identified possible vaccine targets on HA, including a pH-sensitive epitope on H7 HA (100), receptor binding site antibodies for influenza B (79), and the influenza B HA stem (101). Furthermore, recently isolated human antibodies against epitopes in the HA trimer interface provided protection in an Fc-dependent manner (38, 102), and such antibodies were elicited in animal models by vaccination (103). These studies suggest that probing the human immune system could identify additional targets for vaccine design.

Most human studies measure responses to influenza infection or vaccination, and some correlate those responses with epidemiologic data, but far fewer perform experimental infectious challenge to correlate a response with protection. In 2016, Jegaskanda et al. reported that high levels of pre-existing ADCC-mediating antibodies protected against experimental infection (104), suggesting that if ADCC activity is present prior to infection it can protect. However, it is not clear whether ADCC activity alone is protective, and as of this writing, there do not appear to be any reports of human challenge studies investigating the protective capacity of passively administered ADCC-mediating influenza antibodies. Essentially all humans over the age of 6 years have evidence of prior influenza infection (105), meaning that vaccination or passive infusion studies of ADCC-based protection in humans have to be interpreted in the light of prior immunity. Despite this, the studies described in this review and other work are leading to new vaccine designs, and it is hoped that one or more of these new designs will provide long-lasting protection to all people worldwide.

CONCLUSIONS

Influenza remains an important cause of human disease that often resists effective control by current vaccination strategies and there is a current push for the development of "universal vaccine" candidates. Antibody binding to Fc receptors with effector cell activation has been shown to protect in numerous animal models and harnessing this activity could be an important component of universal vaccine designs. ADCC and other activities based on the "other end of the antibody," in combination with traditional activities like neutralization, might be harnessed to contribute to protection from this ever-changing pathogen.

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TV wrote the first draft and participated in editing. MM edited the draft and prepared the final version.

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