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Antibody-dependent cellular cytotoxicity and influenza virus

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Antibodies are a key defence against influenza infection and disease, but neutralizing antibodies are often strain-specific and of limited utility against divergent or pandemic viruses. There is now considerable evidence that influenza-specific antibodies with Fc-mediated effector functions, such as antibody-dependent cellular cytotoxicity (ADCC), can assist in the clearance of influenza infection *in vitro* and in animal models. Further, ADCC-mediating antibodies that recognize a broad array of influenza strains are common in humans, likely as a result of being regularly exposed to influenza infections. The concept that influenza-specific ADCC can assist in the partial control of influenza infections in humans is gaining momentum. This review examines the utility of influenza-specific ADCC antibodies.

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Introduction

Antibody-dependent cellular cytotoxicity

The diverse effector functions of the humoral immune response increase its effectiveness against a wide range of viruses that pose a threat to global health. Antibodies bind viral surface proteins to directly neutralize infectious virions, promote phagocytosis, and promote killing of virally infected target cells by complement and cytotoxic innate effectors. The non-neutralizing functions of antibodies represent a critical link between the innate and adaptive arms of the immune system [1]. Antibody-dependent cellular cytotoxicity (ADCC) is induced when human Fc gamma receptor IIIα (FcγRIIIa) on innate effector cells is engaged by the Fc region of secreted immunoglobulin G (IgG) bound to viral antigens on the surface of an infected cell [2,3]. Natural killer (NK) cells, monocytes and macrophages express FcγRIIIa on their surface [4,5]. The multimeric engagement of FcγRIIIa molecules (often referred to as "crosslinking") on the surface of an effector cell leads to ITAM phosphorylation and subsequent activation of a Ca^{2+}-dependent signaling pathway, causing the release of preformed cytotoxic granules and apoptosis of infected target cells [6–8]. Upon FcγRIIIa crosslinking effector cells also secrete important antiviral cytokines (IFNγ and TNFα) and β-chemokines (MIP-1α and MIP-1β) [9,10]. Together these antiviral cytokines can promote an antiviral environment in which virus replication can be reduced.

ADCC responses have been shown to form a critical component of effective immunity against diverse clinically important human pathogens such as human immunodeficiency virus (HIV), West Nile virus (WNV) and influenza virus. ADCC has been extensively studied in the context of HIV vaccination and infection [11–15]. In the Thai RV144 HIV vaccine trial, which showed a modest efficacy of 31%, ADCC was identified as a key correlate of protection [12,13]. Additionally, a subset of HIV+ controllers, who maintain undetectable levels of virus without antiretroviral therapy, show greater breath of antibody binding to different subtypes of HIV, improved ADCC functionality and higher levels of ADCC activity than HIV+ individuals with progressive HIV infection [11,14,15]. Studies have shown that human flavivirus infection elicits a cross-reactive but poorly neutralizing antibody response against the fusion loop of domain II on the viral envelope protein [16,17]. Monoclonal antibodies (mAbs) against this dominant epitope protect mice from lethal WNV infection in an FcγRIIIa-dependent fashion suggesting a protective role for ADCC against flaviviruses [18].

Influenza infection and the need for better protective immunity

Influenza viruses cause periodic worldwide pandemics and any universal influenza vaccine remains elusive. Seasonal influenza epidemics are responsible for ~500 000 deaths and ~50 million cases of serious disease

each year [19]. Seasonal influenza vaccines are updated annually and are widely administered to high risk groups with the aim of inducing neutralizing antibodies [19]. However, the protection afforded by seasonal influenza vaccination is dramatically reduced if vaccine and circulating strains are mismatched. In the 2014–2015 influenza season, the Center for Disease Control estimated that the influenza vaccine only averted 6.5% of influenza-associated hospitalizations in the United States caused by a vaccine mismatch with the predominantly circulating H3N2 virus [20]. Furthermore, seasonal influenza vaccines are ineffective against potentially pandemic influenza viruses of avian origin (H5N1 or H7N9) [21,22].

There is a critical need to improve our understanding of immune responses that can protect against divergent influenza viruses. During influenza infection viral surface proteins hemagglutinin (HA) and neuraminidase (NA) accumulate on the surface of infected cells prior to budding [23], where they are can be bound by ADCC antibodies and thereby target infected cells for killing. Several recent studies have suggested that influenza-specific ADCC-mediating antibodies recognize more conserved epitopes than neutralizing antibodies and as such may contribute to the development of a universally protective vaccination strategy [24•,25••,26]. Herein we review the significance of ADCC in animal and human models of influenza infection.

Main text of review

Summary of surrogate ADCC assays

A variety of assays can be used to study influenza-specific ADCC *in vitro*. A recombinant soluble human FcγRIIIa dimer ELISA has recently been developed to detect the capacity of immobilized immune complexes to crosslink FcγRIIIa [27–29], which can induce effector cell activation and ADCC *in vivo*. In the future, FcγR dimers from other animal models of influenza infection, such as non-human primates, mice and ferrets, could be generated. The FcγRIIIa dimer ELISA is relatively economical and high throughput compared to cell-based flow cytometry assays. The FcγRIIIa dimer ELISA is, however, less biologically relevant as it is performed with influenza proteins (not necessarily in their native conformations) and without cells. NK cell activation assays with flow cytometric readouts of CD107a (degranulation) and/or IFNγ (antiviral cytokine) expression are frequently used as surrogate ADCC assays for humans and non-human primates [25••,26–34]. NK cell activation assays can be performed with immobilized influenza proteins or influenza-infected cells as targets, and primary NK cells or NK cell lines as effectors. NK cell activation assays are high throughput and use biologically relevant effector cells to assess activation and degranulation. A major shortcoming of the NK cell activation assay is that activation of primary NK cells is donor-dependent and highly variable. An ADCC reporter bioassay (commercially available from Promega) is commonly used to screen mAbs for ADCC activity [24•,25••,35•]. This assay allows mouse or human antibodies to be tested with their respective FcγRs (FcγRIIIa for humans and FcγRIV for mouse) and FcγR engagement results in luciferase production. This ADCC reporter bioassay, however, uses Jurkat cells transfected with FcγRs (and the necessary signalling machinery) as effectors in the place of more biologically relevant cells types like NK cells or monocytes/macrophages. A drawback of all the above-mentioned assays is that they do not directly measure killing of influenza-infected target cells. Chromium-51 (Cr^{51}) and non-radioactive lactate dehydrogenase release (LDH) assays are the most biologically relevant *in vitro* ADCC assays to date, as they measure elimination of influenza-infected target cells by effectors [36–41]. Cr^{51} and LDH release assays that directly measure killing are important to confirm ADCC activity, but they require a large number of controls (spontaneous release, maximum release etc.) making them less high throughput. Each surrogate ADCC assay has different strengths and weaknesses that are summarized in Table 1.

A brief history of influenza-specific ADCC

Influenza-specific ADCC was originally described nearly 40 years ago when Greenberg et al. showed that peripheral blood leukocytes (PBLs) with small amounts of associated anti-HA antibody were capable of mediating cytotoxicity against influenza-infected cells *in vitro* [36]. Maximal cytotoxicity of influenza-infected cells was observed with PBLs isolated from human subjects within 7 days of inactivated influenza vaccination or natural influenza infection, and within 9 days of experimental influenza infection [37]. Greenberg and colleagues also showed that anti-HA antibodies secreted by PBLs from influenza-infected volunteers (on days 7 and 17 post-infection) could mediate increased cytotoxicity of influenza-infected cells when added to heterologous PBLs, from a donor lacking recent influenza exposure [38]. In the early 1980s, Hashimoto et al. detected ADCC activity in sera from children vaccinated against influenza (with either inactivated or live attenuated vaccines) or naturally infected with influenza [39]. Serum ADCC antibodies were generated earlier and were more broadly reactive than hemagglutinination-inhibiting (HI) antibodies. Hashimoto et al. also showed that ADCC was primarily mediated by NK cells and that both influenza envelope proteins (HA and NA) were targeted by ADCC antibodies [39]. For several decades there was minimal study of influenza-specific ADCC, however, in recent years we and others have re-investigated ADCC to better understand its role in protecting against and clearing influenza virus infections.

ADCC as a mechanism of influenza protection *in vivo*

Mouse models of influenza infection have revealed the importance of Fc-mediated antibody functions for

Table 1

Advantages and disadvantages of surrogate ADCC assays

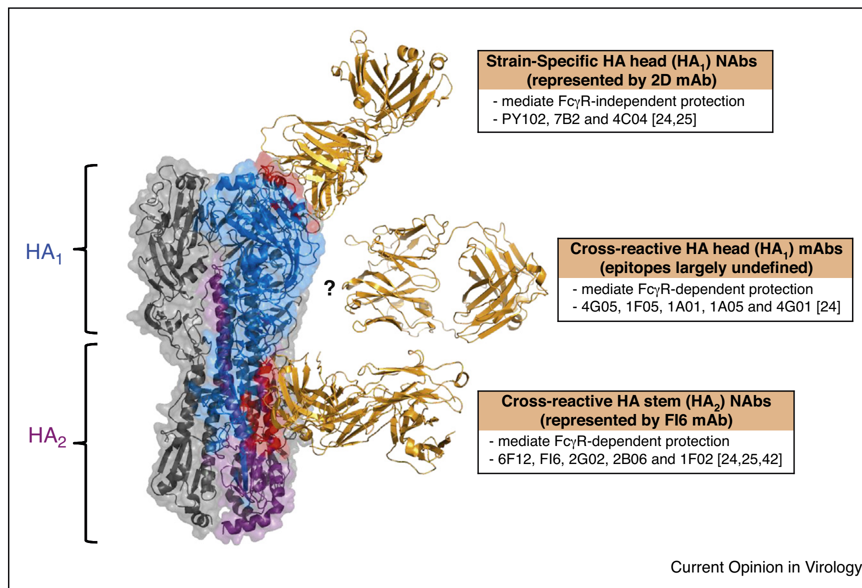
Surrogate ADCC assays	Advantages	Disadvantages	References
FcγRIIIa dimer ELISA	-Very high throughput -Relatively inexpensive -Potential to generate FcγR dimers for animal models of influenza infection	-Less biologically relevant -Influenza protein not in native conformation -No effector or target cells (not cell-based) -Does not directly measure Ab-mediated killing	[27–29]
NK cell activation assay	-High throughput -Biologically relevant effector cells and can readout multiple Ab-mediated NK cell functions (e.g., CD107a and/IFNγ expression)	-Primary NK cell activation is highly donor-dependent -Influenza protein not in native conformation (unless infected cells used as targets) -Does not directly measure Ab-mediated killing	[25••,26–34]
ADCC reporter bioassay	-High throughput -Commercially available -Biologically relevant influenza-infected target cells -Available for mouse and human Abs	-Expensive -Biologically relevant immune effector cells are not used (Jurkat cells are used) -Does not directly measure Ab-mediated killing	[24•,25••,35•]
Cr^{51} and LDH release assays	-Most biologically relevant -Directly measures Ab mediated killing of influenza-infected cells	-Not high throughput -Ab-mediated killing by primary NK cells is donor-dependent and variable	[36–41]

protection *in vivo*. Passive transfer of FI6, a human IgG1 broadly neutralizing antibody (bNAb), protected both mice and ferrets from lethal influenza infections [42]. A significant drop in efficacy was observed for a FI6 mutant (FI6-LALA) engineered to be deficient in FcγR engagement. FI6-LALA protected only 40% of mice from a lethal dose of A/Puerto Rico/8/1934 (PR8) virus suggesting that FI6 antiviral activity is in large part Fc-mediated [42]. This study shows that human IgG1 can interact with murine FcγRs, but this interaction may be less efficient since mouse and human FcγRs differ significantly in expression patterns and binding specificities. Rodents (mice, hamsters, rat) and some nonhuman primates (macaque, gibbon, orangutan) also express a novel activating IgG receptor, FcγRIV, which is not expressed in humans. Murine FcγRIV is the proposed functional homolog of human FcγRIIIa and has a high affinity for mouse IgG2a and IgG2b [4]. In 2014, DiLillo et al. screened a panel of influenza mAbs for *in vivo* protection in mice [25••]. For optimal engagement of murine FcγRs, antibody constructs with mouse IgG2a Fc, which can mediate ADCC via FcγRIV, were used for passive transfer experiments. Five bNAbs targeting the highly conserved HA stem (6F12, FI6, 2G02, 2B06 and 1F02) required Fc-FcγR interactions to protect mice from lethal influenza challenge with PR8 or A/Netherlands/602/2009 (H1N1) viruses (Figure 1), whereas mutant bNAbs null in FcγR engagement (DA265) were not protective [25••]. In contrast, antibodies against variable epitopes in the globular head of HA (PY102, 7B2 and 4C04) conferred strain-specific protection in an FcγR-independent manner (Figure 1) [25••].

To verify that the antiviral activity of HA stem bNAbs was mediated through activating FcγRs, DiLillo et al. showed that 6F12 administration failed to protect Fcγ chain-deficient (*Fcer1 g*$^{-/-}$) or FcRα-null mice from a lethal dose of PR8 virus [25••]. Infusion of FcγRIV knockout mice with an anti-HA stem bNAb (6F12) led to increased weight loss and a 50% reduction in survival compared to wild type mice, confirming that ADCC was necessary for *in vivo* protection [25••]. Interestingly, two human IgG1 anti-HA stalk bNAbs (FI6 and 6F12) were also capable of generating immune complexes that could bind to human FcγRIIIa and mediate NK cell activation *in vitro*, while a strain-specific anti-HA head antibody (PY102) could not [25••]. In 2016, DiLillo et al. showed that broadly reactive anti-HA head mAbs (4G05, 1F05, 1A01, 1A05 and 4G01), targeting conserved epitopes in the globular head of HA, also required FcγR engagement to protect mice from lethal influenza challenge *in vivo* (Figure 1) [24•]. Furthermore, a cross-reactive anti-NA mAb (3C05), but not a strain-specific anti-NA mAb (3C02), conferred FcγR-dependent protection in mice [24•]. Together these studies suggest that an antibody's ability to engage FcγRs and confer ADCC-mediated protection *in vivo* is epitope driven.

The mechanisms that underpin the role epitope localization plays with regard to ADCC activity, and any differential outcomes of influenza immunization/infection, are currently unknown. However, a recent study suggested that for efficient induction of ADCC the interaction between HA on the influenza-infected cell and sialic acid on the effector cell may be an essential second point of contact to stabilize the immunological synapse [35•]. Thus anti-HA head mAbs that block the sialic acid binding site of HA may not be able to efficiently mediate ADCC. Additional studies are warranted to clarify our understanding of this second point of contact for different viruses, FcR functions and effector cell types.

Figure 1



The epitopes bound by influenza HA mAbs dictate their ability to confer ADCC-mediated protection *in vivo*. Strain-specific NAbs bind to highly variable epitopes in the head or HA$_1$ domain (blue), protect mice from influenza challenge in an FcγR-independent manner and do not mediate ADCC *in vitro* [24•,25••]. In contrast, broadly cross-reactive NAbs that bind highly conserved epitopes in the stem or HA$_2$ region (purple) protect mice from influenza challenge through FcγR-mediated effector functions and mediate potent ADCC *in vitro* [24•,25••,42]. A number of broadly cross-reactive mAbs, with currently undefined epitopes in the head or HA$_1$ domain (blue), can similarly provide FcγR-mediated protection to lethal influenza infection in mice [24•]. Representative strain-specific NAb 2D1 (PDB: 3LZF) and stem-specific NAb FI6 (PDB: 3ZTN) are depicted binding the HA trimer of the H1N1pdm09 virus (PDB: 3LZG). The precise epitopes where broadly cross-reactive, HA head antibodies bind have yet to be resolved by crystallography.

Influenza-specific ADCC in humans

Humans are regularly exposed to diverse influenza strains that commonly generate cross-reactive ADCC antibodies. Young adults have serum antibodies that could mediate cross-reactive ADCC against an H3N2 virus from 1968, to which they had no previous exposure or serum HI activity [31]. Similarly, ADCC activity was detected against an avian influenza virus of the H5N1 subtype [31]. Intravenous immunoglobulin G (IVIG) preparations pooled from thousands of human donors and collected over a 6 year period (2004–2010) were screened and found to meditate cross-reactive ADCC to avian H5 and H7 HAs [26]. Titers of ADCC-mediating antibody in IVIG decreased when measured using the HA$_1$ domain instead of a full length HA, suggesting that broadly-reactive ADCC antibodies may be targeting conserved epitopes within the HA stalk [26]. Terajima showed that children $\geq$8 years and adults (but not infants) had high titers of antibodies able to mediate ADCC against A549 cells infected with avian influenza viruses of the H7N9 and H5N1 subtypes [40•]. Interestingly, a positive correlation was observed between serological H7N9 ADCC activity and age [40•]. Similarly, we previously showed that immediately prior to the 2009 pandemic in Australia (November 2008–May 2009) adults aged >45 years had higher baseline concentrations of antibodies mediating ADCC against the pandemic A/California/07/2009 (H1N1pdm09) virus than children (1–14 years) or young adults (15–45) [32], which may have contributed to increased protection of elderly subjects observed during the 2009 pandemic [43–46]. Collectively, serum antibodies able to mediate broadly cross-reactive ADCC are common in human populations, appear to accumulate with increasing age and may contribute to protection against newly emerging influenza viruses.

Influenza vaccination with the trivalent or quadrivalent inactivated vaccines (IIV3 or IIV4) is routine in many developed countries and enables influenza-specific ADCC elicited by immunization to be characterized. Our group demonstrated that IIV3 did not generate ADCC-mediating antibodies in influenza naïve pigtailed macaques [30], however these results do not accurately reflect the outcome of influenza vaccination in humans because (unlike macaques) humans are serially exposed to influenza from early childhood. Recently, Kristensen et al. found that IIV3 immunization boosted ADCC activity to the HA proteins of all three vaccine strains in both HIV− and HIV+ adults [27]. Similarly, Zhong et al. showed that IIV4 immunization of healthy adults increased serum ADCC activity against H3 antigens from the H3N2 vaccine virus and an antigenically drifted

circulating H3N2 virus [47]. Further, a monovalent inactivated subunit vaccine (IIV) targeting the H1N1pdm09 virus significantly increased ADCC-mediating antibody titers against both H1N1pdm09 and a range of heterologous group 1 influenza viruses [33••]. A live attenuated influenza vaccine (LAIV) against the H1N1pdm09 virus did not increase ADCC-mediating antibody titers in vaccinated adults or children [33••]. This study disagreed with previous work by Hashimoto et al. that showed increased ADCC activity following LAIV in children [39], however the LAIVs used in these two studies targeted heterologous influenza viruses from different subtypes and groups. The elderly bear the greatest burden of influenza-related disease and recent studies have shown that elderly adults who seroconverted to the vaccine viruses following immunization with IIV3 also demonstrated a rise in serum ADCC activity [48]. Vaccines targeting avian influenza viruses have been widely studied in preparation for a potential pandemic. Immunization of healthy adults with two doses of an adjuvanted H5N1 avian influenza vaccine has been shown to induce a robust increase in ADCC-mediating antibody titers against the homologous HA protein [49]. Taken together, these studies suggest that vaccination can elicit influenza-specific ADCC activity, but different vaccine types and compositions vary in their ability to induce ADCC-mediating antibodies.

Studying naturally acquired influenza infections in humans is complicated by limited definition of pre-existing influenza exposures, absence of pre-infection samples and variable timing of presentation and outcomes. Nevertheless, Jegaskanda et al. showed that naturally acquired H1N1pdm09 influenza infection did not drive a rise in serum ADCC activity between day of presentation (or the first day of medical attention) and 28 days later [33••]. These results are in agreement with studies of naturally acquired influenza infection from the late 1970s, which showed that anti-HA antibodies secreted 7–17 days post-infection could increase ADCC activity *in vitro*, but by day 35 post-infection ADCC activity had returned to baseline [38]. Subjects experimentally infected with A/Wisconsin/67/131/2005(H3N2) virus showed significant rises in ADCC activity against both infected A549 cells and the HA protein of the infecting virus [33••]. It is not surprising that human studies performed with experimentally and naturally acquired influenza infections gave conflicting results. Experimentally (unlike naturally) infected humans have pre-infection samples to define baseline levels of ADCC activity and the influenza viruses used for experimental infections tend to result in relatively mild disease. It is therefore essential to study both types of influenza infection in order to gain a complete understanding of the relationship between human influenza infection and serological ADCC activity. Two separate studies with subjects similarly infected with A/Wisconsin/67/2005(H3N2) observed that individuals with more clinical symptoms tended to exhibit a greater rise in HA-specific ADCC activity to the homologous HA [28,33••], possibly caused by increased virus replication and antigen availability. A very small subset of subjects (n = 3) with ADCC antibody titers $\geq$320 prior to experimental influenza infection showed lower severity of disease and less total detectable virus (by PCR and $TCID_{50}$) than those with ADCC antibody titers <320 [33••]. Additionally subjects with pre-infection ADCC antibody titers $\geq$320 were more likely to have undetectable virus in nasopharyngeal swabs within the first 24 hours following experimental infection than subjects with lower ADCC antibody titers [33••]. This preliminary study introduces the possibility that high levels of serum ADCC activity prior to infection could decrease viral load and severity of influenza disease. However, larger infection cohorts, including those with more severe naturally acquired infections, are required to draw specific conclusions.

Influenza ADCC to non-envelope proteins

Since influenza envelope proteins HA and NA are prone to antigenic drift, there has been recent interest in antibodies able to elicit ADCC against conserved influenza antigens such nucleoprotein (NP) and the extracellular domain of matrix 2 protein (M2e). NP can be detected on the surface of influenza-infected cells *in vitro* [41,50] and could provide a highly conserved target for ADCC. Supporting this idea, passive transfer of anti-NP antibodies can protect mice from heterosubtypic influenza challenge through an FcγR-dependent mechanism [51,52]. Recent work by our group showed that healthy and influenza-infected humans have anti-NP antibodies that can cross-link FcγRIIIa and activate human NK cells [28]. Future studies with purified anti-NP antibodies (polyclonal or mAbs) and influenza-infected cells will confirm if human NP antibodies have the capacity to mediate ADCC *in vitro*.

Like NP, M2e is on the membrane of influenza-infected cells. Injection of anti-M2e immune serum has been shown to protect wild type mice, but not FcγR knockout mice (FcγR $^{-/-}$) from a lethal PR8 influenza infection [53]. Furthermore, human anti-M2e IgG1 was only protective in mice that expressed a functional FcγRIII [53]. Prophylactic treatment of mice with a human anti-M2e mAb (Z3G1) decreased viral load in the lungs through both FcγR- and complement-dependent mechanisms following a sublethal influenza infection [54]. Simhadri and colleagues recently showed that a human mAb targeting the M2 protein (Ab1-10) was capable of activating NK cells and mediating ADCC of M2 expressing 293FT cells and influenza-infected A549 cells [55]. These studies suggest that both NP and M2 could be important targets of influenza-specific ADCC as they are highly conserved between strains and subtypes of the virus.

Conclusions

There has been a renewed interest in the ability of ADCC to provide a mechanism to partially protect against infections arising from the diverse pool of circulating and potential pandemic influenza strains. The generation of ADCC activity targeting both HA and more highly conserved influenza proteins may be critical in the development of a more universal influenza vaccine.

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