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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