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Progress on adenovirus-vectored universal influenza vaccines

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Abbreviations: flu, influenza; IFV, Influenza virus; HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; M1, matrix protein 1; M2, matrix protein 2; IIVV, inactivated influenza virus vaccine; LAIV, live attenuated influenza vaccine; Ad: adenovirus; rAd, recombinant adenovirus; ITRs, inverted terminal repeats; HDAd, helper-dependent adenoviral; HEK293, human embryonic kidney 293 cell; RCA, replication competent adenovirus; DVD, drug–vaccine duo; HI, hemagglutination inhibition; mAbs, monoclonal antibodies; FcγRs, Fc receptors for IgG; ADCC, antibody-dependent cell-mediated cytotoxicity; IF-γ, interferon-γ; IL-2, interleukin-2; MHC-I, major histocompatibility complex class I; HLA, human leukocyte antigen; VAERD, vaccine-associated enhanced respiratory disease; CTLs, cytotoxic T lymphocytes; APC, antigen-presenting cell; DC, lung dendritic cells; CAR, Coxsackie-Adenovirus Receptor

Influenza virus (IFV) infection causes serious health problems and heavy financial burdens each year worldwide. The classical inactivated influenza virus vaccine (IIVV) and live attenuated influenza vaccine (LAIV) must be updated regularly to match the new strains that evolve due to antigenic drift and antigenic shift. However, with the discovery of broadly neutralizing antibodies that recognize conserved antigens, and the $CD8^+$ T cell responses targeting viral internal proteins nucleoprotein (NP), matrix protein 1 (M1) and polymerase basic 1 (PB1), it is possible to develop a universal influenza vaccine based on the conserved hemagglutinin (HA) stem, NP, and matrix proteins. Recombinant adenovirus (rAd) is an ideal influenza vaccine vector because it has an ideal stability and safety profile, induces balanced humoral and cell-mediated immune responses due to activation of innate immunity, provides 'self-adjuvanting' activity, can mimic natural IFV infection, and confers seamless protection against mucosal pathogens. Moreover, this vector can be developed as a low-cost, rapid-response vaccine that can be quickly manufactured. Therefore, an adenovirus vector encoding conserved influenza antigens holds promise in the development of a universal influenza vaccine. This review will summarize the progress in adenovirus-vectored universal flu vaccines and discuss future novel approaches.

Introduction

Influenza is an acute respiratory infectious disease that leads to serious health problems. Each year, influenza infects 5%–10% of adults and 20%–30% of children globally. Worldwide, 3 to 5 million cases of severe illness and approximately 250 000 to 500 000 deaths due to influenza are reported each year,[1] and the newest statistical data show that influenza activity continues to increase in the southern hemisphere.[2]

Influenza is classified into 3 groups: A, B and C; however, influenza A is responsible for most seasonal influenza infections and all known pandemics.[3] Influenza viruses are divided into 17 HA subtypes and 10 neuraminidase (NA) subtypes based on the expressed surface proteins HA and NA.[4] Influenza evolves through antigenic drift and antigenic shift, resulting in the emergence of new strains; therefore, IIVV and LAIV cannot control emerging pandemic influenza virus threats. Furthermore, the production of a new vaccine cannot be achieved until 4 months after the identification of a pandemic strain[5] because it is not easy to expand vaccine production capacity within a short time due to limited egg supplies. In general, both IIVV and LAIV have limited capacity to prevent and control pandemic influenza; therefore, identifying alternative vaccine strategies for influenza outbreaks is critical. Recent studies have led to progress in the development of a universal vaccine. rAd is a respiratory virus. An adenoviral vector can mimic natural infection[6] and induce long-term cross-protective immunity toward influenza viruses,[7,8] and many studies indicate that rAd induces effective transgene-specific humoral[9] and cellular immune responses.[10,11] Therefore, the adenovirus vector is one of the most promising types of vaccine vectors. This review describes the progress in adenoviral vectored universal flu vaccines and outlines novel future approaches.

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Recombinant Adenoviral Vectors for Vaccines

Adenovirus was first isolated from human adenoid tissue culture nearly 60 y ago,[12] and since then, additional adenoviruses have been isolated from a variety of animal species and humans.[13]

Human Ads are classified into 53 serotypes, which are grouped into 7 subgroups (A-G), based on serological properties and genome DNA sequences.[14] Adenovirus is a non-enveloped, 70–100-nm diameter, icosahedron, DNA virus.[15] The adenovirus capsid is composed of 3 major structural proteins (i.e., hexon, penton base and fiber) and several minor proteins.[16] The viral genome is a linear, double-stranded DNA between 33 and 38 kb that is flanked by 2 inverted terminal repeats (ITRs); the upstream ITR is followed by a packaging signal (ψ).[17] The Ad genes are classified into early transcription units (E1a, E1b, E2a, E2b, E3 and E4) and later transcription units (L1-L5).[17,18]

rAds have many advantages as vaccine delivery vectors. Many clinical and preclinical studies have demonstrated that rAds are safe, and rAd-vectored vaccines can be easily generated and cultured in suspension cells, such as PER.C6, at low cost.[19] The rAd vaccine may retain activity for at least 1 y in lyophilized or liquid form,[20] and new thermostabilization techniques enable the complete recovery of rAd titer and immunogenicity after storage at up to 45°C for 6 months and longer, with minimal losses.[21] rAd vectored vaccines do not require classical adjuvants, which may result in unpredictable side effects,[22] because the Ad hexon protein is a potent adjuvant for the activation of innate immunity.[23] rAd can infect a variety of cells and tissues; therefore, rAd can be administered via nasal, aerosol and intramuscular vaccination.[24,25]

Ad5 has been widely studied, and we now have extensive knowledge of the structure of the virion, the mechanism of the virus-cell interaction, and the replication, transcription, expression and assembly of the virus.[17] Ad5 is primarily used for gene/vaccine delivery vectors,[26] and currently, rAd5 vectors are in at least the third generation of development. Progenitor vectors with the E1 gene deleted can be packaged and cultured in the human embryonic kidney 293 (HEK293) packaging cell line, which provides the E1 gene product in trans.[27] Replication-deficient adenovirus can regain the deleted E1 gene and become replication-competent adenovirus (RCA) as a result of recombination.[28] The appearance of RCA in an Ad vector population raised the possibility of undesired Ad infection. Furthermore, RCA also induces the host immune response, which may result in inflammation and tissue damage.[29] An RCA-free Ad vector can be constructed using the PER.C6 cell line,[30] which has permitted the production of adenovirus for clinical trials using good manufacturing practices.[30,31]

Furthermore, the second-generation rAd vector had the E2 and/or E4 as well as E1/E3 genes deleted from the vector backbone to reduce toxicity and increase the packaging size of the rAd vector.[32,33] The third-generation rAd vector has nearly all capsid-coding sequences deleted, except for the essential cis-acting elements, including the ψ and ITRs. A helper virus provides the viral functions that are required for replication of the vector DNA, produces viral structural proteins and packages the vector DNA into virions. Therefore, third-generation rAd vectors are also called helper-dependent adenoviral (HDAd) vectors.[34] The second- and third-generation rAd vectors avoid pre-existing anti-vector immunity and induce robust immune responses against the encoded transgenes.[35] While major anti-Ad adaptive immune responses focus on the capsid proteins, antigen-presenting cells (APCs) infected by the Ad5 vector deleted for E1 and E2b may be less susceptible to attenuation by pre-existing anti-Ad immunity because deletion of E2b prevents expression of late gene products, including highly immunogenic proteins such as hexon. Thus, the infected DC are not cleared as rapidly by NK cells, allowing more time for immune responses to the antigen (Ag) to develop.[35] However, the problems of manufacturing and purification remain unsolved.

Ad5 is the leading subtype of human adenovirus. Because of natural exposure to wild type Ad5, antibodies against Ad5 pre-exist in the majority of human populations,[36,37] which may severely reduce the immune response to injected Ad5-vectored vaccines.[38,39] Many researchers have attempted to resolve this potential problem by developing rare-serotype rAd vectors (Ad11, Ad26, Ad35, Ad48, Ad49 and Ad50),[40-44] nonhuman rAd vectors (chimpanzee Ads,[45,46] bovine Ad3,[47] canine Ad2,[48] porcine Ad3[49]), and molecularly engineered Ad5 vectors.[50,51] However, studies have shown that novel rAd vectors derived from rare serotypes and nonhuman rAd vectors are less potent than rAd5 vectors.[43,52,53] Furthermore, pre-existing Ad5-specific T cells are cross-reactive with Ad vectors derived from rare serotypes.[54] Hutnick et al. found that Ad-specific $CD4^+$ and $CD8^+$ T cell responses against chimpanzee-derived AdC6 and AdC7 were found in all 17 human subjects, indicating the commonality of cross-serotype reactivity of Ad-specific T cells.[55] This cross-reactivity is due in part to epitopes recognized by Ad-specific T cells conserved across many adenovirus serotypes.[54,56,57] The prevalence and cross reactivity of Ad-specific T-cells in humans may interfere with transgene product-specific immune responses by eliminating vector-infected cells even when rare serotype Ad vectors are employed.[55]

Because of these obstacles, researchers have designed molecularly engineered Ad vectors to induce lower immune responses than wild type Ad.[58,59] These approaches include PEGylation of vectors, using fibers from other serotypes, modification of fibers, and using hexon proteins modified by 'Antigen Capsid-Incorporation'.[59] Because fiber proteins modified with polylysine residues target heparin sulfates on the cellular surface, an Ad in which the fiber protein is modified to contain 7 lysine residues, AdK7, shows reduced spleen distribution, which in turn decreases the production of inflammatory cytokines, compared with conventional Ad.[60] Because fiber binding to Coxsackie-Adenovirus Receptor (CAR) plays a major role in inducing the production of cytokine in non-immune cells,[61] another strategy for reducing innate immune responses is the substitution of Ad5 fiber with the fiber protein of other types of Ad vectors that do not bind to CAR, such as Ad7, Ad35 and Ad4.[62,63] Ad vector modified with monomethoxypoly-ethylene glycol (MPEG) is another approach to avoid the innate immune responses. PEGylation reduces vector uptake in spleen, resulting in the decrease of cytokine production.[64] 'Antigen Capsid-Incorporation' is another novel strategy to circumvent preexisting immunity. This strategy consists of incorporating antigenic peptides within the Ad capsid protein, and offers potential advantages: a strong humoral response against the given Ag

similar to the response generated by native Ad capsid proteins, allowing boosting of the immune response against antigenic epitopes that are part of the Ad capsid[50,51] Antigen capsid-incorporation display platforms based on Ad5[65,66] and Ad3[67] have been used for a variety of vaccines against infectious diseases, including virus infection[66,67] and parasite infection[65]. The results show that this novel Ag capsid-incorporation approach may provide exciting opportunities to circumvent the major limitations associated with Ad vectors. Although some progress has occurred for molecularly engineered rAd, it is difficult to construct and manufacture these new vectors on a large scale. Furthermore, the safety of these new vectors remains unclear.[68]

Administration Route of Adenovirus-Vectored Vaccine

rAd can infect a variety of cells and tissues and can be administered via many delivery routes, such as nasal and aerosol vaccination.[24,69-72] The route and dose of rAd administration impact the phenotype and quality of the transgene-specific immune response.[73-76] The traditional intramuscular route induces robust humoral and cellular immune responses;[77] however, pre-existing Ad5 immunity can weaken the immune responses of rAd vectors.[78] Mucosal immunity may overcome pre-existing immunity against the rAd5 vector. Growing evidence shows that nasal vaccination can effectively avoid pre-existing Ad5 antibodies in mouse, rabbit and primate animal models, induce a potent antibody (Ab) effect against the encoded antigens and protect the vaccinated animal from pathogen challenge.[9,79-81]

Clinical research indicates that nasal vaccines are more potent than epicutaneous administration under adjuvant-free conditions. Nasal Ad5 vaccines induce strong immune responses, even when antibodies against Ad5 exist.[70] Recent research has focused on mucosal immunity, including mucosal immunity in response to nasal and aerosol vaccinations, because there are many advantages to mucosal immunity. Mucosal administration is a pain-free and needle-free systemic delivery that can be performed by non-medical personnel[82]. Therefore, this type of vaccine may be suitable for mass vaccination programs during a crisis because nasal and aerosol administration is simple and economical and these vaccines are well tolerated.

Pre-existing S-IgA, IgG and $CD8^+$ T cells are the keys to broad-spectrum cross-protection.[83] Increasing evidence has shown that nasal vaccination with the rAd-vectored influenza vaccine induces robust antigen-specific IgAs and IgGs during respiratory illness. Compared to other administrations, mucosal rAd induced stronger IgA responses and more virus-specific activated T cells in the lung.[84-86] Because mucosal vaccination can mimic natural infection, it is superior to parenteral administration for inducing cross-protection. Furthermore, mucosal vaccination can induce a cross-reactive IgA and IgG response, resulting in cross-protection against different subtypes of influenza viruses.[87-89]

Mucosal vaccination shows good safety. Nasal-vaccinated rAd seeds into the olfactorius bulbus and central nervous system (CNS),[90-92] and no cytopathic effect (CPE) has been observed in the CNS due to this approach.[90] Nasal administration of an Ad-vectored vaccine encoding influenza HA has also been shown to be safe and well tolerated in human volunteers.[70]

Intranasal spray is an efficacious delivery route for the rAd vector. However, many of the large droplets do not reach the target nasal airway tissues. Aerosol delivery may provide a strategy to improve vaccine efficacy[93] because a fine aerosol regimen of rAd vector induced remarkably high and stable lung T-cell responses and humoral responses of both IgA and IgG isotypes in nonhuman primates.[72] rAd5 encoding influenza HA protected ferrets against challenge with a lethal dose of H5N1 avian influenza via 4-μm aerosol immunization.[72] To achieve better mucosal immunity induced by an aerosol rAd vector, Roy et al. characterized the dynamics of aerosolization and its effects on immune responses, including particle size, vector viability, and the actual delivered dose of the aerosolized adenoviral vector. Because of the clogging effect, a nebulizer can produce smaller aerosolized particles at high rAd concentrations. The particle diameter has an effect on the immune responses of rAd because the smaller particles can reach deep into the respiratory tract and induce robust biological responses.[94]

Mucosal vaccination with rAd5 rapidly induces an anti-influenza state, similar to a prophylactic drug, followed by the elicitation of sustained protective immunity, similar to a vaccine. Therefore, rAd5 confers seamless protection against mucosal pathogens when administered as a drug-vaccine duo (DVD) in a single package by mucosal vaccination.[95] rAd vectors have been shown to activate innate immune responses and induce the production of inflammatory cytokines and chemokines in mouse models.[96] Many factors have been shown to be involved in this process, including type I interferon (IFN-α and β),[97] lung dendritic cells (DCs),[98] natural killer cells[99] and antiviral nitric oxide.[100] The effects induced by DE1E3 Ad5 result in a multi-dimensional defense barrier against infection by IFV, and these protective effects persist for at least 3 weeks and up to 47 d when administered in a single-dose regimen.[95] Therefore, rAd, as a prophylactic drug, may provide protection against IFV infection prior to inducing specific immunity by a rAd encoding IFV Ag. M2 ion channel blockers and neuraminidase inhibitors may contribute mutational pressure for further selection of resistant isolates of IFV.[101] Conversely, Ad5-DVD induces an anti-influenza effect by changing the biological state of the respiratory tract and activating a specific innate immunity to prevent IFV growth without directly attacking the IFV[95]. Therefore, it is conceivable that Ad5-DVD may confers no mutational pressure that could induce drug resistance. Moreover, administration of the neuraminidase inhibitor, oseltamivir (OSV), suppresses respiratory mucosal secretory IgA responses and increases the risk of re-infection, whereas mucosal vaccination with rAd5 enhances mucosal innate immunity against IFV.[102] Unlike LAIV, Ad5-DVD cooperates with contemporary influenza drugs because of its lack of antiviral drug targets.[30,95]

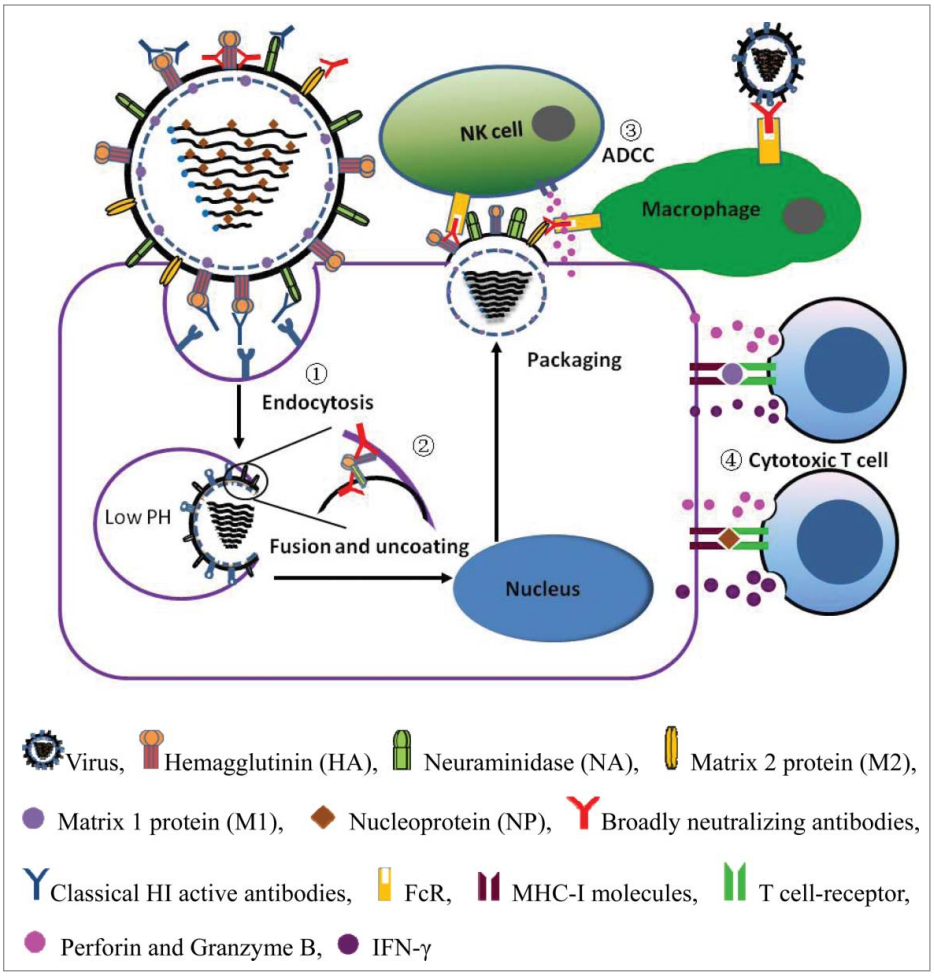

Figure 1. Schematic diagram of influenza virus infection and the adaptive immune responses involved in host defense. (**1**) Classical HI antibodies prevent receptor-mediated endocytosis of the virus by binding to the HA head domains, which are typically variable. (**2**) Broadly neutralizing antibodies prevent membrane fusion by binding to the highly conserved HA stem. (**3**) Broadly neutralizing antibodies specific for HA stem and viral internal proteins mediate ADCC of infected cells, which is dependent on binding to FcR. (**4**) The influenza A virus internal proteins, M1 and NA, induce cytotoxic T lymphocyte-specific responses, which are dependent on MHC-I molecules.

Influenza-Specific, Broadly Neutralizing Antibodies

HA is currently a major target of influenza vaccine research. The HA protein is a trimer of approximately 13.5 nm (135 Å) and is found on the surface of the virus. The trimeric HA ectodomains consist of the HA1 and HA2 domains, which are assembled into a head domain and a stem domain.[103] HA head domains are the major protective antibody-binding site (**Fig. 1**), and neutralizing antibodies can induce a serum hemagglutination inhibition (HI) effect.[104] The HA head domain evolves with a high mutation rate to avoid initial antibody suppression;[103] therefore, the classical influenza vaccine must be continuously updated to defend against the challenge of new mutational viral strains.

Influenza virus A viruses are divided into 17 subtypes based on HA and are further segregated into 2 phylogenetic groups (**Fig. 2**).[4] In 1993, the Japanese researcher Y. Okuno first found a monoclonal antibody, designated C179, that neutralized all H1 and H2 strains of influenza A virus.[120] Further research showed that C179 also neutralized the H5 strain.[121] The monoclonal antibody C179 not only protected a mouse model from challenge with H1 and H2 influenza virus infection but also treated H1, H2 and H5-induced bronchopneumonia in the mouse model.[105,106] Then, new antibodies, CR6261 and F10, were found to neutralize additional influenza viruses in group 1, including H1, H2, H5, H6, H8 and H9.[107-109] In 2010, Wang et al. identified the monoclonal antibody 12D1, which neutralized H3 virus in group 2 strains and protected from challenge by the H3 subtype strain.[110] However, researchers did not identify an antibody that neutralized all group 1 and group 2 viral strains until FI6v3 was found in 2011.[111] Furthermore, CR9114 neutralizes both influenza A and B viruses and protects against lethal challenge with H1N1, H3N2 and influenza B viruses[112] (**Table 1**).

In contrast to the most abundant influenza antibodies that interfere with receptor binding by binding to the head of HA, the above-mentioned, broadly neutralizing antibodies recognize a highly conserved and hydrophobic helical region in the membrane-proximal stem of HA, the 'fusion peptide',[108] which plays a decisive role in the membrane fusion process. When HA undergoes a conformational change at low pH (5~6) in the endosome, the fusion peptide is exposed and inserted into the endosomal membrane, causing the endosome and virus to fuse, followed by the release of viral RNA and successful infection.[113] Universal monoclonal antibodies (mAbs) block infection by inserting their heavy chains into the conserved fusion peptide in the stem region, thereby preventing membrane fusion[109] (**Fig. 1**).

Both x-ray crystallography and electron microscopy models suggest that universal monoclonal antibodies bind to the stem region of HA trimers and block the pH-induced conformational changes in HA.[112] HA is active as a trimer on the viral surface, and the trimeric stem domain is the key to the induction of universal neutralizing antibodies; therefore, mutational escape is not possible due to the critical function and conserved helical structure of the stem. However, it is difficult to simulate the trimeric HA stem domain.

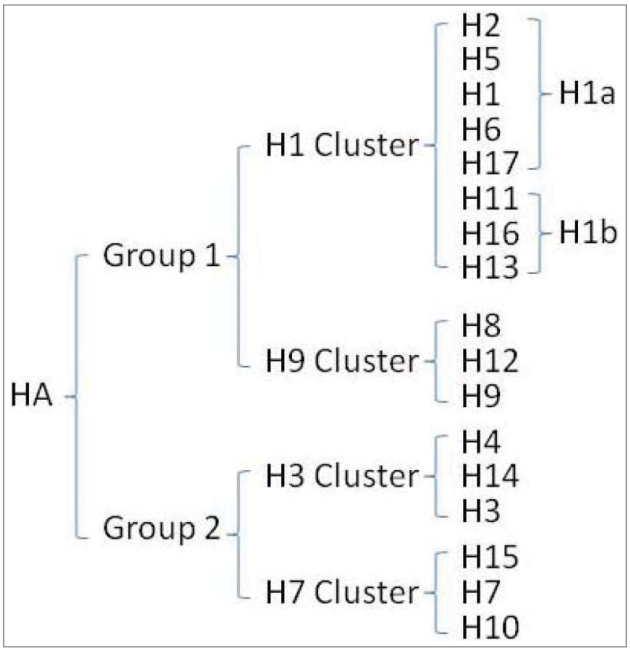

Figure 2. HA Subtypes.

Several research groups have been developing a broadly protective influenza vaccine based on the stem domain.[114–116] However, none of the vaccines have produced a properly folded stem trimer. Wei et al. constructed a rAd vector encoding a stem mutant trimer that was recognized by the mAb C179.[69] Lin et al. used baculovirus-insect cell expression to obtain trimeric HA proteins that resulted in high levels of neutralizing antibodies when coupled with a PELC/CpG adjuvant.[114] Lin et al. also constructed a glycan-masked HA mutant that overlapped with broadly neutralizing epitopes of the mAb CR6261. The trimeric HA mutant induced HA-inhibition and virus-neutralizing antibodies.[115] Because the head covers conserved Ag epitopes on the stem region, the neutralizing antibodies that were induced by the complete trimeric HA recognized the head of HA but only blocked a few subtypes in the same group of influenza A viruses.[103]

In addition to recognizing conformational epitopes, the monoclonal antibody 12D1 also binds to a linear epitope present between amino acids 76–106 in the stem of HA.[110] Hu et al. found three neutralizing mAbs (1F2,1F4, and 1E1) that could neutralize different influenza virus strains between group 1 and group 2, including subtypes of H1(H1N1), H3 (H3N2), H5 (PR8-H5), H7 (PR8-H7), and H9 (H9N2). The three mAbs could specifically recognize a conserved linear epitope that is part of the fusion peptide on HA2.[117] Nevertheless, a number of linear and conformational neutralizing epitopes within the HA stem shows that this region is complicated. Therefore, further research is needed to understand the wide diversity of interaction between neutralizing antibodies and the HA stem region.

Universal Antibody-Dependent, Cell-Mediated Cytotoxicity

Typically, stem-specific Abs prevent membrane fusion between the endosome and virion membranes but do not induce a HI effect via the receptor-binding site, as do classical head-specific Abs. Further research revealed that only anti-stem mAbs were capable of mediating antibody-dependent cell-mediated cytotoxicity (ADCC) of infected cells, which is dependent on the binding of Fc receptors (FcRs) to IgG[116] (**Fig. 1**).

Influenza A virus internal antigens are also involved in potent ADCC effects. Cells infected with influenza virus express nucleoprotein (NP) on their surface,[118] and a natural anti-NP antibody was detected in human serum[119] that specifically promoted heterosubtype influenza virus clearance in mice via ADCC involving FcRs.[120] The high conservation of NP Ag and the ADCC effect may provide a critically necessary component of a universal influenza vaccine. Jegerlehner et al. found that mice immunized with M2 coupled to hepatitis B core (M2-HBc) produce M2-specific protective Abs that failed to neutralize the virus in vitro. NK cells are important for protection induced by M2-HBc. They also found that the dominant M2-specific Ab isotype after infection of vaccinated mice is IgG2b, followed by IgG2a.[121] These 2 isotypes have been shown to be the most important mediators of ADCC in mice.[122] The M2-specific mAb Z3G1 recognizes a broad spectrum of M2 variants from natural viral isolates. Passive immunotherapy with Z3G1 significantly protected mice from influenza A infection via ADCC.[123,124] These results indicate that M2 may also induce protection through an ADCC-dependent mechanism.

Table 1. Broadly neutralizing antibodies against influenza A virus

MAb	Group	Subtype	Challenge	Model	Reference
C179	1	H1, H2, H5	H1N1/H5N2	Mice	105,106
CR6261	1	H1, H2, H5, H6, H8, H9	H1N1/H5N1	Mice	107,108
F10	1	H1, H2, H5, H6, H11, H13, H16, H9	H1N1/H5N1	Mice	109
12D1	2	H3	H3N2	Mice	110
CR8020	2	H3, H7, H10	H3N2/H7N7	Mice	190
FI6v3	1 and 2	All	H1N1	Mice, ferret	111
CR911*	1 and 2	All	H1N1/H3N2	Mice	112

*Also neutralizes influenza B virus and protects a model from challenge with lethal influenza B virus.

Influenza Virus-Specific T-Cell-Mediated Immunity

To effectively prevent influenza virus infection, an ideal influenza vaccine should induce a cell-mediated immune response to limit disease severity when mucosal and humoral immunities are inadequate or are circumvented by a reassortant virus. In cell-mediated immunity, the T-cell response effectively clears the virus and promotes the recovery from influenza virus infection.[125] Mice lacking $CD8^+$ T cells have significantly delayed pulmonary viral clearance and a significantly higher mortality rate than control mice.[126] However, mice devoid of Abs and mature B cells can survive primary influenza infection. These mice cleared virus from the lungs in a process dependent upon $CD8^+$ T cells, and these Ab knockout mice can produce antigen-specific immune protection against challenge infection.[127,128] Adoptive transfer of cytotoxic T lymphocytes (CTLs) to mice challenged with a lethal dose of influenza virus has been shown to cause a significant reduction of the infectious virus levels in the lungs and prevented death.[129] Further studies found that the adoptive cross-reactive CTL clone A7 protects mice from a simultaneously lethal challenge with H1N1 and H2N2 subtypes and promotes complete recovery.[130] In a non-human primate model of influenza, IFN-γ^+ $CD8^+$ T cells mediated the early clearance of an antigenically novel influenza virus.[131] Furthermore, memory CTLs (mCTLs) reduced the titers of heterologous type A viruses 2–3 d earlier than in naïve controls.[132] The frequencies of pre-existing T cells specific for conserved CD8 epitopes have a strong inverse correlation with illness severity and the total symptom score of influenza,[133] demonstrating that cross-reactive T cell responses play an important role in the early clearance of newly emerging pandemic influenza viruses.

Activation of T cells is initiated by major histocompatibility complex class I (MHC-I)-displaying viral epitopes from within the infected cell to T cells. In humans, MHC is also called human leukocyte antigen (HLA). Polymorphic HLA molecules occur at significantly different frequencies in different ethnicities; therefore, a single $CD8^+$ T cell epitope derived from a conserved influenza viral protein may be insufficient to induce strong cellular immunity in different populations.[128,129]

Although hundreds of HLA alleles are present in the human population, a large fraction of HLA Class I molecules have overlapping repertoires of binding specificity. Therefore, HLA Class I molecules can be grouped into 9 supertype families based on overlapping peptide-binding repertories and consensus B- and F-pocket structures.[134–136] It is possible to account for the predominance of all known HLA class I molecules with only 9 main functional binding specificities. Assarsson et al. identified 54 non-redundant conserved epitopes (38 class I and 16 class II) that bind to the common HLA alleles and belong to the corresponding 6 class I (A1, A2, A3, A24, B7, B44) and 1 class II (DR) supertypes that provide high coverage among different ethnicities. The theoretical population coverage for the class I and class II epitopes was high throughout the major different populations, with an average of 98.5%. On average, each individual was calculated to bind 6.5 epitopes.[137]

Influenza virus epitope information can be accessed from the Immune Epitope Database and Analysis Resource (IEDB, http://www.immuneepitope.org/). The IEDB contains data related to both B cell and T cell epitopes from infectious pathogens.[138] Available online since January 2005, the IEDB data are derived from over 4000 literature references and imported from previously developed databases.[139] The IEDB provides various online tools that cover a broad range of research areas relating to epitope discovery and analysis to assist in vaccine discovery and development.[138] Particularly, tools to visualize data are hosted, such as tools for viewing 3D structural data that provides antibody and Ag interaction information.[140] Researchers can easily access relevant epitope information from the IEDB to assist in the development of prophylactic or therapeutic approaches against infectious diseases.

The published data for influenza-derived epitopes indicate that the major highly conserved epitopes broad binding to class I HLA supertype molecules are located within NP, M1 and PB1[137,141-143] (**Fig. 1**). These influenza T cell epitope data were obtained using mice and other mammalian models; however, it is difficult to provide proof-of-concept support for the protective capacity of T cells against influenza illness in humans.[144] Sridhar et al. followed 342 healthy adults through the pandemic waves of influenza and correlated the responses of pre-existing T cells with clinical outcomes. They found that individuals who developed less severe illness had higher frequencies of pre-existing T cells specific for the conserved $CD8^+$ epitopes. The total symptom score had the strongest inverse correlation with the frequency of IFN-γ^+IL-$2^-$$CD8^+$T cells. In the absence of cross-reactive neutralizing antibodies, $CD8^+$T cells specific to conserved viral epitopes play a key role in the reduction of influenza symptoms and cross-protection against influenza. This protective immune response correlation may guide universal influenza vaccine development.[133]

Cooperation Between $CD8^+$T-Cell- and Virus-Specific Non-Neutralizing Antibodies

Laidlaw et al. found that virus-specific $CD8^+$ T cells or virus-specific non-neutralizing antibodies are relatively ineffective at conferring heterosubtypic protective immunity alone. However, both cooperatively elicit robust cross-protective immunity against H1N1 and H3N2,[145] and this synergistic effect is dependent on alveolar macrophages. Therefore, the basis for a potential 'universal' vaccine is the capacity to elicit both $CD8^+$ T cells and antibodies specific for highly conserved influenza proteins. Ad is a respiratory virus and is therefore an ideal vector to activate alveolar macrophages.

rAd-Vectored Universal Influenza Vaccine

Many studies have evaluated the protective effect of Ad-vectored influenza vaccine against various subtypes,[146–148] and some have completed phase I clinical trials.[148] The HA protein plays

critical roles in the early stages of virus infection by binding to viral receptors and mediating membranes fusion between viruses and cells.[149] Therefore, the HA protein is an attractive target of influenza vaccine research. The Ad-vector-based, full-length H5N1 HA (A/Vietnam/1203/04) has been shown to induce homologous and heterotypic (A/Hong Kong/156/197) HI responses[146]. Furthermore, another study assessed the protective efficacy of rAd-HA against challenge with variant H5N1 strains. Immunization of mice with rAd-HA/H5N1/Hong Kong/156/97 provided effective protection from heterologous H5N1 (A/Hong Kong/483/97, A/Vietnam/1203/04, and A/Hong Kong/156/197) disease, death, and primary viral replication, even without a strong humoral neutralizing response against A/Vietnam/1203/04 virus.[147] Two studies of Ad-vectored HA (H3N2) vaccines have revealed that cross-protection from heterotypic challenge can also occur in the absence of neutralizing humoral immunity in swine and mice.[70,150] H1N1 HA has a similar protection efficacy. Vaccination with plasmid DNA encoding H1N1 HA and boosting with a rAd vector encoding HA stimulated broadly neutralizing antibodies that recognized diverse H1N1 strains dating from 1934 to 2007 and conferred protection against divergent H1N1 viruses in mice and ferrets.[69] These studies indicate that cellular immunity likely plays a major role in heterotypic immunity. In addition, $CD4^+$ and $CD8^+$ T cell-mediated immunity may play important roles in protecting against this virus and promoting recovery after influenza infection.[151] However these studies also show that the HA protein provides limited heterotypic protection for the same subtype and is unable to induce cross-subtype and cross-group protection. The HA2 subunit, which comprises most of the HA stem region, shows high sequence conservation among the different HA subtypes. Therefore, the HA2 region would be a very attractive target to induce broader neutralizing Abs then full-length HA.[108,152-154] Results of recent studies that reevaluated the HA stalk subunit are likely to contribute to the development of more effective rAd vectors encoding HA stems. In one study, Ad-vectored HA2 failed to prevent homologous virus infection but partially enhanced viral clearance and recovery from influenza infection.[146] Recent research has shown that glycan-masked H5HA elicits stem-specific antibodies that overlap with broadly neutralizing epitopes of the CR6261 mAb, which neutralizes most group 1 subtypes.[115] In another study, a conserved HA stalk domain (H1N1) expressed in transiently transfected cells induced stem-specific antibodies that were cross-reactive among group 1 HA subtypes (H2N2 and H5N1).[152] Further studies assessed the protective efficacy of HA stem with different heads. The chimeric HA antigens induced high titers of stalk-reactive Abs in mice and ferrets,[155,156] and such humoral immunity broadly protected from lethal challenge by divergent group 1 and group 2 viruses, including H5N1 and H7N9 viruses[156-158]. However, it is important to note that, to date, no cross-group protection has been observed from vaccination with only HA or the HA stem.[155]

These previous reports suggest that the conserved HA stem may provide much weaker protective Ag compared with the whole HA protein and may induce only mild immunity and protection. This mild immunity may be partly caused by the lack of CAR on DCs, which result in resistance of DCs to Ad infection.[159] Moreover, Ag presentation by transduced non-professional APCs may lead to suboptimal T cell activation or even tolerance induction.[160] An alternative method to strengthen the immunity efficacy of Ag is by retargeting Ag or rAd vector to CD40 on APCs such as DCs.[161-163] CD40 and its ligand (CD40L) not only play a crucial role in the expansion and survival of T cells and B cells to initiate and sustain immune responses but also promote DC maturation into fully competent APCs.[164,165] Fan et al. generated a recombinant rAd encoding a secreted and codon-optimized HA2 fusion with murine CD40L. Mice immunized with this recombinant viral vaccine were completely protected against lethal challenge with cross-group influenza A virus subtypes, including H1N1, H3N2, and H9N2.[166] The results also show that codon-optimization of HA2 as well as the use of CD40L as a targeting ligand/molecular adjuvant were indispensable for enhancing HA2-specific mucosal IgA and serum IgG levels.

In addition to the HA stem, conserved internal viral proteins, such as NP and matrix protein 1/2 (M1/2), induce cross-immunity between different subtypes in the same group. Epstein et al. predicted DNA prime-rAd boost vaccination to conserved NP and M2 in ferrets and mice, and this vaccine strategy protected against virulent H1N1 and H5N1 challenges.[86,167] However, antibodies induced by conserved internal viral epitopes failed to replace HA stem-specific neutralizing Abs that play a key role in the prevention of infection and merely reduced the disease symptoms.[121] NP has also been shown to provide limited protection against high challenge doses of H5N1 in ferrets.[168] To enhance the immune-inducing efficacy of NP, Hashem et al. constructed rAd vectors encoding a secreted NP-CD40L fusion protein (SNP40L). SNP40L expressed in rAd-infected cells could be secreted and target CD40 on APCs. CD40L, as an adjuvant and targeting molecule, can enhance the breadth, potency, and durability of NP-specific immune responses involving both $CD8^+$ T cells and anti-NP Abs and provide complete cross-group protection against H1N1 and H3N2 strains in mice.[169] Therefore, secreted Ag fusion with CD40L may be a potential platform to improve the immunogenicity and protective efficacy of other HA stem and conserved internal viral proteins. The present vaccine development strategies include expressing HA Ag combined with other conserved internal viral proteins, such as NP and M1/2, to induce highly effective and lasting $CD8^+$ T cell responses and ADCC effects.

An adenoviral vector-based vaccine that contains HA and conserved NP (H5N1) elicited cell-mediated $CD8^+$ T cell immune responses as well as neutralizing antibodies against clade 1 and clade 2 strains within the same subtype viruses (H5N1),[170] and similar observations were recorded in another study. An Ad-based HA vaccine protected mice from challenge with different clade strains of the same subtype.[171] Furthermore, lung virus titers were significantly reduced in mice that were challenged with the cross-subtype strain after vaccination with rAd-HA and rAd-NP.[171] However, another study showed that co-administration of Ad-based HA and NP did not confer better protection than HA alone.[172] Future research will assess

the relative risks and benefits of different combinations of vaccines.

Kim et al. reported that intranasal vaccination with rAd encoding H5 and M2e induced significant HA- and M2e-specific Ab responses and protected vaccinated mice against heterosubtypic (H1N1) challenge. This cross-subtype protection is based on stalk-specific Abs that prevent the release of viral genetic material into the cells and on M2e-specific Abs that mediate the lysis of virus-infected cells by ADCC.[173]

Holman et al. developed a multi-antigen Ad vector, cAdVax-FluAv, containing the HA, NA and M1 genes. Mice vaccinated with cAdVax-FluAv survived after challenge with lethal clade 1 and clade 2 H5N1 viruses.[174] A single Ad vector encoding a multi-subunit of the influenza virus has many advantages, such as a reduction of the vaccine dose, avoidance of the complexity of production, and induction of an optimized immune response.

Codon optimization was used to elicit immune responses to viral antigens by improving the expression of the protein in host cells.[175] Steitz et al. demonstrated that a single-dose of codon-optimized, Ad-based H1N1 vaccine encoding HA Ag induced more robust cellular and humoral responses than wild type HA Ag in mice.[176] Codon-optimization of HA2 also seems to induce significantly higher levels of local and systemic anti-HA2 Abs than wild-type HA2 in mice.[166]

In addition to adenovirus type 5, Ad type 4 (Ad4) has been evaluated as a candidate vector to circumvent pre-existing adenoviral immunity. A pre-clinical evaluation showed that rAd serotype 4 vaccine expressing HA was safe and induced HA-specific humoral and cellular immunity.[177] Currently, the Ad4-vectored vaccine has been investigated in multiple phase I clinical trials, including for H5N1 influenza, HIV infections, and anthrax infection.[178] Other nonhuman adenovirus vectors include chimpanzee adenovirus AdC7,[45] bovine adenovirus subtype 3,[47] canine adenovirus type 2,[55] and porcine adenovirus.[56]

Vaccine-Associated Immunity Escape

Current study results present a challenge to HA-specific universal antibodies. To et al. found that nonneutralizing Ab titers were significantly higher for patients with severe disease than for those with mild disease during the 2009 H1N1 influenza pandemic. Early IgG response within 2 to 4 d after symptom onset indicated that the nonneutralizing antibody present in patients was likely to be preexisting or was the result of a secondary heterotypic antibody response against conserved epitopes.[179] This study concluded that higher levels of nonneutralizing antibodies in the early stage of infection may be associated with worse clinical severity and poorer outcomes. Khurana et al. evaluated the mismatched influenza vaccine-associated enhanced respiratory disease (VAERD) after pandemic H1N1 (pH1N1) infection in a swine model. Cross-reactive HA2-specific Abs induced by inactivated H1N2 promoted H1N1 virus fusion and enhanced influenza virus respiratory disease.[180] Gauger et al. have also confirmed that high levels of IgG serum Abs targeting the mismatched pH1N1 HA2 stalk domain were exclusively detected in IIVV-vaccinated swine and associated with increased pH1N1 virus infectivity in MDCK cells. IIVV-vaccinated swine challenged with mismatched pH1N1 were not protected from infection and demonstrated severe respiratory disease consistent with VAERD.[181] Conversely, infection-enhancing HA2 Abs were detected at minimal levels in the serum of intranasal LAIV vaccinates, and VAERD was not observed, though both IIVV and LAIV vaccinates induced low and similar mean levels of Abs against mismatched pH1N1 HA1.[181] Therefore, when challenged with mismatched virus, pigs lacking protective Abs in the presence of high titer anti-HA2 Abs may have an increased risk of VAERD. Although the mechanism of the differences in the type of Abs elicited by WIV and intranasal LAIV is still unknown, the results of this study suggest that the immune balance among globular-specific protective Abs, stalk-targeting Abs and local IgA may play an important role in the infection outcome. However, VAERD did not interfere with the adaptive immune response following challenge with H1N1.[178]

There have been no reports of HA2-specific Abs related VAERD in other animal models. However, Dougan et al. found another mechanism of VAERD in a mouse model. Influenza virus infects HA-specific B cells via its receptor, disrupting antibody secretion and causing HA-specific B cell death in mice. Infection and killing of antigen-specific B cells impair the kinetics of the memory response that is established by infection or vaccination.[182] Therefore, it is necessary to further investigate the possibility of establishing a balanced immune response induced by a combined vaccine.

Furthermore, a vaccine based on conserved internal viral proteins induces T cell immunity, which may lead to selective immune pressure on the influenza virus, similar to Ab-mediated antigenic drift.[183] Under this selective pressure, virus escape mutants arise at the residues that anchor the epitope peptide to MHC.[184] Gras et al. demonstrated that influenza virus escapes $CD8^+$ T-cell immunity through mutations at highly conserved $NP_{418\text{-}426}$peptides.[185] Therefore, this type of theoretical vaccine-associated T-cell immune escape must be further estimated.

Prospect of a Universal Influenza Vaccine

There is no doubt that great progress has been made in the development of a rAd-vectored influenza vaccine. However, it is too early for the use of a rAd-vectored vaccine as an alternative to the classical IIVV and LAIV because many problems are still unresolved. There are many difficulties in the development of a universal vaccine that induces a cross-reactive CD8+ T-cell immune response, including time-related attrition of immune competence,[125,186] the protective capacity in different HLA populations, and vaccine-associated immune escape and immunopathology. Early studies have shown that cytotoxic T-cell memory has a half-life of approximately 2–3 y.[125] Therefore, a booster immunization is needed every 2–3 y to maintain an adequate level of memory T-cells. Furthermore, the future design of a universal vaccine should consider major HLA allele populations, as well as rare-allele ethnicities.

The ideal influenza vaccine should induce universal and efficient cross-reactive antibodies to conserved antigens, such as the HA stem, and this strategy has been assessed in mouse and ferret models. Furthermore, the ideal influenza vaccine should also induce a strong T-cell immune response and maintain long memory potential. Significant progress has been made to identify a universal antibody and produce cross-reactive T-cell immunity, but generating an effective universal vaccine remains difficult.

Moreover, cross immunity-associated immune evasion and its effect on virus evolution must be further assessed. Another major problem is that the relationship between the vaccine dosage and immune effect remains unclear.[187] Serum HI antibody titers of more than or equal to 1:40 reduce the risk of influenza infection by at least a 50%.[188] However, no such correlation of protection exists for a rAd-vectored vaccine encoding the HA,[174] HA1/HA2,[146] NP or M2[86] genes. ADCC activity is impaired in neutrophils from aged subjects;[189] therefore, an ADCC-dependent universal vaccine may be ineffective in the elderly.

Future directions for the universal influenza vaccine should focus on multi-faceted based on cross-reactive antibodies, T-cell immune responses and long memory potential. Standardized virus strains and animal models are necessary to develop standard methods for evaluating different candidate vaccines. Additional studies will probe the relationship between the dosage of the vaccine and the immune effect. Overall, the development of a universal influenza vaccine based on the Ad vector is early in its development, but the approach holds great potential in the fight against influenza.

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No potential conflicts of interest were disclosed.

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