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Review

Progress on the development of universal influenza vaccines

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ABSTRACT

Influenza A virus is a prominent viral pathogen that infects between 5-20% of the U.S. population annually, and is a major contributor to life-threatening respiratory illness in the young, old, and immunocompromised. In addition, sporadic outbreaks of highly virulent avian H5N1 influenza and the recent outbreak of a pandemic H1N1 virus have heightened concerns about the eventual emergence of a particularly lethal pandemic virus. Although commercial influenza vaccines have been available since the mid-1900s, a number of key challenges continue to limit the efficacy of these vaccines. Foremost, current strain-specific vaccines must be revised annually due to the rapid mutation rate of immunodominant viral proteins. Despite this drawback, some recent research investigations have indicated the potential for developing universally protective immune responses against influenza viruses. Over the past decade, an array of conserved influenza virus epitopes have been identified, and the ability of both cell-mediated and humoral immune components to elicit cross-protective immunity to heterologous influenza A viruses has been documented. Moreover, improvements in influenza vaccine immunogenicity via novel delivery methods, molecular adjuvants, and modification of vaccine modality have also been widely reported, and will likely contribute to further progress on the development of a maximally efficacious universal influenza vaccine. This report reviews some of the literature on universal influenza A viral vaccine development and provides a comprehensive account of recent progress that has been made in this field.

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INTRODUCTION

Influenza A virus represents one of the most prominent viral pathogens of modern times. Infection with this microbe results in an estimated 36,000 deaths [1] and over 200,000 hospitalizations [2] in the U.S. annually, with a projected total economic burden in excess of 80 billion U.S. dollars per year [3]. According to the CDC, the incidence of influenza virus infection in the U.S. population may reach 20% during a typical flu season; however, this figure can increase substantially during periods of pandemic influenza. Although influenza is typically a self-limiting disease, serious complications can occur, including primary viral pneumonia, secondary bacterial pneumonia, myositis, and neurologic syndromes. The risk of mortality and disease complications is elevated for certain populations, including the young, old, and immunocompromised. The ability

of influenza A virus to infect millions of people each year speaks to the resilient nature of this pathogen and to the necessity for developing improved methods of disease prevention.

Current influenza vaccines: Characteristics and limitations

Commercial influenza vaccines have been available since the mid-1900s; however, immunity afforded by these approaches is typically limited due to the continual emergence of antigenically distinct viral strains. Current vaccine preparations incorporate influenza viruses in a trivalent inactivated (IIV) or live attenuated (LAIV) form, and are delivered via intramuscular injection or intranasal spray, respectively (Table 1). The viral strains utilized in these vaccines are predicted based upon extensive influenza virus surveillance data, and currently consist of two influenza A virus strains (H1N1 and H3N2) and one influenza B virus strain. Immunity elicited upon vaccination is associated with the production of antibodies to the viral surface proteins hemagglutinin (HA) and neuraminidase (NA) [4, 5], which are critical mediators of viral attachment and release from host cells, respectively. The structure and composition of the influenza virus particle is shown in Figure 1. Due to the rapid antigenic drift of these immunodominant influenza proteins, the influenza vaccine must be updated on an annual basis. While antigenic drift results in minor mutations that contribute to seasonal influenza epidemics, antigenic shift can result in the emergence of dramatically reassorted influenza viruses that may spread as influenza pandemics. Notable pandemics include the Spanish Flu outbreak of 1918, which resulted in an estimated 50 million deaths worldwide [6], as well as the recent 2009 outbreak caused by the H1N1 virus subtype. The possibility of an influenza pandemic derived from the highly virulent H5N1 avian influenza virus has also remained a key concern of the scientific community over the past decade [7-9]. Importantly, H5N1 viruses are not a component of current seasonal influenza virus vaccines, and a time lag of several months would likely occur between the emergence of a pandemic H5N1 virus and mass distribution of strain specific vaccines [10-12]. Such factors contribute to concerns about current emergency preparedness protocols that incorporate status quo vaccination techniques for pandemic influenza [13-15].

A number of key challenges continue to limit the overall efficacy of seasonal influenza virus vaccines. Current vaccines are only 30-50% effective in preventing hospitalization and pneumonia in the elderly, and about 70% effective in preventing illness in healthy adults [16]. The process of monitoring circulating strains of influenza is costly and laborious, requiring a broad network of independent laboratories to coordinate information effectively. Furthermore, predictions regarding the dominant influenza virus strains for a given flu season are occasionally incorrect, or are rendered inaccurate due to rapid virus evolution, resulting in seasonal vaccines with low efficacy [17]. Such mishaps are costly

Table 1. Currently available licensed, seasonal influenza virus vaccines in the USA.

Name	**Type**	**Manufacturer**	**Predominant Immune Response**
Afluria	IIV	CSL Limited	Serum Antibody
FluLaval	IIV	ID Biomedical Corporation of Quebec	Serum Antibody
Fluarix	IIV	GlaxoSmithKline Biologicals	Serum Antibody
Fluvirin	IIV	Novartis Vaccines and Diagnostics Limited	Serum Antibody
Agriflu	IIV	Novartis Vaccines and Diagnostics S.r.l.	Serum Antibody
Fluzone	IIV	Sanofi Pasteur, Inc.	Serum Antibody
FluMist	LAIV	MedImmune, LLC	Mucosal, Antibody and Cytotoxic T cell

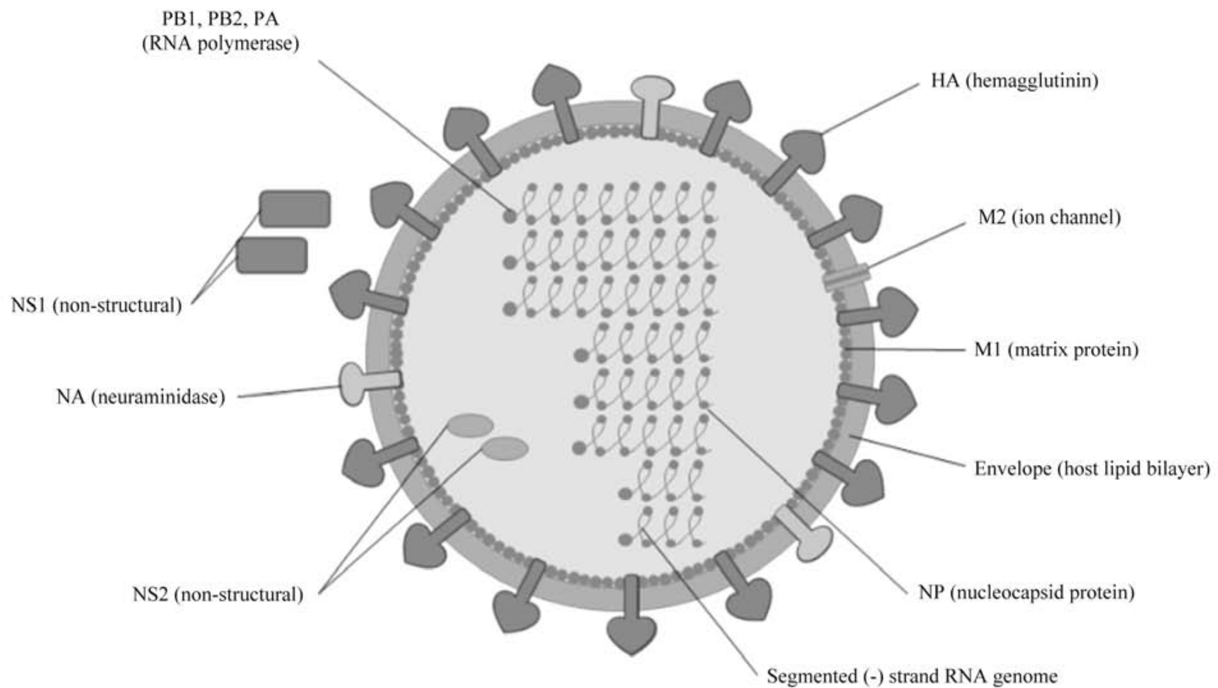

Figure 1. Structure and composition of the influenza virus particle.

both for vaccine manufacturers, which must offer sub-optimal vaccines for a given season, and for the general public, which must contend with an increased incidence of infection. Additionally, individuals in particularly susceptible populations, such as the very young and the elderly, must rely solely on the IIV vaccine to provide protection. While the IIV vaccine is proficient in eliciting humoral immune responses, the vaccine is lacking in its ability to stimulate cell-mediated immunity [18]. Cell-mediated immune responses, characterized by antigen specific CD8+ T cells and Th1 CD4+ T cells, play important roles in resolving viral infections, and may function as critical mediators of heterotypic immunity to influenza virus infection [19].

Potential for development of a universal influenza virus vaccine

Despite the extensive variability in strains of seasonal influenza, and the general inability of the immune system to produce broadly cross-reactive antibodies, some research investigations have indicated the potential for developing universally protective immune responses against influenza viruses. These investigations have focused on components of the influenza virus that are highly conserved, including internal viral proteins and structural regions of the surface glycoprotein HA. In particular, efforts to categorize influenza virus epitopes have revealed numerous antigens recognized by B cells, CD4+ T cells, and CD8+ T cells that bear considerable sequence identity amongst distinct viral strains [20]. The influenza virus epitopes exhibiting the highest degrees of conservation are found within the matrix (M), nucleocapsid (NP), and polymerase B (PB) proteins, and are subject to recognition by T cells [20, 21]. In one relevant example, Assarasson and colleagues found that the $M1_{59-67}$ epitope sequence was conserved at 100% across a panel of 23 distinct influenza A virus strains [22]. Interestingly, this peptide was originally identified as a human leukocyte antigen (HLA) class I-restricted T cell epitope; however, it is embedded within another epitope that is recognized by HLA class II. This HLA class II-restricted epitope sequence ($M1_{58-72}$) was also found to be conserved at 100% amongst 23 influenza A virus strains, suggesting that certain expanded epitopes may contain a dual potential for activating CD4+ T cells via HLA class II and CD8+ T cells via HLA class I [23].

The importance of eliciting CD4+ T cell responses in tandem with B cells or CD8+ T cells has been documented in multiple studies of influenza virus infection [23, 24], and remains an important consideration of current attempts to develop a universal influenza vaccine.

Role of cell-mediated immunity in eliciting cross-protection

The potential for components of cell-mediated immunity to elicit cross-protection to distinct influenza virus strains was first demonstrated in a study conducted by Webster and colleagues in 1980 [25]. In this study, mice infected with live influenza A virus were capable of generating cross-reactive cytotoxic T cells, and were protected from secondary challenge with a different influenza virus subtype. In contrast, mice immunized with a subunit vaccine containing isolated HA and NA did not develop cytotoxic T cell memory, and succumbed to secondary infection with a different influenza virus subtype. Despite the elicitation of cross-protective immunity in this model, primary infection with virulent influenza A virus does not represent a viable option for vaccinating the general public; thus, the investigation of other strategies that incorporate non-pathogenic viral components is necessary. Vaccination with γ-irradiated influenza preparations has been reported to induce cross-reactive T cell responses [26], and this technique was recently shown to elicit cross-protective immunity to avian H5N1 influenza virus in a murine model of intranasal vaccination and viral challenge [27]. In addition to whole virus vaccines, administration of structurally conserved viral proteins, such as the M2 protein, has been shown to induce heterotypic immunity against influenza A viruses [28, 29]. One particularly appealing viral peptide for incorporation into a universal vaccine is the aforementioned M1 epitope at position 58-66. This immunological determinant is highly conserved amongst influenza A virus strains, is immunodominant, and binds to an HLA-A allele (HLA-A*0201) with a high prevalence in the human population [30]. Despite the intense pressure imparted on this peptide by the human immune system, functional constraints within the M1 molecule appear to severely restrict its mutation [31]. Moreover, Boon and colleagues reported that HLA-A*0201-restricted CD8+ T cells were consistently present within individuals expressing the HLA-A*0201 allele, and were capable of producing IFN-γ and lysing target cells upon stimulation with virus [32]. This study also identified two HLA-B alleles, HLA-B*2705 and HLA-B*3501, as preferential mediators of CD8+ T cell immunity to influenza viruses. An NP epitope at position 418-426 has been identified as a ligand for HLA-B*3501 [33], as well as HLA-B*0702 [34], although mutations at specific positions within the epitope sequence can result in virus escape [35]. Aside from cell-mediated immune effectors, humoral immune mechanisms may also contribute to the protection afforded by certain conserved antigen influenza vaccines, such as those directed towards NP [36] and M2 [37]. Collectively, these findings indicate that structural influenza proteins, or epitopes derived from them, may represent good candidates for inclusion in a universal influenza vaccine.

Role of humoral immunity in eliciting cross-protection

From the standpoint of humoral immunity, the use of modified ‘headless’ HA molecules has shown promising results in generating broadly neutralizing antibodies in murine models [38]. These headless HA molecules contain only the stalk domain of the HA molecule, which is highly conserved amongst members of the same HA group. Two basic HA groups have been described, which encompass all 16 HA subtypes. In an assessment of the protection afforded by modified HA molecules, mice immunized with truncated HA derived from an H2N2 influenza virus exhibited superior protection to infection with H1N1 influenza virus versus mice receiving a mock vaccine [39]. Similarly, a recent study with ferrets has indicated that immunization with plasmid DNA encoding HA can modulate immune responses towards cross-protective immunity targeting the HA stalk domain [40]. The ferrets utilized in this study were protected from virus challenge with two diverse H1N1 strains upon primary immunization with a plasmid containing HA and a booster immunization with the 2006-2007 seasonal influenza vaccine. This study also indicated that cross-reactive immune sera could be

raised against distinct H1N1 influenza viruses in mice, ferrets, and non-human primates. These studies highlight the efficacy of incorporating such conserved B cell epitopes into novel vaccine modalities and warrant continued investigation.

Improvement of vaccine efficacy via adjuvants and novel vaccine modalities

Numerous attempts have been made to improve the immunogenicity of vaccine formulations through the inclusion of molecular adjuvants, and a number of these immune modulators have been investigated directly within the context of experimental influenza vaccines. A thorough evaluation of these vaccine components may prove particularly important for the development of universal influenza vaccines, as many of the conserved influenza antigens targeted by such formulations are not inherently very immunogenic. In 2008, Radosevic and colleagues used a murine model to analyze the Th1 versus Th2 proclivity of various chemical adjuvants administered alongside a virosomal adjuvanted H9N2 avian influenza virus vaccine [41]. The findings from this study indicate that chemical adjuvants have a distinct impact on the dominant antibody isotype produced upon vaccination, with aluminum salts (alum) heavily skewing the immune response towards IgG1 production (i.e., a Th2 response) and a novel immunostimulating complex (ISCOM)-based adjuvant heavily skewing the immune response towards IgG2a production (i.e., a Th1 response). In conjunction with these findings, administration of the ISCOM-based adjuvant, but not alum, significantly enhanced *in vitro* production of IFN-γ by CD8+ T cells upon stimulation with virus. Presently, alum and the Adjuvant System 04 (AS04), which combines monophosphoryl lipid (MPL) A and aluminum salt [42], are the only FDA approved adjuvants for use in the USA. With standard alum adjuvants, however, the concomitant induction of Th2 responses may prove counterproductive for preventing influenza virus infection. In one relevant example, Bungener and colleagues found that inclusion of alum in an inactivated influenza virus vaccine enhanced inhibitory antibody titers to HA, but resulted in decreased viral clearance from mouse lungs compared to the inactivated virus vaccine alone [43]. Unlike classical alum adjuvants, the MPL component of AS04 can engage Toll-like receptor (TLR) 4 and skew the immune response towards Th1. Other reports have also identified unmethylated CpG-containing oligodeoxynucleotides (CpG-ODN) as potent inducers of Th1 responses [44-47], and our laboratory has previously shown that poly(I:C) can redirect immune responses from Th2 to Th1 [48]. The functions of some common influenza vaccine adjuvants and the associated induction of a specific Th-type response are summarized in Table 2.

Table 2. Currently licensed and experimental adjuvants commonly utilized in influenza virus vaccine studies.

Adjuvant-Licensed	**Active Component**	**Target**	**Associated Immune Response**
Alum	Aluminum salt crystals	General	Th2
MF59*	Squalene and surfactants	General	Mixed Th1/Th2
AS03*	Squalene and surfactants	General	Mixed Th1/Th2
Adjuvant-Experimental	**Active Component**	**Target**	**Associated Immune Response**
AF03	Squalene and surfactants	General	Mixed Th1/Th2
ISCOMs	Quillaja saponins	General	Th1
CpG-ODNs	CpG motifs	TLR-9	Th1
Flagellin-antigen fusion proteins	Bacterial flagellin	TLR-5	Mixed Th1/Th2
Cytokines and immune signaling molecules	Inherently immunogenic	Variable	Variable

*Not licensed in the USA.

In addition to molecular adjuvants, certain vaccine modalities have demonstrated a propensity for augmenting immune responses to targeted antigens. In particular, investigations of DNA vaccination have shown that this modality is capable of engaging both the humoral and cell-mediated arms of the immune system [46-52]. The ability of DNA vaccines to amplify immune responses and mobilize an assortment of immune effectors can likely be attributed to the self-adjuvating capacity of plasmid DNA [53, 54], as unmethylated CpG motifs present within bacterial DNA vectors have been shown to bind TLR 9 and initiate innate immune responses. In general, DNA vaccines delivered intramuscularly tend to polarize immune responses toward a Th1 phenotype [55, 56]; however, the precise mechanism of delivery can have a profound impact on the polarity of the ensuing immune response [57]. As mentioned by Wei and colleagues, DNA vaccines utilized in a prime-boost scenario have the ability to elicit cross-reactive immune responses to distinct influenza viruses in multiple host species [40]. Furthermore, our laboratory has previously shown that DNA vaccines can induce immunological memory to influenza virus in baboons primed as neonates [58]. In addition to DNA vaccines, adenovirus vectors and virus like particles have demonstrated promising results when utilized as vehicles for conserved influenza antigens, including centralized HA [59], M2 [60, 61], and NP [61]. Such findings provide an impetus for continued evaluation of the effects of distinct adjuvants and vaccine modalities on influenza vaccine efficacy.

Improvement of vaccine efficacy via nanotechnology

In recent years, advances in nanobiotechnology have greatly enhanced experimental vaccine formulations and delivery methods targeting infectious disease and cancer. Since vaccine antigens are composed of nano-sized material, the manipulation of biological compounds at this level represents a logical approach to improving vaccine efficacy. Indeed, various nanoparticle and nanocapsule carrier systems have been shown to enhance the immunogenicity of experimental vaccines, most notably within the context of mucosal immunization [62]. Common nanocarrier systems used in the induction of mucosal immune responses include natural and synthetic polymers, aqueous or oil-based emulsions, liposomes, and ISCOMs. Although this technology has a broad-based potential for use in microbial and cancer vaccines, specific reports have indicated that nanocarriers can improve the efficacy of cross-protective immune responses to influenza viruses [63]. In addition to novel carrier systems, micro-nanotechnology has contributed to the development of next-generation vaccination devices, such as microneedle arrays [64-67]. Microneedles have been shown to induce potent immune responses to various viral pathogens, including influenza viruses, while requiring minimal amounts of the vaccinating agent [68-71]. As the field of micro-nanotechnology continues to grow, the exploration of nanotechnology solutions to current vaccination challenges will clearly contribute to the development of more efficacious vaccines.

CONCLUSIONS AND FUTURE DIRECTIONS

The development of a universal influenza vaccine represents one of the scientific community's most ambitious endeavors to improve the control of widespread infectious disease. Within the past few years, substantial progress has been made on the construction and evaluation of relevant vaccine components, including various vaccine antigens, adjuvants, modalities, and delivery systems. Future work in this field will continue to provide insight into an ideal influenza vaccine formulation, and will likely point toward a combination of novel vaccine components as a means of achieving a maximally efficacious immune response. Perhaps in the foreseeable future, the development of a universal influenza vaccine will lead to the eradication of this notorious human pathogen.

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