# INFLUENZA VIRUS VACCINES AND IMMUNOTHERAPIES

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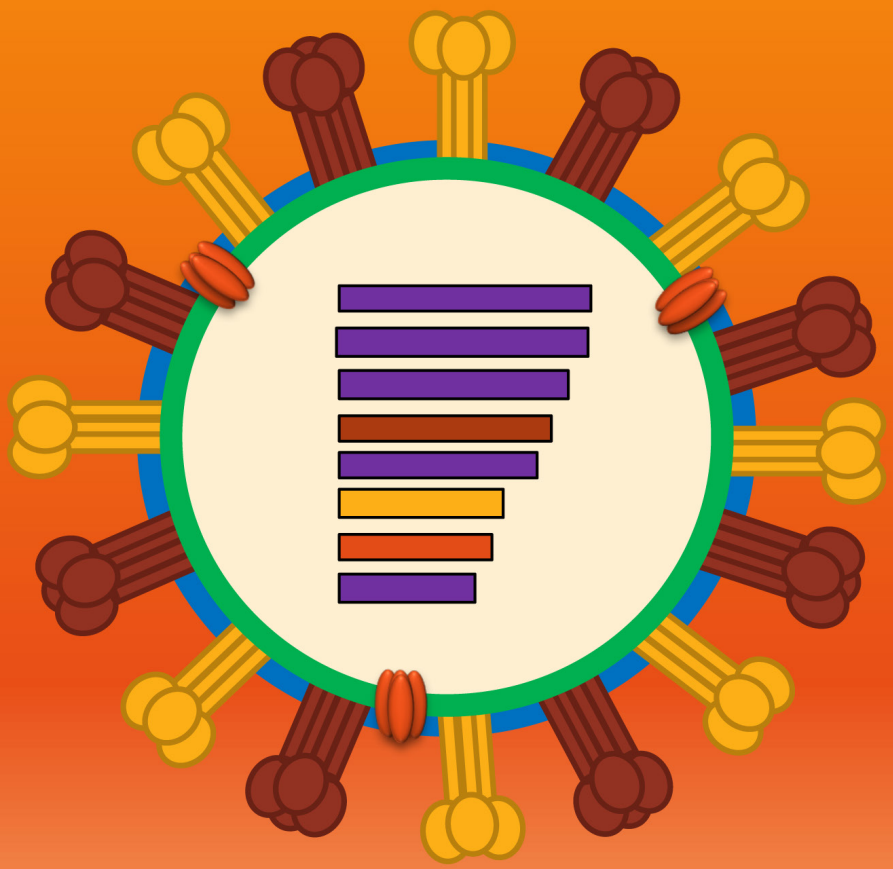

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# INFLUENZA VIRUS VACCINES AND IMMUNOTHERAPIES

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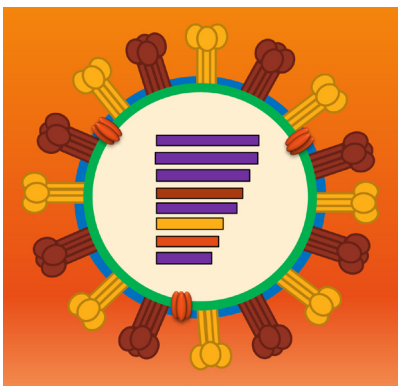

Schematic diagrams of influenza A virus and surface hemagglutinin protein. The segmented negative-sense RNA genome of influenza A virus encodes three envelope proteins (hemagglutinin, neuraminidase, and ion channel M2 protein), and internal nucleoprotein (NP), polymerases (PA, PB1, and PB2), matrix protein 1 (M1), and non-structural proteins (NS). The lipid bilayer is derived from host cell membrane (Modified from: Lofano G, Kumar A, Finco O, Del Giudice G and Bertholet S (2015) B cells and functional antibody responses to combat influenza. Front. Immunol. 6:336. doi: 10.3389/fimmu.2015.00336).

Influenza virus infections lead to thousands of deaths worldwide annually and billions of dollars economic burden. Despite continuing advances in our understanding of the immune evasion mechanism, the disease remains one of the foremost threat for human being. Traditional vaccines (attenuated and inactivated) mainly provide protection by inducing virus neutralizing antibodies, targeting ever changing surface antigens: Haemagultinin (HA) and Neuraminidase (NA). Due to genetic shift and immune selection pressure, prevalence of circulating influenza virus subtypes changes every year. Therefore, mismatch between circulating strain and vaccine strain can critically affect the success rate of these conventional flu vaccines, and requires continuous monitoring of circulating influenza virus subtypes and change in the vaccine formulations accordingly.

The collective limitations of existing flu vaccines urgently call for the development of a novel universal vaccines that might provide the required protective immunity to a range of influenza virus subtypes. New approaches are being investigated mainly targeting conserved regions of flu proteins. Some of these approaches include universally conserved epitopes of HA, nucleoprotein (NP), capsid protein (M1) and ion channel protein (M2) that induced strong immune responses in animal models. Some attention and progress appears to be focused on vaccines based on the M2 ectodomain (M2e) employing a variety of constructs, adjuvants and delivery systems, including M2e-hepatitis B core antigen, flagellin constructs, and virus-like particles (VLP). Animal studies with these M2e candidate

vaccines demonstrated that these vaccine candidates can prevent severe illness and death but not infection, which may pose difficulties in both the evaluation of clinical efficacy and approval by the regulatory authorities. VLP vaccines appear to be promising, but still are mostly limited to animal studies.

The discovery and development of new and improved vaccines have been greatly facilitated by the application of new technologies. The use of nucleic acid-based vaccines, to combine the benefits of in-situ expression of antigens with the safety of inactivated and subunit vaccines, has been a key advancement. Upon their discovery more than 20 years ago, nucleic acid vaccines promised to be a safe and effective mean to mimic immunization with a live organism vaccine, particularly for induction of T cell immunity. In addition, the manufacturing of nucleic acid-based vaccines offered the potential to be relatively simple, inexpensive and generic. Reverse Vaccinology and in-silico designing of vaccines are very innovative approaches and being considered as future of vaccines. Furthermore, various immuno-therapeutic agents also being developed to treat and minimize immuno-pathological damage in patients suffering from life threatening complications. For the treatment of such pathological conditions, various novel approaches such as administration of immune suppressive cytokines, blocking co-stimulatory signals or activating co-inhibitory signal of T cell activation, are being tested both in lab and clinics.

The Research Topic on influenza virus vaccine and therapeutics will give an insight in to the current status and future scope of these new innovative approaches and technologies. Moreover, these new methods will also serve as a reference tool for the development of future vaccines against several other pathogens.

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EDITORIAL
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# Editorial: Influenza Virus Vaccines and Immunotherapies

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**Keywords: influenza, human, influenza A virus, vaccines, influenza vaccines, immunotherapy**

Influenza virus infections are responsible for billions of dollars of economic burden annually worldwide. Regardless of advances in our understanding of the immune evasion mechanism, the disease remains one of the foremost threats for human being (1). Currently available vaccines and therapeutic agents are not very potent against the deadly flu infections because of the frequent mutations in influenza virus subtypes. The mismatch between the circulating strains and vaccine strains critically affects the success rate of the conventional vaccines and requires continuous monitoring of circulating influenza strains. These collective limitations of existing flu vaccines urgently call for the development of novel vaccines with a wide range of cross-protective immunity (2). The discovery and development of new and improved vaccines have been greatly facilitated by the application of new technologies. The articles included in this research topic explain the key methods of improvement in existing vaccines, therapeutics, and mechanism of protection.

The opening articles of the present topic discuss about the novel methods of improving existing influenza vaccines and enhancing immune responses. Various strategies including use of adjuvants, heterologous prime/boost and unique antigen design have shown to induce protective influenza-specific neutralizing antibodies. Lofano et al. reviewed recent advancements in the flu vaccine development and highlight the role of B-cells in controlling influenza virus infections (3). Soema et al. discussed the recent developments and design of T-cell based vaccines based on novel peptide and protein-based vaccine formulations (4). Adjuvants play a critical role in the induction of rapid, effective, and durable immune responses when administered with vaccine antigens. Recent progress in the adjuvant formulations allows existing vaccines to reduce the number of booster doses, increase dose-sparing ability, induce potent T and B-cell responses and enhance breadth of the immune responses against heterotypic antigens (5). Vogel and Brown reported that only single dose of CpG adjuvanted influenza vaccine can induce robust memory T-cell responses and confer protection against heterosubtypic challenge (6). Similarly, in the presence of oil-in-water emulsion adjuvant system (AS03), even a very small dose of influenza vaccine can induce significantly strong immune responses (7). Alternative strategies of immune protection such as use of immuno-modulatory agents like TLR agonist can activate protective non-specific antiviral immune responses. Mifsud et al. demonstrated that mice pre-treated with TLR-2 agonist PEG-Pam2Cys were able to mount specific B- and T-cell responses and also protect mice against heterologous virus challenge (8). These types of alternative strategies can provide immediate immune protection in the absence of effective vaccine without compromising the antiviral specific immunity.

The next generation universal influenza vaccine targets the most conserved structure of the virus and hence confers heterotypic protection, for example, new vaccine strategies target stalk of the HA instead of the globular head. Mallajosyula et al. designed a potent immunogen comprised of HA-stem-fragment from H3N2 strain (A/Hong Kong/1/68) and trimerization motifs: coiled-coil isoleucine zipper and globular β-rich. Immunization of mice with the immunogen induced cross-reactive antibodies and provided only partial protection against homologous virus challenge (9).

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Li et al. engineered a dual vaccine by incorporating botulinum neurotoxin A (BoNT/A) receptor-binding subdomain in universal influenza vector based on PR8 strain of influenza virus. This vaccine induced protective immunity against both BoNT/A and Influenza virus. Potent immune responses can also be generated by exploiting receptors on antigen presenting cells (APCs) (10). Grodeland et al. demonstrated that a novel DNA vaccine candidate specifically induced Th2 and IgG1 antibody responses or Th1 and IgG2a responses by targeting hemagglutinin to MHC class II molecules or chemokine receptors (XCR1 or CCR1/3/5), respectively (11).

The historical 1918 influenza pandemic caused very high mortality in adult population and the immunological parameters in this population still remain speculative. In light of current understanding of influenza immuno-pathogenesis, McAuley et al. claimed that, the high mortality rate in the adult population is due to the dysfunctional or excessive cross-reactive memory T-cells, induced by previous influenza infections, which render these individual susceptible to the 1918 pandemic influenza (12). Peng et al. provided the evidence that pre-existing seasonal influenza virus HA-specific cross-reactive T-cells can be boosted by a heterologous vaccine (13). Pregnant women are at paramount priority for influenza vaccination due to influenza virus related complications during the pregnancy. Modulation of maternal immunity during the pregnancy can influence the influenza vaccine specific immune responses. Kay and Blish summarize the immunogenicity and efficacy of the influenza vaccine and discuss impact on T and B-cell responses during the pregnancy (14).

Apart from prophylactic vaccine, therapeutic approaches also required for those severely infected with the influenza virus. The use of antibodies seems to be an attractive immunotherapeutic approach for the treatment of various infectious diseases. Sasisekharan et al. reviewed different antibody based strategies to prevent and treat the influenza infections (15). The cells of innate immune system are triggered by signaling pathways during influenza virus infection that causes extensive damage to lung tissues and in airway lining, resulting in severe immunopathology. Ramos and Sesma suggest that these damages can be minimized by selectively modulating the innate signaling pathways using immuno-modulatory drugs while maintaining the ability of the host cells to mount an antiviral response to control virus replication (16). Further, Graham et al. described the role of mast cells in immunopathology during influenza A virus infection and suggested them as a potential drug target in viral infections (17).

The new strategies for vaccine design also require more sensitive and efficient methods for the evaluation of the vaccine potency. Carnell et al. reviewed comprehensive methods for influenza virus neutralization assays, based on the pseudotype viral particles, which utilize chimeric viruses bearing influenza glycoproteins, and depict how such assays can replace the traditional HA neutralization assays for the evaluation of new age influenza vaccines (18). To detect the incidence of Influenza virus with high efficiency and accuracy, Rajput et al. generated HA specific high affinity recombinant single chain variable fragment (scFv) antibodies showing high sensitivity (83.9%) and specificity (100%) for three different strains of influenza virus (19).

The compilation of research articles included in this research topic should help reader to have an overview of different strategies for improving existing influenza vaccines and immunotherapies. This research topic also highlights the progress made in understanding of the immune protection and pathogenic mechanisms of influenza virus.

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MINI REVIEW
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# B cells and functional antibody responses to combat influenza

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Vaccination against influenza is the most effective way to protect the population. Current vaccines provide protection by stimulating functional B- and T-cell responses; however, they are poorly immunogenic in particular segments of the population and need to be reformulated almost every year due to the genetic instability of the virus. Next-generation influenza vaccines should be designed to induce cross-reactivity, confer protection against pandemic outbreaks, and promote long-lasting immune responses among individuals at higher risk of infection. Multiple strategies are being developed for the induction of broad functional humoral immunity, including the use of adjuvants, heterologous prime-boost strategies, and epitope-based antigen design. The basic approach is to mimic natural responses to influenza virus infection by promoting cross-reactive neutralizing antibodies that directly prevent the infection. This review provides an overview of the mechanisms underlying humoral responses to influenza vaccination or natural infection, and discusses promising strategies to control influenza virus.

**Keywords: influenza, hemagglutinin, functional antibody responses, universal influenza vaccine, neutralizing antibodies, vaccination strategies**

## Introduction

Influenza virus alone causes over 40,000 deaths every year in the United States, and even more during pandemics, like in 2009 with pandemic A/California/07/09 H1N1 virus strain (1, 2). Influenza viruses contain eight single stranded RNA segments and are classified in three different types (A, B, and C), on the basis of major antigenic differences; only influenza A and B are responsible for annual human epidemics. All influenza virus subtypes circulating in non-human species have the potential to infect humans, and transmissions from animals to humans may occur, albeit rarely, with dramatic scenarios for the public health; this was the case of the avian H5N1 strain that appeared for the first time in human in 1998 and re-appeared in 2004–2005 with a mortality rate of 50% among infected patients and thousands of deaths are reported until today (3, 4). Treatment of influenza infections is a major challenge for clinics and public health institutions because available antiviral drugs are often ineffective due to antigenic mutations or are given too late after infection (5). The most effective intervention that we have today to combat influenza is the vaccination that reduces virus infection and spreading, even if some levels of morbidity and mortality remain due to the suboptimal efficacy of the current vaccines and mismatch between the vaccine and the circulating virus strain.

Most of the current seasonal influenza vaccines are produced with live attenuated or inactivated (split or subunit) virus and both types of vaccines reduce virus infectivity and restrict viral replication by inducing functional antibodies against the virus. The antibodies generated against the virus

represent the primary correlate of immunity, whereas cell-mediated immunity can contribute to reduce the clinical symptoms (6). Although existing vaccines confer acceptable levels of protection in the general population, they are suboptimal, and are associated with some important limitations: (i) antigen composition needs to be updated every year in order to match the new seasonal circulating viruses, (ii) mismatch between the vaccine and the circulating virus can always happen, and (iii) people with a reduced ability to mount an immune response, infants, the elderly, and pregnant women respond suboptimally to these vaccines, requiring a tailored vaccine formulations (7–11).

Current influenza vaccines consist of three different virus strains: two influenza A strains (usually H1N1 and H3N2) and one influenza B strain. More recently, quadrivalent influenza vaccines have been developed, which are composed of influenza B strains of both lineages (12). Unfortunately, influenza strains acquire mutations every 1–3 years in their genome segments expressing the antibody-binding regions, a process named antigenic drift, and give raise to new circulating strains. Antigenic drift represents the principal immune evasion mechanism of influenza virus and has two major consequences: first, the humoral immunity developed in response to previous infections/vaccinations is usually non-fully effective against the new emerging strains, and second, manufacturers need to update the vaccine every year with increasing costs and risks of delays in the release of the lots. The virus can also undergo major antigenic changes in his hemagglutinin (HA) and neuraminidase (NA), referred to as antigenic shift, which consists of an ample reassortment of viral gene segments between different viruses of human or zoonotic origin, leading to the emergence of totally new and potentially dangerous virus strains, as happened during the pandemics of the last century and more recently in 2009 with the H1N1 virus of swine origin (13).

In this review, we summarize the mechanisms eliciting humoral responses against influenza infection or vaccination, and discuss the approaches that are today under evaluation to develop broadly protective and, hopefully, universal vaccines against influenza.

## Learning from Antibody Responses Against Influenza

Immune responses, generated against influenza by vaccination and by natural infection, consist of neutralizing and non-neutralizing antibodies. Non-neutralizing antibodies make the most part of the antibody pool generated during the immune response, but only a small fraction is functional and participates in the clearance of infected cells through interaction with other immune cells. On the other hand, neutralizing antibodies specifically bind epitopes crucial for viral function and are extremely important to confer immunity. Most of the neutralizing antibodies recognize surface proteins of the virus, in particular, the trimeric HA, which is critical during the process of cell invasion. The overall structure of HA can be segmented in a globular head and a stem region (**Figure 1**). The globular head is responsible for the sialic acid-dependent binding on the extracellular surface of target cells, and allows for a conformational change of the protein for membrane fusion. Neutralizing antibodies against HA interfere during both steps of the process, in particular, they bind to the sialic acid-binding site (or in close proximity) of the globular head, thus preventing attachment of the virus to the cells. Antibodies against the stem region may restrict the conformational changes required for the membrane cell fusion. Although both kinds of antibodies are functional, only those against the stem region can have the intrinsic ability to confer broad protection against different influenza strains because this region is much less susceptible to antigenic changes as compared to the globular head (**Table 1**). Unfortunately, stem-specific neutralizing antibodies are rare and difficult to induce because vaccination with the seasonal vaccine formulations typically skews

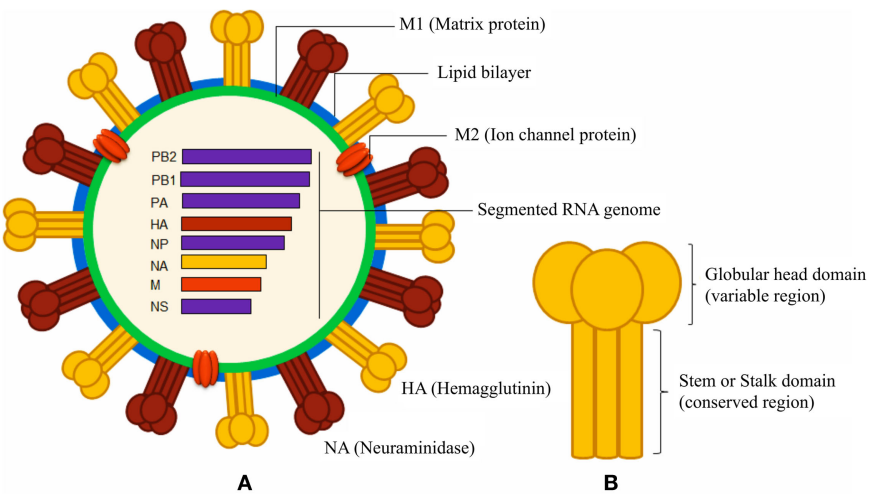


**FIGURE 1 | Schematic diagrams of influenza A virus and surface hemagglutinin protein. (A)** The segmented negative-sense RNA genome of influenza A virus encodes three envelope proteins (hemagglutinin, neuraminidase, and ion channel M2 protein), and internal nucleoprotein (NP), polymerases (PA, PB1, and PB2), matrix protein 1 (M1), and non-structural proteins (NS). The lipid bilayer is derived from host cell membrane. **(B)** The cylindrical HA is a homo-trimeric protein consisting of a variable globular head and a conserved stem domain.

**TABLE 1 | Target site of important cross-reactive neutralizing antibodies on HA stem domain**.

| Neutralizing antibodies | Epitope location on HA stem | Breadth | Reference |
|---|---|---|---|
| CR6261 | Helical region in the membrane-proximal stem of HA1 and HA2 | A/H1, H2, H5, H6, H8, H9 | (15–22) |
| CR9114 | F subdomain | A/H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, and influenza B viruses | (16, 22) |
| F10 | Helical region in membrane-proximal stem | A/H1, H2, H5, H6, H8, H9 | (21–25) |
| CR8020 | Base of stem in close proximity to the viral membrane | A/H3, H7, H10 | (26, 27) |
| C179 | Amino acid sequences 318–322 and 47–58 of HA1and HA2, respectively | A/H1, H2, H5, H6, H9 | (28, 29) |

the specificity of B cell responses toward non-neutralizing epitopes of the stem region or, depending on the formulation of the vaccines, toward immunodominant epitopes of the HA globular head (14).

Natural responses against influenza elicit also non-neutralizing antibodies, which are specific not only to HA and NA (30) but also against M1, M2, and NP proteins (**Figure 1**). Such non-neutralizing antibodies, typically, can promote the clearance of the virus, relying on their Fc portion after the interaction of the variable-region of the antibody with its epitope. Several cell populations, including phagocytic cells and natural killer cells, express Fc receptors (FcRs) and may mediate the clearance of virions or virus-infected cells (31, 32). Natural killer cells express FcRs and may participate in the killing of the virus-infected cells by a mechanism called antibody-dependent cell-mediated cytotoxicity (ADCC) (33); ADCC has been observed to participate in the control of the H1N1 influenza virus infection in macaques, and a mix of intravenous antibodies that may mediate ADCC has been suggested as therapeutic for humans (34, 35). Also, the complement system may participate in clearing the virus by a mechanism called complement-dependent cytotoxicity (CDC) involving influenza-specific antibodies; in particular, IgG antibodies that bind to target surface proteins may activate complement factors in the host serum that ultimately puncture the lipid membrane of pathogen or infected host cells. Two studies have clearly shown that C3, a critical component of the complement system, may participate in reducing viral titers and in clearing the virus with a mechanism that involves M2-specific antibodies of the IgG1 or IgG2a subclasses (36, 37).

## B Cell Responses to Influenza

Most of the responding B cells after influenza vaccination or infection are specific to HA, and they are difficult to isolate and characterize, especially by flow cytometry (38), because of the binding of HA to any sialic acid residue on the host cell. Taking advantage of *ex vivo* ELISpot assay, several studies have shown that adults or older children possess low but consistent base line levels of influenza-specific IgG memory B cells, in the range of 0.1–0.6% of the total IgG memory B cells (39). Those cells respond to further antigen encounter by quickly differentiating in antibody-secreting cells, they mostly produce isotype switched antibodies and show high frequencies of mutation in their Ig genes (40, 41). Pre-existing immunity in adults makes the characterization of the responses after seasonal vaccination challenging, so the 2009 H1N1 pandemic (pH1N1) influenza virus was a great opportunity to better understand the immune responses to influenza. Indeed, the pH1N1 HA was remarkably divergent from the HAs of the seasonal vaccines (even with a stem region quite conserved). Surprisingly, the highest numbers of deaths during the 2009 H1N1 pandemic were registered among the younger population, while the older population showed pre-existing protective immunity. How to explain the unexpected level of deaths among adults that is typically the most resistant group to influenza infections? It was suggested that adults had too low frequencies of cross-specific B cells to generate protective levels of cross-neutralizing antibodies against HA (42). On the contrary, the older population (over 65 years old) showed a very low incidence of infection and hospitalization (42–45), probably due to their life-long accumulation of an expanded reservoir of stem-specific cross-reactive memory B cells that efficiently responded to the 2009 pH1N1 virus (42). In addition, a close antigenic relation was found between the HA of the 2009 pH1N1 virus and the HA of influenza viruses that had circulated before 1950; hence, neutralizing antibodies against the HA globular head may also have contributed to protect the elderly population (46, 47).

In 2010, Lanzavecchia et al. reported that some individuals who received the seasonal influenza vaccine developed cross-reactive antibodies able to neutralize viruses belonging to different HA subtypes (H1, H2, H5, H6, and H9), including the pH1N1 isolate. By immortalizing IgG-expressing B cells, Lanzavecchia et al. showed that heterosubtypic monoclonal antibodies bound to acid-sensitive epitopes in the HA stem region, used different VH genes and carried high frequency of somatic mutations (24, 48, 49). More recently, the same group showed that most of the HA stem-specific antibodies are characterized by the use of the heavy-chain variable-region VH1-69 gene, only few polymorphisms are functional, and that few single somatic mutations are sufficient to promote high-affinity HA-specific antibodies (50).

The above studies have enhanced our understanding of influenza-specific B cell responses, and helped to set the primary goals in the development of next-generation anti-influenza therapies and vaccines. A major objective is to promote the generation of HA-specific broadly neutralizing antibodies in order to target cross-protective epitopes that are present among multiple strains. A second objective is to promote long-lasting memory B cells and plasma cells, hopefully for the entire life. Several strategies are today evaluated to achieve such goals including the use of adjuvants in vaccine formulation, heterologous prime-boost strategies, and antigen design with a "minimalistic-approach."

## Cutting-Edge Strategies for Inducing Protective Anti-Influenza Immune Responses

How to translate our knowledge of the influenza-specific humoral responses into novel strategies that specifically elicit the ideal protective immunity? As primary goals, successful vaccination strategies should confer cross-protection against multiple strains of influenza virus, and should boost long-lasting protective immunity in subjects with weakened immunity, as well as in younger and elderly populations.

A very promising strategy to meet those purposes is based on the use of particular adjuvant formulations. Adjuvants have been used in influenza vaccines for decades, usually in combination with split or subunit vaccines with the major goal to enhance their intrinsic immunogenicity (51). Although aluminum salts are potent adjuvants for most of the subunit antigens present in licensed vaccines, they seem not to be good adjuvants for influenza antigens. Instead, oil-in-water emulsions, like MF59, have been successfully used in influenza vaccines for the past 20 years with outstanding results (52, 53). MF59 not only induces high titers of influenza-specific antibodies but also cross-reactive responses against different clades of influenza viruses (54–56). Khurana et al. showed that MF59 adjuvant promotes high titers of HA-specific antibodies and expands the overall diversity of the influenza-specific antibody repertoire (14, 57). MF59 also promotes persistence of long-lasting memory B cells and increases the affinity of the antibody responses, not only in adults but also in younger and elderly (14, 52, 56–58); such evidences have shed light on the use of oil-in-water emulsion as adjuvants for influenza vaccines. Furthermore, oil-in-water adjuvants may prevent the effect of the "original antigenic sin" that is the propensity of the immune system to preferentially utilize immunological memory instead of inducing novel responses, hence limiting the development of an expanded B cell repertoire (59–61). Although their mechanisms of action are still not fully understood, MF59 and AS03 (62), the other oil-in-water adjuvant used for pandemic vaccines, represent an important tool on the way to develop broadly protective influenza vaccines. An increased risk of narcolepsy was found few years ago following vaccination with AS03-adjuvanted split influenza vaccine used in several European countries during the A/California/07/09 H1N1 influenza pandemic, but multiple subsequent studies have not confirmed any possible association between vaccination and narcolepsy (63–66).

An alternative strategy consists of heterologous prime/boost vaccinations. When the immune system encounters for the first time an influenza antigen, it generates specific antibodies and long-lasting memory B cells. Many influenza epitopes shift every year, so a second encounter with the antigen will recruit naïve B cells, which are specific for the new shifted epitopes and will also expand the pre-existing pool of memory B cells that is specific for the most conserved epitopes (30, 67, 68). Subsequent immunizations with divergent influenza antigens, the "prime/boost strategy" might expand the memory B cells specific for the most conserved epitopes that usually are under-represented in the B cell repertoire, hence inducing cross-protective immunity. This approach has been shown to be successful by Wang et al. who used a gene-based heterologous prime/boost strategy to induce cross-protection. Mice were sequentially immunized with DNA coding for the HA of different influenza A H3 virus strains (A/Hong Kong/1/1968, A/Alabama/1/1981 or A/Beijing/47/1992) and boosted with another H3 virus, A/Wyoming/3/2003; mice developed cross-neutralizing antibodies and protective capacity against multiple subtypes of H3 viruses (69). In a similar study, Wei et al. immunized mice twice with the same HA strains, but using a different delivery system for priming and for boosting. Mice primed with a DNA plasmid encoding H1N1 HA or H3N2 HA from the 2006/2007 vaccine strains and boosted with the trivalent 2006/2007 seasonal vaccine, developed enhanced neutralizing antibodies against diverse H1N1 strains compared to mice receiving only DNA or seasonal vaccine and showed higher levels of protection after infection (18). The above studies provided the proof-of-concept that a prime/boost strategy can increase the production of broadly neutralizing antibodies, and suggested that a combined strategy involving nucleic acids/proteins may have the benefit of expanding the antibody repertoire as well as inducing a different type of cellular immunity (18, 70). We further speculate that sequential immunizations with different HA proteins properly formulated with oil-in-water emulsion adjuvants (MF59 or AS03), may truly maximize the broadly neutralizing repertoire against influenza compared to non-adjuvanted vaccine formulations.

The minimalist approach is an innovative strategy that is evaluated today to promote cross-protective humoral responses. It is based on the design of antigens composed only of cross-protective epitopes, in order to focus the immune system on the desired response and generate cross-protective immunity. This approach is strongly supported by the fact that most of the broadly neutralizing antibodies identified until today are directed against the stem region of HA, and very few against its globular head (17, 23, 24, 26, 28). The minimalistic approach for antigen design has demonstrated to be successful in mice immunized with a "headless" HA, an antigen composed by the complete HA2 polypeptide and some regions of HA1 that both form the stem part of HA. Such antigen maintained the structural integrity of the conserved stem domain, but lacked the globular head with its immunodominant strain-specific epitopes (71). Sera form mice receiving the "headless HA" showed broader reactivity against heterologous strains than sera from mice vaccinated with the full-length HA and were protected against lethal virus challenge. Similar findings were obtained by using a stabilized HA2 peptide (72). Furthermore, Wang et al. designed a 60-amino-acid peptide to reproduce a long α-helix (LAH) of HA2 recognized by a broadly neutralizing monoclonal antibody, the clone 12D1 (73). The LAH peptide was not much immunogenic by itself, but when coupled to a carrier protein (KLH)-induced protection in mice challenged with divergent subtypes of influenza viruses, including H3N2, H5N1, and H1N1 strains; this work represents the most important proof that a carefully designed immunogen can be used in influenza vaccines to skew the B cell responses toward the epitope of interests. However, some concerns have been raised regarding the

development of a vaccine to elicit HA2 stem-targeting antibodies, not only because the stem region is poorly immunogenic by itself (requiring further optimization of the formulation with adjuvants or protein carriers) but also, in some circumstances, anti-stem antibodies have been observed to be detrimental for the host. Indeed, in a swine experimental model, Khurana et al. showed that a vaccine inducing anti-stem antibodies may have the risk to worsen the outcome of the influenza infection (74, 75).

Other groups have characterized cross-protective epitopes included in the HA globular head; in particular, Whittle et al. have identified a broadly neutralizing antibody that recognizes the receptor-binding pocket of HA and have suggested that a modified HA globular head could be used for epitope-based antigen design to promote broadly neutralizing antibodies (76, 77).

Although not strictly related to the "minimalistic approach" for antigen design, some work recently published by Giles et al. described a new computationally optimized broadly reactive antigen (COBRA) based on the structure of the HA from H5N1 subtype; mice and non-human primates immunized with this antigen develop broadly reactive antibodies and are protected from H5N1 challenge (78–80).

## Conclusion

Current influenza vaccines confer limited cross-protection against different strains of influenza and often fail to promote protective immunity in high-risk populations. Scientists are today evaluating multiple strategies to develop a universal influenza vaccine able to confer cross-protection, long-lasting immunity, and to be effective in subjects with weakened immunity. Such strategies include the use of oil-in-water emulsion adjuvants, heterologous prime/boost strategies, and antigen design. All these new strategies aim at inducing influenza-specific neutralizing antibodies that would confer sterilizing immunity in vaccinated hosts, and HA is the ideal antigen candidate to meet this purpose. Some groups are also evaluating alternative antigen candidates, such as NA, NP and M2, which are well conserved in multiple influenza strains and generate protective immunity through non-neutralizing antibodies helping to control the infection; hence, a multi-component vaccine not limited to HA antigen can be also considered. Each of the above strategies is promising to be successful, and most likely a combination of them will provide a universal influenza treatment in the future.

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# Development of cross-protective influenza A vaccines based on cellular responses

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Seasonal influenza vaccines provide protection against matching influenza A virus (IAV) strains mainly through the induction of neutralizing serum IgG antibodies. However, these antibodies fail to confer a protective effect against mismatched IAV. This lack of efficacy against heterologous influenza strains has spurred the vaccine development community to look for other influenza vaccine concepts, which have the ability to elicit cross-protective immune responses. One of the concepts that is currently been worked on is that of influenza vaccines inducing influenza-specific T cell responses. T cells are able to lyse infected host cells, thereby clearing the virus. More interestingly, these T cells can recognize highly conserved epitopes of internal influenza proteins, making cellular responses less vulnerable to antigenic variability. T cells are therefore cross-reactive against many influenza strains, and thus are a promising concept for future influenza vaccines. Despite their potential, there are currently no T cell-based IAV vaccines on the market. Selection of the proper antigen, appropriate vaccine formulation and evaluation of the efficacy of T cell vaccines remains challenging, both in preclinical and clinical settings. In this review, we will discuss the current developments in influenza T cell vaccines, focusing on existing protein-based and novel peptide-based vaccine formulations. Furthermore, we will discuss the feasibility of influenza T cell vaccines and their possible use in the future.

**Keywords: influenza vaccines, T cell vaccines, influenza A virus, cross-reactive immune response, peptide vaccines, correlates of protection**

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## Introduction

Several million people worldwide are infected with influenza viruses annually, which can result in hospitalization and even death from complications in severe cases. Vaccination is the preferred method to prevent influenza virus infections. Two types of influenza, influenza A and B, currently circulate among the human population. The influenza A virus (IAV), however, can be further divided in several subtypes and strains. The surface of antigens of IAV, hemagglutinin (HA), and neuraminidase (NA), frequently alter due to antigenic drift and sometimes alter due to antigenic shifts. Seasonal influenza vaccines need to be updated accordingly to match the circulating IAV strains. While seasonal influenza vaccines are effective against their matched IAV strains, they are unable to cross-react with unmatched strains. The lack of cross-reactivity of vaccine-elicited immune responses, mainly antibodies, is a major limitation of current influenza vaccines.

Several novel concepts for the development of cross-reactive IAV vaccines have been pursued in recent years. One concept is a vaccine that induces mucosal IgA responses, which can induce strong cross-protective antibody responses against closely related IAV strains (**Figure 1**). However, the cross-reactivity of these IgA responses with respect to more divergent strains is modest (1). Alternatively, vaccines that induce (IgG) antibody responses against conserved antigens, such as HA stalk-reactive- or M2e-specific antibodies, might be promising (2, 3). Studies, however, indicate that these approaches mostly lead to cross-reactive responses within the same phylogenetic group of IAV, such as H5N1 and H1N1 (4), with some exceptions (5, 6). Finally, vaccines inducing influenza-specific T cell responses can offer broad and long-lasting immune responses. Since T cells recognize epitopes that are mostly derived from viral proteins located in the nucleocapsid, which are conserved between IAV strains, T cell responses can be effective against a broad range of influenza strains. This averts the necessity of seasonally changing the influenza vaccine composition, and thus could be a significant improvement over the current influenza vaccines. A drawback of a purely T cell-inducing vaccine for the prevention of seasonal influenza could be that, unlike IgA antibodies, T cell responses cannot prevent infection but prevent (severe) disease. For the application as a universal vaccine, currently T cell responses are thought to have the highest potential to induce such broad heterosubtypic responses that can react to any IAV subtype.

Natural IAV infections induce, next to antibody responses, T cell responses that are potentially cross-reactive. Indeed, it is assumed that memory T cell established by previous IAV infections prevent subsequent IAV infection in some instances; most individuals experience severe IAV-induced symptoms only a few times in their life. However, there are indications that the cytotoxic T cell (CTL) activity of T cell recall responses wanes over times in humans, suggesting that T cell responses established by IAV infections can only protect for a few years (7). Additionally, the number of available influenza-specific memory T cells should be large enough to be able to rapidly respond to IAV infection without excess additional expansion of the T cell pool (8). Furthermore, it is known that regulatory T cells suppress T cell responses during IAV infections, which can have a negative effect on the subsequent formation of a memory T cell pool (9). Natural IAV infections therefore do not mount a T cell response potent enough to provide long-lasting protection against all heterologous IAV strains. T cell-inducing influenza vaccines might overcome this shortcoming by establishing long-lasting, cross-reactive T cell responses. In this review, we will focus on the latest developments in T cell-inducing influenza vaccine research. The selection of antigen, formulation and administration strategies, as well as possible risks and limitations of T cell-inducing vaccines are evaluated.

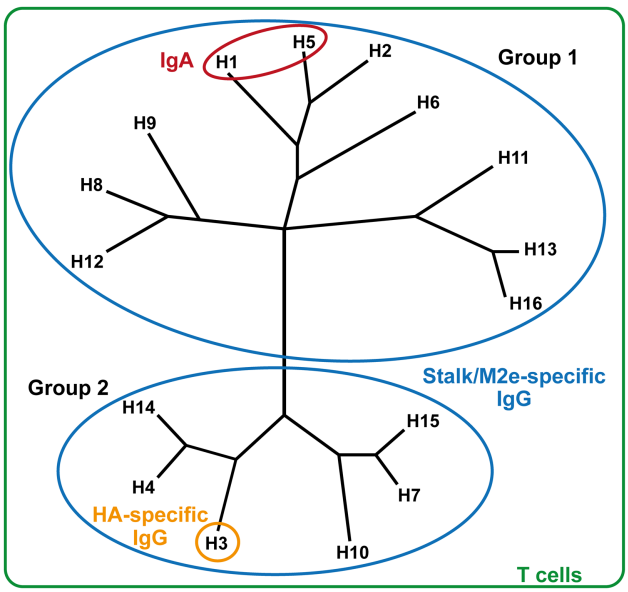


**FIGURE 1 | Reactogenicity of immune responses against influenza strains**. Influenza A strains are displayed in their respective phylogenetic groups. HA-specific IgG responses (orange) react only with homologous influenza strains. Mucosal IgA responses (red) can provide heterosubtypic reactivity against related influenza strains. Stalk- or M2e-specific antibodies (blue) are cross-reactive within either group 1 or group 2 influenza strains. T cells react universally against all influenza strains, regardless of subtype or group.

## CD8$^+$ T Cells

Primed CD8$^+$ T cells, otherwise known as CTLs, are able to lyse influenza-infected cells. Via the endogenous antigen presentation pathway, infected cells will present influenza-derived epitopes on their cell surface, which are recognized by influenza-specific CTLs. The CTLs then induce apoptosis of the target cell either through the secretion of perforins and granzymes or through the Fas ligand pathway. Furthermore, CTLs produce proinflammatory cytokines such as TNF-α and IFN-γ that exert antiviral activity, which further aids viral clearance (10, 11).

Several recent studies have elucidated the importance of CD8$^+$ T cells during IAV infections in humans. Sridhar et al. showed that individuals who possessed a higher frequency of CD8$^+$IFN-γ$^+$IL-2$^-$ T cells experienced a decreased clinical illness during infection with pandemic 2009 H1N1 IAV (12). CD8$^+$IFN-γ$^+$IL-2$^-$ T cells were correlated with a decreased risk of fever, an absence of viral shedding and reduced influenza-like illness (ILI) symptoms. These cells also expressed the lung-homing marker CCR5, which might explain their effectivity. CD8$^+$ T cells induced by seasonal IAV strains were shown to be cross-reactive with several influenza A strains such as 2009 H1N1, swine-origin H3N2, and the recently emerged H7N9 IAV (13–15). Indeed, when cellular responses were studied in individuals infected with pandemic 2009 H1N1 IAV, rapid recall responses of CD8$^+$ T cells were observed, which peaked within 1 week after infection (16). These responses were thought to originate from lymphoid memory CD8$^+$ T cells established from prior seasonal IAV infections. Memory T cells were demonstrated to last for at least several years in a study, which assessed IAV-specific T cell responses in PBMCs of individuals collected from 1999 to 2012 (17). PBMCs from several donors were stimulated with Resvir-9 (a H3N2 reassortant strain), and IAV specificity and CTL activity were subsequently determined by intracellular staining with several labeled, highly conserved CTL peptides and IFN-γ.

Taken together, these studies indicate that $CD8^+$ T cells can play a role in the protection against IAV infections, that these T cells are long-lived and are able to cross-react with multiple IAV strains. Thus, the induction of these T cells may be the basis of broadly reactive universal influenza vaccines.

## $CD4^+$ T Cells

$CD4^+$ T cells also play an important role in IAV infections, but contrary to $CD8^+$ T cells, have not been studied extensively yet in humans. In animal models, activated $CD4^+$ T cells can exert different roles in relation to IAV infections. $CD4^+$ T cells can act as T helper cells ($T_H$), providing costimulatory signals by CD40/CD40L signaling to antigen presenting cells (APCs) during the priming of B cells and $CD8^+$ T cells (18, 19). Interestingly, reactivation of adoptively transferred $CD4^+$ $T_H$ (from IAV challenged mice) increased the recall capacity of both memory $CD4^+$ and $CD8^+$ T cell responses in receptive mice after IAV infection (20). While $CD4^+$ $T_H$ is not necessarily required for the induction of effector CTLs, it is crucial for the transition of $CD8^+$ T cells to the memory phase, which is essential for the maintenance of long-lived immunity (21, 22). Surprisingly, $CD4^+$ T cells can also acquire cytotoxic activity through the release of perforin in mice, providing direct protection against IAV infection (23).

In humans, it was found that pre-existing $CD4^+$ T cells were reactive to pandemic 2009 H1N1 peptides, and were correlated with lower virus shedding and reduced illness during IAV infection (24). Unexpectedly, $CD8^+$ T cell responses were not associated with reduced illness in this study. Nonetheless, it can be concluded that preclinical and clinical studies indicate that targeted induction of $CD4^+$ T cell responses, next to $CD8^+$ T cell responses, may be an attractive goal for novel vaccines.

## T Cell-Inducing Vaccines

Immune responses, and in particular, the antibodies elicited by current seasonal influenza vaccines are limited in their effectiveness against heterologous IAV infections. From the current knowledge on T cell responses during IAV infections in preclinical and clinical studies, as described above, it is believed that T cell-inducing influenza vaccines have the potential to result in broadly reactive, universal influenza A vaccines. While most vaccines are still in preclinical development, a few concepts have recently entered the clinical phase. In **Table 1**, the most recent developments in T cell-inducing vaccines are listed. Recently, the potency of viral vector-based influenza vaccines has been reviewed (25). In the following paragraphs, several other potential T cell-inducing influenza vaccines are highlighted.

### Live Attenuated Influenza Vaccines

Live attenuated influenza vaccines (LAIV) are currently on the market as intranasal (i.n.) IAV vaccines. LAIV induce next to humoral responses both $CD4^+$ and $CD8^+$ T cells in children (26, 27). Remarkably, no cellular immune responses are detected in adults receiving LAIV; the cause of this discrepancy might be related to the naïve status of children. Furthermore, LAIV are more effective than current seasonal trivalent inactivated influenza vaccines (TIV) in children but not in adults, suggesting that the induction of cellular immune responses increases the efficacy of LAIV (48). The encapsulation of LAIV in a biopolymer of alginate and subsequent subcutaneous (s.c.) administration-induced $CD8^+$ T cell responses that protected mice from a heterologous IAV challenge (49), indicating that LAIV can induce T cell responses via immunization routes other than i.n. by use of formulation strategies. The induction of cellular responses by LAIV might be explained by the "live" state of the vaccine antigen; it can still infect after vaccination. During the viral replication, many viral proteins containing $CD8^+$ and $CD4^+$ epitopes are produced within the infected host cell, leading to efficient antigen processing via the endogenous pathway, which leads to MHC class I presentation and subsequent T cell activation.

### Whole Inactivated Influenza Virus

Like LAIV, whole inactivated influenza virus (WIV) contains internal proteins such as nucleoprotein (NP), matrix proteins 1 and 2 (M1 and M2, respectively), polymerase basic proteins 1 and 2 (PB1 and PB2, respectively) and polymerase acidic protein (PA), which possess conserved T cell epitopes. WIV vaccines were replaced by subunit and split vaccines due to incidence of adverse events associated with WIV (50), but have been given increased attention the past few years in the search for cross-reactive vaccines (51). Improvements on WIV production and purification methods have decreased WIV-associated side effects, making this vaccine acceptable for use again, especially for the induction of broadly reactive immune responses. At normal clinical dose, which typically does not exceed 15 μg of HA protein, WIV induces adequate neutralizing antibody titers, but generally fail to induce any cellular responses regardless of administration route (52). However, studies by Budimir et al. showed that multiple high doses of WIV, such as two times 6 μg, were able to induce significant amounts of IAV-specific CTLs in mice (53–55). The critical roles of membrane fusion activity and the presence of viral ssRNA for the induction of CTLs were established (53, 55). Intramuscular (i.m.) administration of WIV proved to be more effective at inducing CTLs than i.n. administration (54). This was confirmed by Takada et al., who found that intranasal vaccination with WIV failed to induce T cell responses (56). By contrast, one study utilizing gamma-irradiated WIV showed that the protective effect of WIV was mainly mediated by T cell responses (29). It is suspected that the method of WIV inactivation can have an effect on its immunogenicity. Aside from increased dosage, WIV-induced cellular responses can also be boosted by the addition of adjuvants. For instance, a dose of 2.5 μg WIV adjuvanted with cationic lipid/DNA complex (CLDC) was able to induce influenza-specific $CD4^+$ and $CD8^+$ T cell responses in mice, whereas alum adjuvanted WIV only induced high-antibody responses (57). Similar to studies with WIV, the addition of alum to virosomes proved to be detrimental to cellular responses in mice (58), since it skewed the $T_H$ to a $T_H2$-type response.

### Virosomes

Virosomal vaccines can also induce influenza-specific CTL responses. The addition of adjuvants to virosomes is necessary to induce T cell responses, since unadjuvanted virosomes only

**TABLE 1 | T cell-inducing influenza vaccines in recent development**.

| Class | Concept name | Antigen(s) | Adjuvant(s) | Immune response | Status | Reference |
|---|---|---|---|---|---|---|
| Whole virus or protein vaccine | Live attenuated influenza vaccine | Live attenuated influenza vaccine (various strains) | None | Induces $CD4^+$ and $CD8^+$ T cell responses in unprimed children | Licensed | (26, 27) |
| | | Single-cycle live attenuated influenza vaccine (H3N2) | None | Induced $CD8^+$ T cell responses in mice that protected against heterologous challenge | Preclinical | (28) |
| | Gamma-irradiated whole inactivated influenza vaccine | Whole inactivated influenza vaccine (H3N2) | None | Induces robust influenza-specific T cell responses in mice | Preclinical | (29) |
| | Influenza virosomes | Virosomes (H5N1) | Matrix-M | Induces good influenza-specific $CD4^+$ T cell responses in healthy adults, but $CD8^+$ T cell responses were limited | Phase I trials | (30) |
| | Multimeric-001 | Synthetic protein containing B and T cell epitopes from HA, M1, and NP | Montanide ISA 51VG | Induces cellular responses in healthy adults and elderly that are reactive against multiple IAV strains | Phase I trials | (31, 32) |
| Peptide vaccine | Lipopeptides | Minimal T cell epitopes from M1, PA, and NS1 | Pam2Cys | Induces $CD8^+$ T cell responses that protect mice against heterologous IAV challenge | Preclinical | (33) |
| | | Minimal T cell epitopes from HA and NP combined with seasonal influenza vaccine | Pam2Cys | Induces $CD8^+$ T cell responses that reduces lung viral load in mice after heterologous challenge | Preclinical | (34) |
| | | Minimal T cell epitope from NP | Phosphatidylserine | Induces peptide-specific $CD4^+$ and $CD8^+$ T cell responses in mice | Preclinical | (35) |
| | Liposome-conjugated peptides | Minimal T cell epitopes from M1, NP, PA, PB1, or PB2 | Liposomes, CpG-ODN 5002 | Induces T cell responses that protect mice from IAV challenge | Preclinical | (36, 37) |
| | Peptide-loaded virosomes | Minimal T cell epitope from M1 | Virosome, CpG-ODN 1826 | Induces peptide-specific $CD8^+$ T cells that reduce body weight loss of mice after heterologous IAV infection | Preclinical | (38) |
| | FP-01.1 | Long peptides containing T cell epitopes from M1, NP, PB1, and PB2 | Peptides conjugated to fluorocarbon moiety | Induces $CD4^+$ and $CD8^+$ T cells in healthy adults that are cross-reactive against IAV-infected target cells | Phase I trials | (39) |
| | Flu-v | Long peptides containing T cell epitopes from M1, M2, and NP | Montanide ISA 51VG | Induces peptide-specific $CD8^+$ T cells in healthy adults | Phase I trials | (40) |
| Virus-like particle/viral vector vaccine | Peptide fused to PapMV nanoparticles | T cell epitope from NP | Papaya mosaic virus nanoparticles | Induces peptide-specific $CD8^+$ T cells in mice | Preclinical | (41) |
| | DdFluM1 | T cell epitopes from M1 | Adenoviral dodecahendron particles | Induces peptide-specific $CD4^+$ and $CD8^+$ T cells in chickens | Preclinical | (42) |
| | PIV5-NP | T cell epitope from NP | Parainfluenza 5 | Induces $CD8^+$ T cells in mice that reduce morbidity and lethality after IAV challenge | Preclinical | (43) |
| | MVA-NP + M1 | T cell epitopes from M1 and NP | Modified vaccinia virus Ankara vector | Induces influenza-specific cellular responses in healthy adults and elderly that reduce viral shedding and reduction of symptoms | Phase II trials | (44–46) |
| DNA vaccine | DNA plasmids encoding for T cell epitopes | DNA encoding for B and T cell epitopes from HA and NP | None | Induces T cell responses that reduce body weight loss of mice after IAV challenge | Preclinical | (47) |

induce humoral responses. The incorporation of LpxL1, a detoxified lipopolysaccharide, in virosomes significantly increased IFN-γ secretion in mice (59). Madhun et al. showed that addition of the saponin-based Matrix-M adjuvant to virosomes significantly increased the production of $T_H1$-associated cytokines IL-2 and IFN-γ when administered i.m. to mice (60). Strikingly, a significant induction of multifunctional $CD4^+$ T cells was also observed in a murine model after the addition of Matrix-M to the virosomal vaccine. In a similar study, Radosevic et al. screened multiple adjuvants (i.e., aluminum phosphate, aluminum hydroxide, MF59, and Matrix-M) in combination with virosomes in mice (61). Unlike the study by Madhun et al., virosomes were readily able to induce $CD4^+$ T cells, and addition of any adjuvant, including Matrix-M, did not increase these responses. However, only MF59 and Matrix-M adjuvanted virosomal vaccines were able to induce IAV-specific $CD8^+$ T cell responses. Furthermore, addition of any aluminum salt-based adjuvants proved to be ineffective at eliciting any cellular responses, which was probably due to $T_H2$-skewed immune responses by aluminum salts.

The ability to induce cellular immune responses by some marketed influenza vaccines is of great value in order to offer limited cross-reactivity against non-matched influenza strains. These vaccine formulations can play a role as an intermediate solution until the next generation of cross-protective influenza vaccines is developed.

## Peptide Antigens

Peptides are another type of antigen that can be used in T cell-inducing influenza vaccines. However, short peptides that consist of a minimal epitope are generally not immunogenic, and thus require additional modification or formulation to be able to induce T cell responses (62).

Several preclinical studies have used minimal epitope peptides as their main antigen to induce influenza-specific cellular responses. Short influenza peptides conjugated to phosphatidylserine were able to induce $CD8^+$ T cell responses in mice (35). The conjugation of lipids to peptides opens up several possibilities; a PA-derived peptide conjugated to Pam2Cys, a lipid and TLR2 ligand, and efficiently induced peptide-specific CTL responses in mice (63). Furthermore, peptides conjugated to liposomes were able to minimize morbidity in IAV-infected mice through the induction of $CD8^+$ T cells (36, 37). Remarkably, these peptide–liposome conjugates were able to induce $CD8^+$ memory T cells without the contribution of $CD4^+$ T cells. Liposomes act as a delivery system for the peptides, which are then internalized more efficiently by APCs than unformulated peptides. Direct conjugation of the peptide to a lipid or liposome is, however, not required. $NP_{366–374}$ peptide encapsulated in liposomes was able to induce potent T cell responses when mixed with anti-CD40 mAbs, and reduced viral lung titers of influenza-infected mice (64).

Aside from liposomes, virosomes have also been used as delivery systems for short peptide antigens. These virosomes utilize the membrane fusion activity of HA proteins to deliver the loaded peptide to the cytosolic compartment of the APC. An early study showed that virosomes loaded with the $H\text{-}2K^d$ binding influenza $NP_{147–155}$ peptide-induced CTLs that were able to lyse IAV-infected target cells (65). The addition of the adjuvant CpG-ODN 1826 to influenza $M1_{58–66}$ peptide-loaded virosomes was shown to increase peptide-specific $CD8^+$ T cell responses even further (38), which resulted in a faster recovery of vaccinated mice after heterologous influenza virus infection.

Long peptide vaccines consisting of multiple epitopes are, opposed to short peptide vaccines, already in the clinical testing phase. Flu-v consists of an equimolar mixture of four synthetic polypeptides derived from M1, M2, and NP IAV proteins, formulated with the adjuvant Montanide (40). Flu-v-induced peptide-specific T cells in healthy subjects; unfortunately, reactivity against actual IAV strains was not determined. However, vaccination studies in mice showed that $CD8^+$ T cell responses induced by Flu-v did reduce mortality after IAV infection (66).

Similar to Flu-v, FP-01.1 consists of six polypeptides derived from M1, NP, PB1, and PB2, which were conjugated to a fluorocarbon moiety. The vaccine was able to induce $CD4^+$ and $CD8^+$ T cells in healthy subjects (39). Moreover, these T cells were cross-reactive with H1N1 and H3N2 IAV-infected target cells. This is the first study that shows a peptide vaccine capable of inducing cross-reactive T cells in humans, which is very encouraging for the development of cross-reactive T cell-inducing vaccines.

The studies described above suggest that peptide-based approaches are very promising in the development of T cell-inducing IAV vaccines. However, an important challenge is the genetic variability among the human population in relation to epitope recognition and presentation. $CD4^+$ and $CD8^+$ T cells recognize IAV epitopes displayed on MHC molecules, which are called human leukocyte antigen (HLA) molecules in humans. Different HLA polymorphisms occur in the human genome, resulting in a host of varying HLA molecules in the human population. Each HLA can only bind specific viral epitopes, which means that multiple epitopes of the same antigen need to be in a peptide-based vaccine to cover the human population (67). *In silico* prediction methods can be employed to determine the potential T cell immunogenicity of conserved epitopes across multiple IAV strains (68). Furthermore, several transgenic mouse strains have been bred that express HLA molecules, which can be used in preclinical development. Nonetheless, there remains a significant challenge for peptide-based vaccines to include enough epitopes to cover each HLA type, which would be required for a vaccine to be effective in the entire population.

## Other T Cell Influenza Vaccine Concepts

Aside from the vaccine strategies described above, several other concepts are currently in clinical development (**Table 1**). Multimeric-001 is a synthetic recombinant protein composed of nine T cell and B cell epitopes derived from HA, NP, and M1 influenza proteins (31). The vaccine in combination with the adjuvant Montanide ISA 51VG was able to induce cellular responses in healthy subjects. The cellular responses showed limited reactivity to multiple IAV strains. In a follow-up study, the Multimeric-001 vaccine showed an induction of humoral and cellular responses in elderly subjects similar to responses observed in healthy adults (32). While the results of these studies are encouraging, the true effectiveness of the induced cellular responses against homologous and heterologous IAV infections has yet to be determined.

Another concept, which has advanced to the clinical stage of development, is the modified vaccinia virus Ankara vectored vaccine MVA-NP + M1 (45). This vaccine consists of a vaccinia virus Ankara expressing the influenza proteins NP and M1. Several clinical trials, including a phase II study, were conducted with this vectored vaccine. MVA-NP + M1 was able to expand pre-existing memory $CD8^+$ T cells in both healthy adults and elderly, and also increased the IAV-specific $CD4^+$ T cell population (44, 46).

### T Cell-Based Influenza Vaccine Concepts in the Clinical Phase

The protein-based influenza vaccines such as LAIV, WIV, and virosomes currently have the advantage that they are already licensed and have been widely used. Such vaccines might be excellent candidates to prime naïve populations for both cellular and humoral responses.

Peptide-based vaccine concepts have the advantage that they can be easily engineered and produced synthetically. However, as mentioned above, selection of the right epitopes remains vital. These vaccines also require additional formulation with adjuvants to increase their immunogenicity. Nonetheless, several peptide-based vaccines have entered the clinical phase.

Vectored T cell-inducing vaccines are a sophisticated concept. They include both antigen and adjuvant in a single particle. Since they express whole proteins rather than epitopes, vectored vaccines might have a higher coverage among different populations compared to peptide-based vaccines. A recent study also combined a seasonal influenza vaccine with MVA-NP + M1 to increase the breadth of the immune response (69). Such an approach is a major improvement and might be an ideal solution to induce both humoral and cellular immunity with a single vaccine. Other concepts, such as peptide-based influenza vaccines, are also eligible to be used simultaneously with seasonal influenza vaccines, as demonstrated recently (34). This is a good step toward a universal influenza vaccine.

## Vaccine Priming

The IAV-naïve status and age of persons may influence the immunogenicity of T cell-inducing IAV vaccines. This was already observed with LAIV vaccines, which effectively induce cellular responses in naïve children, but not induce such responses in adults, who already established an immunological memory to IAV (26, 27). A study in mice reported that $CD8^+$ T cells primed by LAIV rapidly differentiated to IAV-specific memory T cells after short-interval boosting, and were able to protect against heterologous challenge (70). Several T cell-inducing vaccine concepts consider the potency of the prime-boost approach; a DNA–protein prime-boost concept enhanced the T cell responses to IAV in mice (71), and in a clinical trial priming with Multimeric-001 before a seasonal influenza vaccine boost greatly increased IAV-specific cellular responses in elderly subjects (32). Priming at an early age in naïve mice with IAV resulted in the induction of long-term memory $CD8^+$ T cells with the broadest reactivity, while priming at an older age resulted in a $CD8^+$ T cell population with a reduced diversity (72). Thus, T cell priming at an early age, when the subject is still naïve, should be considered before immunization with an influenza vaccine that only induces humoral responses. As a result, the intended target population of a vaccine is key for vaccine design and development (73).

## Resident Lung T Cells

Many T cell-inducing vaccine concepts aim for the induction of systemic IAV-specific T cell responses. However, local T cell responses at the site of IAV infection are potentially more effective. The presence of IAV-specific resident memory T cells ($T_{RM}$) in the lungs was correlated with clearance of heterologous IAV infection in mice (74). $CD4^+$ T cells mediated the formation of $CD8^+$ $T_{RM}$ cells, adding yet another important function for $CD4^+$ $T_H$ (75). Current knowledge on the establishment of $T_{RM}$ cells has been reviewed recently (76). While the process of $T_{RM}$ induction is not completely unraveled, some possible mechanisms can be exploited to induce IAV-specific $T_{RM}$ responses with vaccines. A recent study specifically targeted an antigen to resident lung DCs using antibodies, and were able to generate IAV-specific $CD8^+$ $T_{RM}$ cells in mice that provided protection against a lethal influenza challenge (77). Furthermore, it is known that CXCR3-expressing $CD8^+$ T cells play an important role in the establishment of $CD8^+$ $T_{RM}$ cells in the lungs (78). The near future may learn us whether specific targeting of certain T cell populations, e.g., by adjusting the route of administration to the lungs (79, 80), may add to the potential of T cell-inducing influenza vaccines.

## Preclinical Cellular Correlates of Protection

There is clear evidence that cellular responses correlate with a reduction of symptoms after IAV infection. However, current correlates of protection (CoP) for influenza vaccines are all based on the induction of antibodies, such as the presence of hemagglutination inhibition- or virus neutralization titers, which are inadequate CoPs for T cell-inducing vaccines. Instead, responses that indicate the presence of effector T cells such as IFN-γ and IL-10 cytokines, combined with cytotoxic effector molecules like granzyme B may be more suitable as CoP for T cell-inducing vaccines (81). These parameters also need to be further evaluated in epidemiological studies in order to define their efficacy. For instance, it is still unclear what quantitative levels of IAV-specific $CD8^+$ or $CD4^+$ T cell responses are required for protection against an IAV challenge. Furthermore, an adequate translation from animal models to the human setting has to be made. While there is quite some experience with humoral responses against IAV in animal models and their relation to the clinic, such experience has not been established yet for cellular responses. Establishing these responses as human CoPs, and translating study findings from animal models to humans remain important tasks for the development of T cell-inducing IAV vaccines.

## Concerns and Limitations of T Cell-Inducing IAV Vaccines

There are some concerns whether IAV-specific T cells can provide the same level of protection compared to IAV-specific antibodies. While T cells have a broader reactivity, they can only recognize and lyse IAV-infected host cells. Most likely, an IAV infection is

already spreading before an efficient T cell response is mounted. It can therefore be debated whether T cells responses actually provide protection (i.e., sterilizing immunity) or only shorten the length and severity of influenza symptoms (i.e., decreased morbidity). The difference between these two can be very hard to distinguish. Therefore, elucidation of T cell responses after influenza infection in humans is of critical importance to determine the efficacy of T cell-inducing influenza vaccines. Nonetheless, reduction of morbidity of IAV infections would already be a great success in situations where seasonal influenza vaccines would be ineffective, such as a mismatched influenza epidemic or an influenza pandemic. The definition of protection should therefore not only be limited to sterilizing immunity but also to reduction of disease morbidity.

Another concern is the possibility of excessive T cell responses to IAV infections, which could cause immunopathology in the lungs (82). There are indications that excessive T cell responses mediate severe lung inflammation and subsequent lung damage after IAV infection in mice. Only one study describes the phenomenon in humans; elevated IAV-specific $CD8^+$ and $CD4^+$ T cell responses were found in pandemic 2009 H1N1-infected children with severe pneumonia (83). It was, however, unclear whether these T cell responses were the cause of pneumonia or simply present due to the infection.

It is yet unknown whether T cell-inducing influenza vaccines can mount long-lasting T cell responses after a limited number of immunizations. As already discussed above, natural IAV infections are able to induce T cell responses, but their effectivity is limited. Studies suggest that local inflammation and inflammatory cytokine production caused by IAV infection suppress $CD8^+$ T cell responses in mice. This was partly attributed to an increased expression of PD-L1 on the $CD8^+$ T cells, which cripples the functionality of these T cells (84, 85). T cell-based vaccines, however, should not experience the effects of these immunosuppressive pathways, since inflammation after immunization is generally limited. It is thus likely that these vaccines can induce T cell responses, which are more potent than those elicited by natural IAV infections. Nonetheless, it is important that T cell-inducing vaccines elicit balanced T cell responses, and special interest should be given to T cell-mediated immunopathology during safety studies of these vaccines.

Aside from the intensity of T cell responses, special attention should be given to the selection of target epitopes derived from IAV. A recent study described the existence of tolerizing epitopes in certain influenza strains, which are recognized by autologous regulatory T cells and may suppress protective T cell responses (86). Another study found that T cells against certain immunodominant epitopes such as $M1_{58–66}$ have a poor functionality, and are unable to clear IAV-infected cells (87). It was hypothesized that these immunodominant epitopes are actually a decoy of IAV to evade T cell-mediated immunity and to prevent the generation of more potent T cells against other epitopes. It is therefore important that such epitopes, which could lead to decreased or impotent T cell responses, are identified and excluded in any prospective T cell-inducing IAV vaccines.

## Conclusion

Humoral immune responses elicited by current IAV vaccines do not provide sufficient cross-protection against non-matched IAV infections. IAV-specific T cells recognize conserved epitopes of IAV and thus have to potential to be cross-protective. Many different T cell-inducing vaccines are currently under development, and some have even reached clinical phases. Selecting suitable preclinical testing models and clinical CoPs are vital for further development of such vaccines. In addition, proper understanding the effectiveness of each T cell response and their possible pathological effects is of great importance. The current developments with T cell-inducing IAV vaccines, including novel formulations and extended immunological insight, are fast evolving and may ultimately result in universal influenza vaccines.

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# Single-dose CpG immunization protects against a heterosubtypic challenge and generates antigen-specific memory T cells

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Despite extensive research, influenza A virus (IAV) remains a major cause of morbidity, mortality, and healthcare expenditure. Emerging pandemics from highly pathogenic IAV strains, such as H5N1 and pandemic H1N1, highlight the need for universal, cross-protective vaccines. Current vaccine formulations generate strain-specific neutralizing antibodies primarily against the outer coat proteins, hemagglutinin and neuraminidase. In contrast to these highly mutable proteins, internal proteins of IAV are more conserved and are a favorable target for developing vaccines that induce strong T cell responses in addition to humoral immunity. Here, we found that intranasal administration with a single dose of CpG and inactivated x31 (H3N2) reduced viral titers and partially protected mice from a heterosubtypic challenge with a lethal dose of PR8 (H1N1). Early after immunization, vaccinated mice showed increased innate immune activation with high levels of MHCII and CD86 expression on dendritic cells in both draining lymph nodes and lungs. Three days after immunization, CD4 and CD8 cells in the lung upregulated CD69, suggesting that activated lymphocytes are present at the site of vaccine administration. The ensuing effector Th1 responses were capable of producing multiple cytokines and were present at least 30 days after immunization. Furthermore, functional memory responses were observed, as antigen-specific IFN-$\gamma^+$ and GrB$^+$ cells were detected early after lethal infection. Together, this work provides evidence for using pattern recognition receptor agonists as a mucosal vaccine platform for inducing robust T cell responses capable of protecting against heterologous IAV challenges.

**Keywords: immunization, CpG, influenza A virus, nasal mucosa, vaccines, cytotoxic T lymphocytes, CD4 T lymphocytes, CD8 T lymphocytes**

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## Introduction

Influenza A viruses (IAV) cause annual outbreaks of upper respiratory tract infection and induce severe infections in nearly three to five million people per year (1). In addition, IAV induces occasional pandemics, making it a prominent pathogen today. Influenza A virus outbreaks occur due to high rates of viral mutations in the outer coat proteins and reassortment of viral RNA segments stemming from antigenic drift (2) and antigenic shift (3),

**Abbreviations:** CTL, cytotoxic T lymphocyte; GrB, granzyme B; IAV, influenza A virus; IFN-γ, interferon gamma; PR8, influenza A/Puerto Rico/8/34; TNF-α, tumor necrosis factor alpha; x31, influenza A/Hong Kong/1/1968-X31.

respectively. Due to these unpredictable events and poor predictive modeling, protection by current vaccines can be greatly reduced in some influenza seasons (4). For example, vaccine effectiveness has been shown to be 70–90% when circulating strains are well matched, but <50% if antigenic mutations occur (5). Influenza has increased deleterious effects on people at both ends of the age spectrum, with increased morbidity in the elderly and health complications in young children. Thus, the ability of the influenza virus to mutate and evade host immunity coupled with inadequate vaccines for at-risk populations highlights the need to develop a more universal influenza vaccine.

Current influenza vaccines are designed to generate humoral immunity against hemagglutinin (H) and neuraminidase (N) proteins in the vaccine strain. Master seed stalks are developed from either cold-adapted live virus or inactivated virus consisting of prevalent H1N1, H3N2, and one or two influenza B strains. Thus, the generation of high antibody titers and seroconversion to outer coat proteins in the vaccine strains are the main mechanisms of protection. However, antibodies induced by these vaccines have significant limitations if antigenic variation occurs. Therefore, universal vaccine approaches are currently being explored based on the fact that immunization with conserved influenza proteins elicits protection in many animal models following a lethal heterosubtypic challenge (6–8).

One approach to universal influenza vaccines is based on broadly neutralizing antibodies (9, 10). The majority of these studies focus on generating antibody responses to the stalk region of the HA protein that interfere with the ability of receptor-binding motifs to bind to sialic acid or inhibit fusion with the endosomal membrane, thus preventing infection. While broadly neutralizing antibodies can mediate sterilizing immunity, problems still persist with these approaches including low titers of stalk neutralizing antibodies after infection and/or overcoming the immune response to the immunodominant globular head of HA (11).

A second approach is to target T cells by vaccination. Successful universal IAV vaccines not only induce T cell responses with cytokine-producing and cytotoxic capabilities but also induce helper T cells responsible for generating optimal antibody responses (12) and maintaining CD8 memory (1). Furthermore, influenza-specific T cells not only respond to surface antigens but are also capable of mounting strong protective responses to much less variable regions of the influenza proteome including the nucleoprotein (NP), matrix (M), and polymerase proteins (13–15). Thus, T cell-based vaccines can be directed toward internal antigens that are well conserved between IAV subtypes and can circumvent outer coat mutations and viral escape variants to initiate protective immune responses.

As natural infection generates high levels of antigen-specific cells (16), CD8 cytotoxic T lymphocytes (CTL) responses have been established as a key cell type important for heterosubtypic infection. However, CD8 CTL is not the only cell type implicated in heterosubtypic protection. A role for CD4 cells is now becoming increasingly appreciated in establishing protection against heterologous IAV infections. Supporting observations for the importance of CD4 T cells include a role for cytolytic CD4 cells (17). These cells, via perforin dependent cytotoxicity in the absence of antibodies, have been shown to be important for protection against a lethal viral challenge (12, 18). Furthermore, vaccine platforms that induce T cell responses have translational applications as pre-existing memory T cells specific to internal influenza proteins have been associated with less virus shedding and lower symptom scores in humans (13, 19). Therefore, cell-mediated immunity should be able to provide a broad range of protection against serologically distinct viruses.

Consistently boosting and maintaining high levels of antigen-specific memory T cells still have not been achieved in humans as current influenza vaccines do not universally boost T cell responses across vaccinated individuals. One way to enhance vaccine efficacy and boost cellular responses is through the addition of adjuvants (20, 21). Unmethylated CpG, a TLR9 agonist, is currently being examined as an adjuvant in a number of clinical applications ranging from vaccine development to cancer immunotherapy (22). CpG is an attractive vaccine adjuvant not only for its potent immunostimulatory properties but also for its excellent stability, tolerability, and metabolism within a host.

Like IAV infection, CpG administration induces a Th1-biased response. Many of the immunological reactions that CpG induces result in adaptive responses that share a common mechanism with IAV infection. Indeed, both human and mouse studies have demonstrated that administration of CpG allows for enhanced T cell interferon-gamma (IFN-γ) production and CTL responses with antiviral capabilities *in vitro* and *in vivo*. (23–25). Because of the adjuvanticity in inducing robust T cell responses, CpG combined with inactivated influenza makes for a promising vaccine candidate. Here, we show that a single dose of CpG and inactivated influenza via the mucosal route promotes the development and differentiation of effector T cells that persist into memory and confer partial protection against a heterosubtypic influenza challenge. The current study highlights the evidence for the generation of a universal influenza vaccine that could not only provide protection against seasonal IAV variants but also highly virulent, potentially pandemic infections.

## Materials and Methods

### Mice

Male BALB/cByJ mice were purchased from Jackson Laboratories (RRID:IMSR_JAX:001026). Mice 6–8 weeks old were used in all experiments. Experimental animal procedures using mice were approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska-Lincoln.

### Mouse Immunizations

For all immunizations, mice were under anesthesia using an isoflurane vaporizer. Mock immunized animals received 30 µl of PBS intranasally. For some groups, IAV was heat inactivated at 70°C for 1 h. One cohort of mice received 10 µl of inactivated virus containing $10^7$ $EID_{50}$ A/HKx31-OvaII (x31/Ova), diluted in PBS for a total volume of 30 µl. A separate cohort of mice received 50 µg of CpG (ODN1826; Invivogen San Diego, CA, USA) combined with $10^7$ $EID_{50}$ inactivated x31/Ova diluted in PBS. For x31/Ova priming, mice were anesthetized with isoflurane

and infected with 950 $EID_{50}$ x31/Ova virus also administered intranasally.

## Influenza Virus Challenge

For challenge experiments, mice were anesthetized with isoflurane, and A/Puerto Rico/8/1934 (PR8) was diluted in PBS and administered intranasally in a total volume of 30 µl. PR8 viruses were used at a sublethal dose of 0.1 $LD_{50}$ or at a challenge dose of either 1 $LD_{50}$ or 10 $LD_{50}$. For challenge experiments, mice were infected with a lethal dose of PR8 4–6 weeks post immunization. Influenza viruses were generously provided by Dr. Paul Thomas, St. Jude Children's Research Hospital (x31/Ova) or the Trudeau Institute (PR8).

## Extraction of RNA and Real-Time qRT-PCR

Mice were euthanized at various times following intranasal inoculation, lungs placed immediately in RNAlater (Ambion, Austin, TX, USA) and frozen at −20°C. The samples were weighed and homogenized in TRIzol (Ambion) at 1 ml/100 mg of lung tissue using a Tissue Tearor homogenizer (Biospec Products Inc., Bartlesville, OK, USA). RNA was isolated from lung homogenates, reverse-transcribed into cDNA, and amplified by quantitative real-time PCR (Step One Plus, Applied Biosystems) as previously described (26). Specific primers for murine TLR9 (Mm00446193_m1), IL-6 (Mm0044619_m1), TNF-α (Mm00443258_m1), MIP-1β (Mm00443111_m1) were purchased from Applied Biosystems. The following murine primer/probe sets were purchased from Integrated DNA Technologies (Coralville, IA, USA):

IFN-$\alpha_4$
5′-/56-FAM/TTTGGATTC/ZEN/CCCTTGGAGAAGGTGG/3IABKFQ/-3′ (probe),
5′-GCCTTCTGGATCTGTTGGTTA-3′ (forward)
5′-GCCTCACACTTATAACCTCGG-3′ (reverse)

CXCL10
5′-/56-FAM/ATCCCTCTC/ZEN/GCAAGGACGGTC/3IABKFQ/-3′ (probe),
5′-TGATTTCAAGCTTCCCTATGGC-3′ (forward),
5′-ATTTTCTGCCTCATCCTGCT-3′ (reverse)

To determine the viral titer, the following acid polymerase (PA) probe and primers were used:

5′-/56-FAM/CCAAGTCAT/ZEN/GAAGGAGAGGGAATACCGCT/3IABkFQ/-3′ (probe)
5′-CGGTCCAAATTCCTGCTGAT-3′ (forward),
5′-CATTGGGTTCCTTCCATCCA-3′ (reverse)

A known concentration of PA-containing plasmid was used to generate a standard curve in all reactions. PA copies per lung were then calculated based an initial concentration of 100 ng of cDNA as described (12, 26).

## Isolation of Lung and Lymph Node Cells for Flow Cytometry

Mice were euthanized at various times post infection, and lungs, draining lymph node (DLN) cells (a pool of mediastinal and cervical lymph nodes), or spleens were processed as described for flow cytometry analysis (26). Briefly, lungs were perfused with PBS, treated with collagenase D, and filtered through a 70-µm filter. DLN and spleens were dissociated into single cell suspensions and stained with fluorochrome-conjugated antibodies to CD4 (eBioscience Cat# 45-0042-80, RRID:AB_906231), CD8 (eBioscience Cat# 11-0081-82 RRID:AB_464915), CD49b (eBioscience Cat# 14-5971-85, RRID:AB_467767), TLR9 (eBioscience Cat# 11-9093-80 RRID:AB_465443), F4/80 (eBioscience Cat# 45-4801-80, RRID:AB_914344), CD103 (BioLegend Cat# 121405 RRID:AB_535948), CD69 (eBioscience Cat# 12-0691-82 RRID:AB_465732), CD11c (eBioscience Cat# 45-0114-82 RRID:AB_925727), CD11b (eBioscience Cat# 17-0112-81 RRID:AB_469342), I-$A^d$ (BD Biosciences Cat# 553548 RRID:AB_394915), and CD86 (BD Biosciences Cat# 553692 RRID:AB_394994) for 30 min at 4°C. In some experiments, isolated lymphocytes were surface stained, fixed in 4% paraformaldehyde, and stained with anti-human GrB (Invitrogen Cat# MHGB05 RRID:AB_1500190) antibody to measure intracellular levels of GrB protein in effector T cells. Cells were acquired using a FACS Calibur (BD Biosciences) or Cytek DxP10 (Cytek Development, Fremont, CA, USA) flow cytometer and analyzed using FlowJo software (FlowJo, RRID:nif-0000-30575).

## Restimulation with Peptides for Cytokine Analysis

For intracellular cytokine assays, cells were isolated from the lungs as described above and restimulated with IAV peptide pulsed A20s (ATCC, Manassas, VA, USA) as antigen-presenting cells (APCs) in RPMI 1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Cellgro, Manassas, VA, USA), 7% FBS (Phenix Research Products, Candler, NC, USA), 10 mM HEPES (Fisher Scientific, Fair Lawn, NJ, USA), and 50 µM 2-ME (Sigma-Aldrich, St. Louis, MO, USA). Peptides used for *ex vivo* restimulation NP peptide 216–230 (RIAYERMCNILKGKF), NP peptide 146–159 (ATYQRTRALVRTGM), matrix peptides 164–179 (SHRQMVTTTNPLIRH), and matrix (M) peptide 211–226 (QARQMVQAMRTIGTH) were synthesized by New England Peptide (New England Peptide Inc., Gardner, MA, USA). Following restimulation for 2 h, Brefeldin A (Sigma-Aldrich) was added to T cell cultures at 10 µg/ml and maintained throughout the final 2–4 h of incubation. In some experiments, T cells were restimulated for 4–6 h, and Brefeldin A was added over night. After a total of 4–18 h in culture, T cells were surface stained with anti-CD4 and anti-CD8 antibodies as described above and fixed in 4% paraformaldehyde. Cells were then stained in saponin buffer (PBS containing 1% BSA, 0.1% $NaN_3$, and 0.25% saponin) containing antibodies to IFN-γ (eBioscience Cat# 17-7311-82 RRID:AB_469504) and TNF-α (eBioscience Cat# 12-7321-81 RRID:AB_466198) for 40 min at room temperature in the dark. Cells were then washed and resuspended for FACS analysis. Cells were analyzed as described above.

## ELISA for Detection of Anti-Influenza IgG2a Antibodies

Ninety-six well plates were coated with x31/Ova virus ($5 \times 10^6$ $EID_{50}$/ml) diluted in PBS overnight. Plates were washed with PBS

and blocked for 1 h with PBS containing 2% FBS and 10 mM HEPES. Serum was added to the plates in blocking buffer and serially diluted twofold. After 2–3 h incubation at room temperature, alkaline phosphatase-conjugated goat anti-mouse IgG2a was added (Southern Biotech Associates, Birmingham, AL, USA) for 1 h at room temperature. Plates were developed using *p*-nitrophenyl phosphate (*p*-NPP) after a 15-min incubation in the dark. Absorbance was read at 405 nm and end point titers were calculated based on the dilution that gave two times the background optical density using serum from a naïve mouse as described (12).

### Statistics

Statistical significance between experimental groups was determined by one-way ANOVA followed by Tukey's test using Prism 6.0 (Graph Pad Software).

## Results

To demonstrate the *in vivo* protective efficacy of a single-dose intranasal vaccine using CpG and inactivated x31/Ova (CpG + Inact) mice were immunized and then challenged with a heterologous virus 4 weeks later. Subsequently, viral titers were measured 7 days post challenge. Viral burden in unimmunized groups were significantly higher than in CpG + Inact immunized mice (**Figure 1**). As expected, mice immunized with live x31/Ova had no appreciable levels of virus by day 7 (27). Sterilizing immunity was not observed with either immunization, as priming with H3N2 virus and challenging with H1N1 avoid contribution of antibody-mediated protection to H and N. Thus, the results suggest that a single immunization with CpG + Inact provides partial protection and may induce memory T cell responses capable of reducing viral titers and morbidity associated with a lethal heterosubtypic challenge.

To investigate the immunostimulatory capacity of this mucosally administered CpG-based vaccine, inflammatory cytokines, and chemokines were analyzed in the lung 1 day after intranasal administration. For inactivated influenza and x31/Ova cohorts, modest cytokine induction was observed characterized by IL-6 and tumor necrosis factor alpha (TNF-α) transcript upregulation. In contrast, immunization with CpG + Inact induced high levels of nearly all inflammatory and chemotactic transcripts tested (**Figure 2A**). TLR9 transcripts were also upregulated in the lung indicating a positive-feedback loop of cells capable of inducing inflammation. Next, the amount of viral PA copies present in the lung was determined 1 day after immunization. As shown in **Figure 2B**, only administration of live virus generated appreciable levels of the PA gene, confirming the attenuation of the vaccination strain.

Innate immune cells, including APC, play important roles in initiation and maintenance of appropriate immune responses to influenza infection (28–31). To determine whether innate immunity was modulated after immunization, a number of APC populations were examined in lymphoid tissues and the lung.

In contrast to x31/Ova infection, CpG + Inact immunization resulted in increased frequencies of innate immune cells in the DLN 1 day after immunization (**Figure 3A**). Rapid migration of $CD11c^+/MHCII^+$ cells as well as $CD11b^+$ macrophages was observed in the DLNs (**Figure 3B**). Immunization also induced

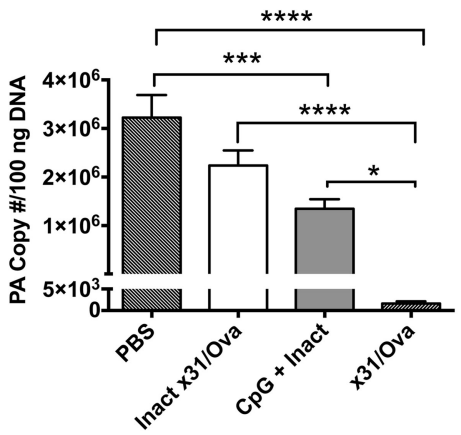


**FIGURE 1 | CpG immunization lowers viral titers after a heterosubtypic influenza challenge**. BALB/cByJ mice were immunized intranasally and 4 weeks later challenged with $10LD_{50}$ of PR8. Seven days post challenge mice were euthanized and viral titers were determined via qRT-PCR. The amount of influenza RNA is shown as the PA copy number/100 ng of cDNA and is relative to a standard curve generated using known amounts of the IAV PA gene as described in the section "Materials and Methods." Results are representative of two independent experiments ($n = 4$–5 mice per group), mean PA copy number + SEM. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

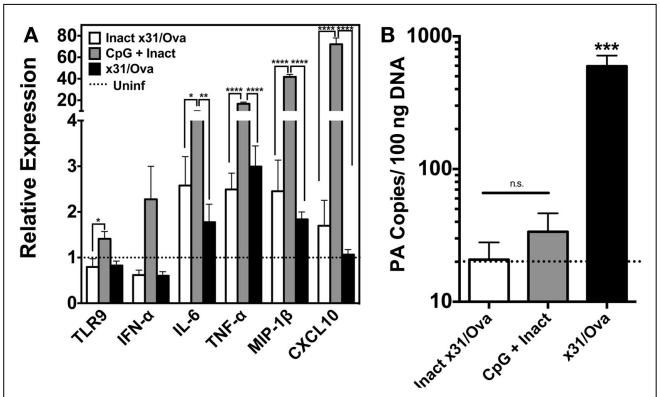


**FIGURE 2 | CpG immunization induces the transcription of cytokine and inflammatory genes in the lung**. **(A)** BALB/cByJ mice immunized with the indicated regimens were euthanized 1 day after intranasal administration. RNA was extracted from whole lungs and qRT-PCR performed. Amount of mRNA transcripts for TLR9 and cytokines/chemokines is shown as arbitrary units relative to the amount of GAPDH mRNA present in each sample. Dashed line represents uninfected controls. **(B)** Influenza PA gene copy number was assessed in whole lungs 1 day after immunization as in **Figure 1**. Dashed line represents the limit of detection for the assay. Results represent the mean + SD of three to four mice per group $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

significant increases in the lung-resident $CD103^+$ migratory DCs to the DLN; however, there were no detectable changes in the frequency of $CD8^+$ DCs after any treatment (**Figure 3C**). To determine if vaccination induced APCs had the capacity to prime adaptive immune responses, expression of the costimulatory molecule CD86 was measured in the DLN populations. Upregulation of CD86 in $CD11c^+/MHCII^+$ and $CD8^+$ DCs was observed, indicating that these cells may initiate naïve T cell responses (**Figure 3D**).

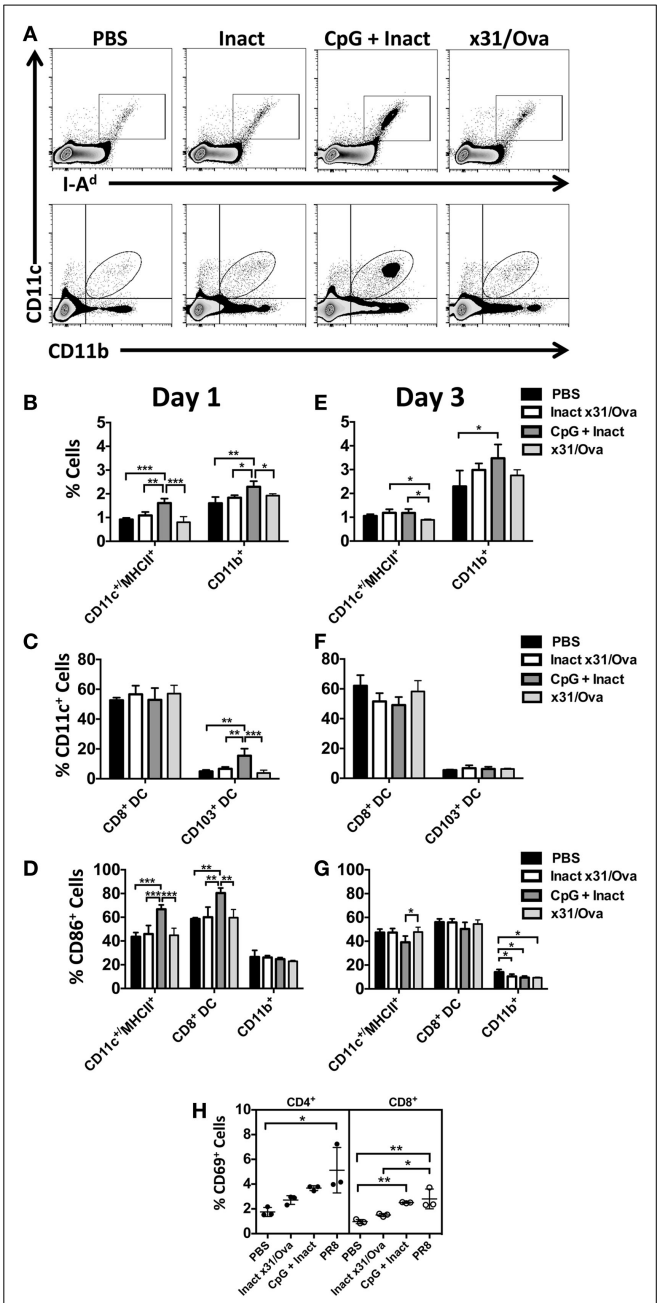


**FIGURE 3 | CpG immunization induces APC maturation in the DLN**. BALB/cByJ mice were immunized intranasally. **(A)** Representative FACS plots from the DLN at day 1 post immunization. Innate immune populations were enumerated at day 1 **(B)** and day 3 **(E)** by flow cytometry. $CD8^+$ DCs and $CD103^+$ DCs were enumerated at day 1 **(C)** and day 3 **(F)**. Gated innate immune populations were assessed for their surface expression of the costimulatory molecule CD86 **(D,G)**. CD69 upregulation on the surface of CD4 and CD8 T cells in the draining lymph nodes 3 days after immunization **(H)**. Graphs represent two separate experiments ($n = 3$–5 mice per group) with data given in mean percentage ± SD. $^*p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$.

The results here are consistent with other published studies suggesting that the intranasal administration of CpG induces APC maturation in the DLNs (32). In contrast to the diverse influx of cells seen in the DLN at day 1, only the frequency of $CD11b^+$ cells was increased after CpG immunization (**Figures 3E,F**). By day 3, minimal changes were observed in CD86 expression (**Figure 3G**). To determine the functional consequences of APC maturation, CD69 (a surface marker typically upregulated after T cell activation) expression on T cells was measured at day 3 in the lymph node (33, 34). Here, PR8 infection was used as a positive control. In the DLNs, CD69 expression was differentially upregulated in CD8 cells after CpG + Inact or influenza immunization while only PR8 infection resulted in increases in CD4 cells (**Figure 3H**). Interestingly, the mature APC phenotype observed in the $CD8^+$ DC subset did correspond with the activation status of the CD8 cells (**Figure 3H**). These results suggest that CpG + Inact immunization induces a population of activated cells derived from naïve precursor cells in the DLN.

In contrast to the DLN, APC influx and activation were sustained in the lung until at least day 3. In the lung, we have identified three populations of cells: (i) interstitial macrophages (ii) alveolar macrophages, and (iii) conventional $CD11c^+$ cells each with unique responses to CpG immunization (**Figure 4A**). While increases in the frequency of $CD11c^+$ cells were not observed until day 3, interstitial macrophage influx was significantly higher at both time points in CpG + Inact immunized groups (**Figures 4B,F**). CpG + Inact treatment reduced the percentage of alveolar macrophages ($F4/80^+/CD11c^+$) in the lung similar to what has been reported for PR8 infection (35). Furthermore, we observed clear increases in the percentage of $CD86^+$ cells (data not shown) as well as CD86 median fluorescence intensity (MFI) in $CD11c^+$ and interstitial macrophages at day 1 and day 3 (**Figures 4C,G**). Interestingly, infection with x31/Ova demonstrated relatively low CD86 MFI levels, a similar profile to that of inactivated virus. $CD103^+$ migratory dendritic cell populations were also examined in the lung. At day 1, increases in $CD103^+$ DCs were observed (**Figure 4D**); however, these cells were decreased in frequency by day 3 (**Figure 4H**). Next, we wanted to determine the frequency of cells expressing the receptor for CpG (TLR9) in the lung. Significant increases in TLR9-expressing $CD11c^+$ dendritic cells were observed over the time course compared to both inactivated and live virus immunizations (**Figures 4E,I**). Lastly, CD69 expression on T cells was examined in the lung 3 days after immunization. Compared with PBS and inactivated virus, both CpG + Inact and PR8 immunization induced significant increases in $CD69^+$ T cells. We conclude that immunization with CpG and inactivated virus leads to a phenotypic maturation of APCs where favorable conditions exist for and early T cell activation in the DLN (**Figure 3**) and lung (**Figure 4**).

Cytotoxic T cells have been shown to be important effectors in IAV infections in heterosubtypic protection in both humans (19) and mice (16). Thus, we sought to determine if T cells with cytolytic potential could be generated after vaccination. Six days after immunization, mice were euthanized and Granzyme B (GrB) expression in T cells was measured. As a positive control for GrB expression, infection with a sublethal dose of PR8 was used and induced >97% $GrB^+$ CD4 and CD8 cells as expected (26, 36). Interestingly, compared to the x31/Ova infection, CpG + Inact immunized mice had a much higher percentage of GrB positive cells (**Figure 5A**). While the percentage of CD4 and

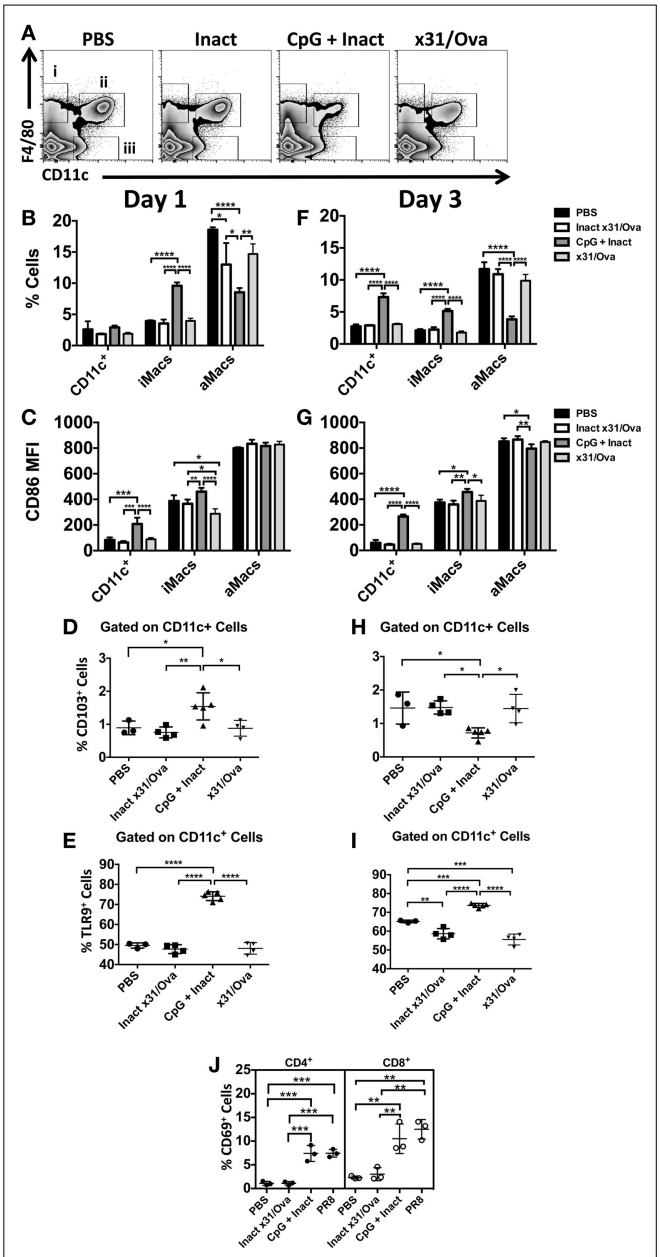


**FIGURE 4 | CpG immunization induces APC maturation in the lung.** BALB/cByJ mice were immunized intranasally. **(A)** Representative FACS plots from the lung at day 1 show (i) interstitial macrophages (F4/80$^+$), (ii) alveolar macrophages (F4/80$^+$ CD11c$^+$), and (iii) conventional DCs (CD11c$^+$). DCs, interstitial macrophages, and alveolar macrophage populations were enumerated at day 1 **(B)** and day 3 **(F)** in the lungs. Innate immune cell populations were assessed for their surface expression of the costimulatory molecule CD86 by measuring the median fluorescence intensity (MFI) **(C,G)**. The influx of CD103$^+$ dendritic cells **(D,H)** and TLR9$^+$ **(E,I)** cells was also enumerated after immunization. CD69 upregulation on CD4 and CD8 T cells in the lungs 3 days after immunization **(J)**. Graphs represent two separate experiments ($n = 3$–5 mice per group) with data given in mean percentage ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

CD8 T cells expressing GrB in inactivated or x31/Ova cohorts was <5%, immunization with CpG + Inact resulted in 40% GrB$^+$ CD4 cells and 20% GrB$^+$ CD8 cells (**Figure 5B**). Similarly, the GrB MFI was highest in CpG- and PR8-immunized mice. As expected, the CD8 MFI was universally higher than the CD4 GrB MFI. These results suggest that while inactivated influenza weakly induces T cell responses in the lung, addition of CpG enhances the effector profile and induces T cells with cytotoxic capabilities.

To further characterize the effector T cell response after immunization, cytokine production in the lung was assessed by intracellular staining. Previously, we have shown that restimulation with a peptide cocktail consisting of NP epitopes induces strong T cell cytokine production during the influenza response (26) and allows us to assess cross-reactive T cells after vaccination. At day 6, most cytokine-producing T cells were TNF-$\alpha^+$ while low frequencies of IFN-$\gamma^+$ cells were observed after peptide restimulation (**Figure 5C**). A small cohort of these cells was dual IFN-$\gamma$ and TNF-$\alpha$ producing cells in CpG + Inact and x31/Ova-primed groups (**Figure 5D**). Collectively, these results demonstrate that effector CD4 and CD8 cells were recruited to the lung during immunization and acute infection, and that antigen-specific responses were generated by day 6. Furthermore, these data suggest that different priming events occur between a natural infection and CpG + Inact immunization, which lead to differences in quality and quantity of effector T cells.

Next, we sought to determine if the effector cells identified in the lung persisted into memory. Expression of CD44 has been shown as a memory T cell marker and is present on the surface of resting memory cells (37). Thus, 28 days after immunization, CD44$^+$ cells were enumerated in the lungs and spleens. Although no differences in CD44 expression were observed in splenocytes (data not shown), CpG + Inact induced significant increases in the number of CD8$^+$/CD44$^+$ T cells in the lungs (**Figure 6A**). Next, *ex vivo* cytokine analysis was performed on cells isolated from the lungs and spleens. Unexpectedly, only increases in antigen-specific responses were detected in x31/Ova-immunized groups (**Figures 6B,C**). Therefore, CpG + Inact immunization induced CD44$^+$ cells in the lungs; however, in the absence of a secondary challenge, cytokine responses were only detected in x31/Ova-infected animals. To confirm that the protection generated by our vaccine was in the absence of pre-existing antibodies, serum was collected 5 weeks after immunization and anti-x31/Ova IgG2a titers were measured. Antibody responses to the virus were only generated by prior infection (**Figure 6D**). Indeed, endpoint titers were very low after CpG + Inact immunization, and only one out of four mice generated any anti-influenza antibody response (**Figure 6E**). Thus, the protection mediated by CpG + Inact immunization does not depend on IgG2a antibodies.

Based on the requirement for secondary effector T cell responses to mediate protection in highly pathogenic infections (38), it was important to determine the frequency of GrB$^+$ and antigen-specific cytokine-producing cells after influenza challenge. Mice were immunized and 4–5 weeks later challenged with a lethal dose of PR8. Five days after challenge, lungs were harvested and cells were analyzed for GrB expression. In contrast to mock or inactivated immunizations, administration of CpG + Inact or x31/Ova significantly enhanced the number of GrB$^+$ T cells within the lung (**Figure 7A**). To

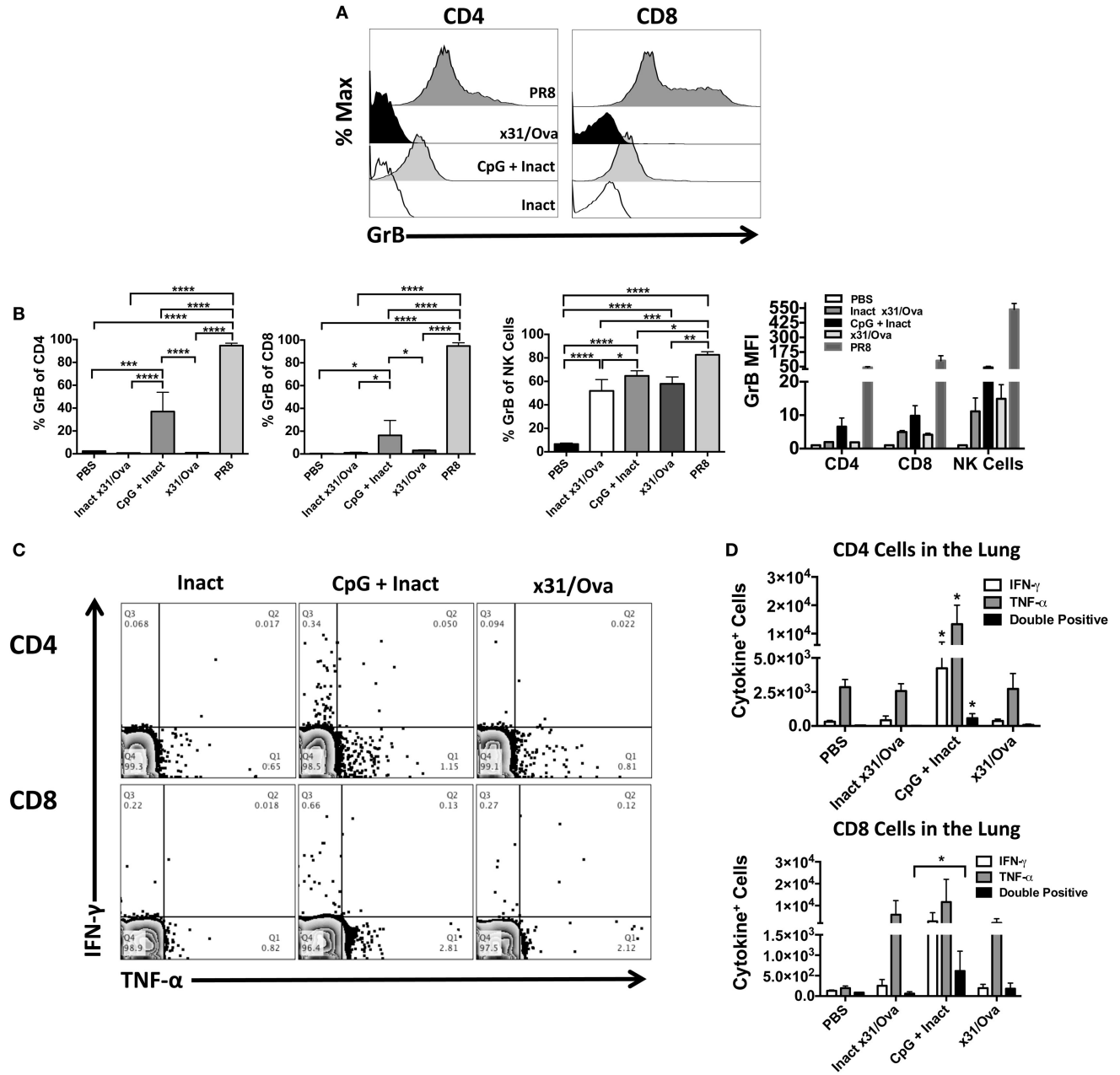


**FIGURE 5 | CpG immunization and IAV infection induce effector T cell responses in the lung**. BALB/cByJ mice were immunized intranasally, mice were euthanized, and lungs harvested at day 6. **(A)** Representative overlay histograms show GrB expression after gating on $CD4^+$, $CD8^+$ T cells, or NK cells in the lung. **(B)** Shown are the percentages of CD4, CD8, or NK cells expressing GrB as well as the GrB MFI of each population. **(C)** Representative FACS plots in the lung after *ex vivo* restimulation for 4–6 h with NP and M peptides. **(D)** Absolute number of cytokine-producing CD4 and CD8 cells were enumerated after restimulation. Single * in the CD4 quantification denotes significance over all other groups as analyzed by ANOVA. Data represent two independent experiments ($n = 4$–5 mice per group) given in mean percentage of cytokine positive cells + SD $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

further quantify memory T cell responses after IAV challenge, we sought to determine the number of cytokine expressing cells at the site of infection. In mock- and inactivated-immunized mice, the frequency of cytokine positive cells was low in both CD4 and CD8 cells at day 5 post challenge indicative of the lack of a primed T cell response (**Figure 7B**). In CpG + Inact immunized mice, T cell responses were apparent, characterized by IFN-γ and TNF-α-positive cells (**Figure 7B**). Furthermore, a robust memory response was initiated with high frequencies of dual cytokine positive T cells after x31/Ova immunization and PR8 challenge (**Figure 7B**). CpG + Inact immunization produced increased trends of cytokine responses compared to PBS groups; however, significance was not achieved (**Figure 7C**). These results suggest the presence of a naïve T cell response

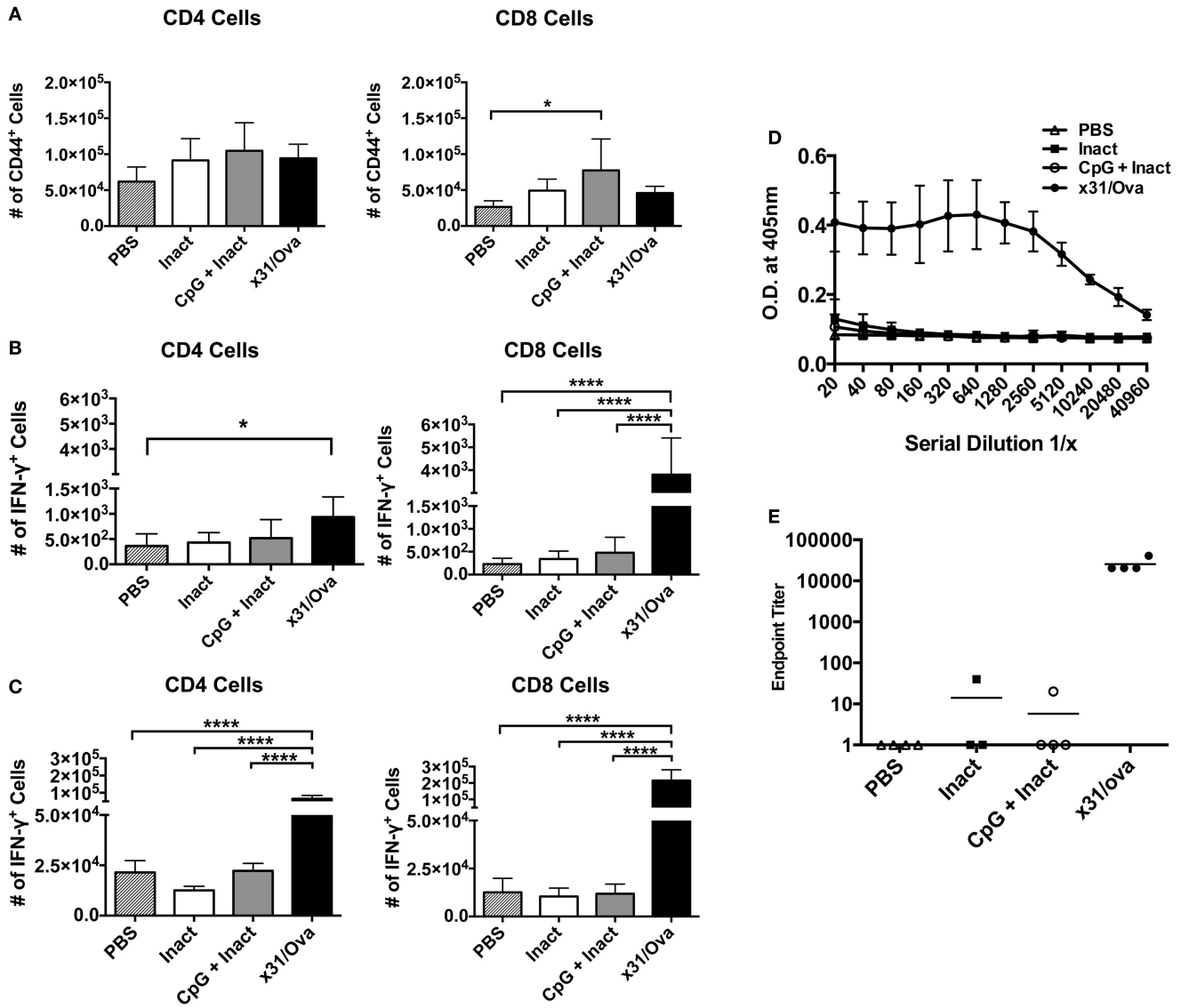


**FIGURE 6 | Low-dose influenza infection generates antigen-specific memory cells in the spleen and lung**. BALB/cByJ mice were vaccinated with the indicated regiments and euthanized 4 weeks later. **(A)** Absolute numbers of $CD44^+$ T cells were quantified in the lungs. Intracellular cytokine staining on cells isolated from the **(B)** lung or **(C)** spleen was performed after an 18-h incubation with NP and M peptides. Shown is the number of IFN-γ positive cells. Data represent two independent experiments ($n = 4$–5 mice per group) with data given in mean percentage of $CD44^+$ or cytokine positive cells + SD. $^*p < 0.05$, $^{****}p < 0.0001$. **(D)** Five weeks post immunization, serum was collected and serially diluted and the optical density at 405 nm was determined. **(E)** Endpoint titers for the anti-x31/ova IgG2a from samples in **(D)**. Data are given in mean ± SD. $^*p < 0.05$, $^{****}p < 0.0001$.

after challenge in mock or inactivated groups, highlighted by the lack of robust T cell responses at day 5. In contrast, the recall of memory T cells in CpG + Inact immunized or x31/Ova-infected animals was seen by the increased frequencies of GrB and cytokine positive T cells early after influenza challenge.

## Discussion

The use of pattern recognition receptor (PRR) agonists is becoming increasingly widespread as potential vaccine adjuvants for a number of diseases including IAV. Here, we describe a novel influenza vaccine platform based on the generation of antigen-specific CD4 and CD8 T cells capable of reducing viral titers after lethal IAV challenge. Single dose, intranasal administration of CpG + Inact resulted in the production of proinflammatory cytokines, mobilization of APC populations, and the establishment of effector T cell responses by day 6 post administration. Our results suggest that effector T cells transitioned into memory cells capable of rapidly responding in the lung upon reinfection to reduce viral replication in an antigen-specific manner.

The vaccine platform described here has numerous advantages compared to other IAV vaccines. First, the use of the

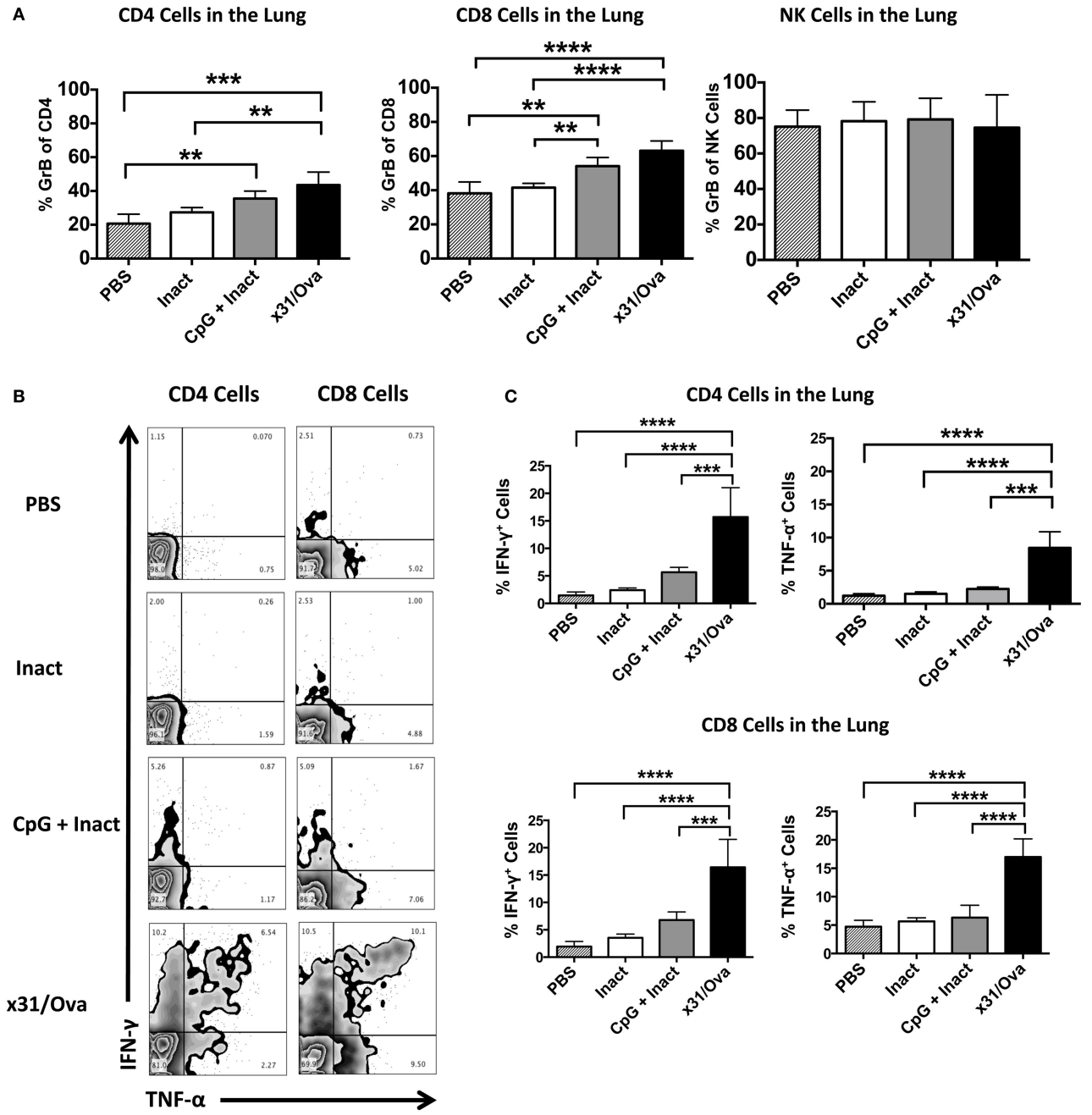


**FIGURE 7 | CpG immunization induces functional memory responses at day 5 post challenge with a heterosubtypic IAV infection**. BALB/cByJ mice were vaccinated as indicated. Four to five weeks post immunization, mice were challenged with a 10 $LD_{50}$ of PR8. **(A)** Lungs were harvested 5 days after infection, and the absolute number of $GrB^+$ T and NK cells were enumerated via flow cytometry. **(B)** Representative FACS plots in the lung after *ex vivo* restimulation for 4–6 h with NP and M peptides. **(C)** Percentage of cytokine-producing CD4 and CD8 cells were enumerated after restimulation. Data represent two independent experiments ($n = 4$–5 mice per group) given in mean percentage of cytokine positive cells or absolute number of $GrB^+$ cells + SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

PRR agonist, CpG, as an innate immune modulator has been approved for use in humans, but is not yet incorporated into current IAV vaccine preparations. In contrast to vaccines administered via the intradermal, subcutaneous, or intramuscular route, intranasal immunization induces immunity at mucosal surfaces where an effective local immune response is required following IAV infection. Additionally, this vaccine induces memory T cell responses in the lung capable of responding to internal conserved viral epitopes, which are thought to greatly aid in protection against heterosubtypic IAV challenges. Lastly, a single dose administered without boosting is a clear advantage for vaccine compliance in human populations.

Pattern recognition receptor agonists, especially CpG, can protect against infectious agents in two ways. One is non-specific immune activation involving the modulation of macrophages, NK cells, inflammatory cytokines, and polyreactive IgM molecules. Numerous studies have demonstrated the protective activation of innate immunity by exposing mice to a single dose of CpG against a number of pathogens including Ebola, anthrax, and malaria (39), which can induce non-specific protection lasting between 1 and 3 weeks. Additionally, we have observed protection against weight loss after IAV infection in an antigen-independent manner using CpG (Brown and Swain, unpublished). Many studies that examine the efficacy of CpG-based vaccines report survival, weight loss, and viral replication of IAV 1–3 weeks after the final boost or immunization (40–44). Even though in the majority of these studies antigen-specific T cell responses are quantified, the experiments performed under these conditions do not allow sufficient time after immunization for the non-specific effects of CpG immunization to wane. In contrast, our study allows the development of T cell memory responses (**Figures 5** and **6**) that participate in partial inhibition of viral replication (**Figure 1**).

Following an infection with a homotypic virus, strain-specific humoral immunity is induced and prevents reinfection upon subsequent challenges. To date, IAV vaccines use this approach to generate strain-specific antibodies against outer coat proteins in the common seasonal influenza strains. In a heterotypic influenza challenge model, T cells reactive to conserved viral proteins, such as NP and M, are capable of providing protection and persist in the tissues and secondary lymphoid organs as a source of memory cells to respond to serologically distinct IAV infections (19). However, it is unlikely that cross-reactive CTLs provide sterilizing immunity, as some degree of antigen processing and presentation must occur to initiate a memory response (45). Here, effector functions provided by CTLs (**Figure 7**) may have substantial impacts on viral replication (**Figure 1**) and therefore morbidity and mortality. Similar to infection with x31/Ova, vaccination with CpG + Inact does not provide sterilizing immunity after heterosubtypic challenge but reduces viral burden. Previous work from our lab suggests that reducing viral replication is one of the determinants for a positive survival outcome (26). Therefore, the protection provided by CD4 and CD8 T cells generated by vaccination is significant to the outcome of a heterotypic challenge.

Other factors likely contributed to the lack of total viral clearance by day 7. Upon challenge, a considerable population of dual cytokine-positive T cells was present in x31/Ova-immunized mice but not CpG + Inact-immunized mice. This suggests differences in priming between the two cohorts results in differences in quality of protection. Surprisingly, little innate immune modulation was observed in x31/Ova mice compared to CpG + Inact mice (**Figures 2–4**), suggesting that while inducing inflammation and APC modulation is important in a vaccine setting, low levels of viral replication may be the best strategy to induce robust T cell memory responses (46). Alternatively, T cell responses generated by CpG vaccination could be below a certain threshold, and thus vaccination would fail to fully protect from infection (47). We have attempted to enumerate the resting population of memory T cells in CpG + Inact-immunized mice 28 days after infection. While differences in CD44 expression were observed CD8 cells in the lung (**Figure 6A**), *ex vivo* restimulation and intracellular cytokine analysis revealed little difference between mock and CpG + Inact immunized groups. However, a trend of increased cytokine-positive cells combined with increases in CD44 expression in the lung suggests that CpG + Inact vaccination could be inducing resident memory T cells poised for effector function (48).

Conversely, the inability to detect the significant levels of cytokine-producing cells by peptide restimulation in CpG + Inact-immunized mice could be due to the low frequency of memory cells. The experiments here specifically look at responses to two peptides derived from M and NP each. Thus, the peptide restimulation might not be activating the full memory T cell repertoire, as memory responses could be generated to multiple conserved T cell epitopes (M, NP, PB1, PA) as well as non-conserved epitopes (H and N) present in the inactivated whole virus preparations. Furthermore, as the memory T cell population induced by vaccination may make up <0.5–2% of the total cells in the lung, isolating and staining these cells may be inefficient and not reflective of the true population. Nonetheless, the data suggest that memory responses are generated in CpG + Inact vaccinated mice as antigen-specific T cells are detected in the lungs 5 days post challenge (**Figure 7**).

Unexpectedly, the preparation of antigen played an important role in assessing the contribution of memory T cells to heterosubtypic protection. IAV can be inactivated in many different ways, each inducing unique immune responses. While heat-inactivated virus is less antigenic than other methods of viral inactivation (49), effector and memory T cell responses can clearly be generated when it is combined with CpG (**Figures 5** and **7**). Interestingly, very little influenza-specific class-switched IgG2a antibody was detected in the serum of immunized mice 4 weeks after immunization (**Figures 6D,E**), further supporting the pronounced role of memory T cells in heterosubtypic protection. One study found immunizing with a single dose of CpG and formalin-inactivated IAV increased anti-IAV antibodies 4 weeks post immunization in the serum and saliva (50). However, the modulation of the cellular immune response, measured by proliferation assays and CTL responses, was not observed. The difference between our study and the Moldoveanu report are likely due to antigen processing and presentation of the viral peptides.

Nonetheless, partial immune protection can serve as a framework to enhance and modify the existing platform to generate a vaccine with satisfactory efficacy and safety. For example, nanoparticle conjugation could be used for a dose sparing effect and enhanced protection. Other ways of modulating immunity could be through using multiple PRR ligands to generate a synergistic T cell response when combined with CpG. Our findings demonstrate a single dose of CpG, administered intranasally, can control viral replication and represents a possible strategy for developing vaccines against heterosubtypic infections in the future.

## Author Contributions

Conceived and designed the experiments: AV, DB. Performed the experiments: AV, DB. Data analysis and acquisition: AV. Drafted

the work: AV. Critical revisions and final approval of the version to be published: DB.

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# AS03-adjuvanted, very-low-dose influenza vaccines induce distinctive immune responses compared to unadjuvanted high-dose vaccines in BALB/c mice

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During the 2009–2010 influenza pandemic, an adjuvanted, dose-sparing vaccine was recommended for most Canadians. We hypothesize that differences exist in the responses to AS03-adjuvanted, low antigen (Ag) dose versus unadjuvanted, full-dose vaccines. We investigated the relationship between Ag dose and the oil-in-water emulsion Adjuvant System AS03. BALB/c mice received two IM doses of $AS03_A$ or $AS03_B$ with exaggerated dilutions of A/Uruguay/716/2007 H3N2 split virion vaccine Ag. Immune responses were assessed 3 weeks after the booster. Unadjuvanted "high" (3 μg) and low-dose (0.03–0.003 μg) vaccines generated similar serum antibody titers and cytokine secretion patterns in restimulated splenocytes. Compared to unadjuvanted "high-dose" vaccination, both $AS03_A$ and $AS03_B$-adjuvanted low-dose vaccines tended to elicit higher serum antibody titers, broader induction of cytokine secretion and generated more influenza-specific antibody secreting cells and cytokine-secreting CD4 and CD8 T cells in splenocytes. We show that varying Ag and/or AS03 dose in this influenza vaccination mouse model can strongly influence both the magnitude and pattern of the immune response elicited. These findings are highly relevant given the likelihood of expanded use of adjuvanted, dose-sparing vaccines and raise questions about the use of "standard" doses of vaccines in pre-clinical vaccine studies.

**Keywords: influenza, vaccine, AS03, adjuvant, dose-sparing**

## Introduction

Vaccines are the most cost-effective method to prevent influenza virus-associated morbidity and mortality (1); however, they are also least effective in high-risk populations (1). To improve vaccine efficacy, manufacturers are increasingly turning to adjuvants. During the 2009–2010 A/California pandemic H1N1 influenza outbreak, the World Health Organization recommended the use of antigen (Ag)-sparing vaccines (2). In Canada, an Ag-sparing vaccine formulated with AS03 and 25% of the adult hemagglutinin (HA) dose in unadjuvanted seasonal influenza vaccines was selected for administration to the majority of Canadians (*Arepanrix*™; GSK Vaccines, Mississauga, ON, Canada) (3). A similar vaccine was used in Europe (*Pandemrix*™, GSK Vaccines, Rixensart, Belgium).

AS03 is an oil-in-water adjuvant consisting of squalene, alpha-tocopherol, and polysorbate-80. The greatest experience with AS03 to date has been with vaccines targeting influenza. This work demonstrated that an AS03-adjuvanted vaccine formulated with inactivated monovalent influenza A/Vietnam/1194/2004 H5N1 at low Ag dose (3.8 μg) was able to meet CHMP/FDA licensure criteria (4). There are two formulations of this adjuvant: $AS03_A$ containing 11.86 mg alpha-tocopherol, 10.69 mg squalene, and 4.86 mg polysorbate-80 per 0.5 ml dose; and $AS03_B$, containing 50% of each of the $AS03_A$ components. Human studies have shown that vaccine formulations with $AS03_A$ and $AS03_B$ generate comparable short-term antibody responses (5), and that low Ag doses (3.75 or 1.9 μg) adjuvanted with $AS03_A$ or $AS03_B$ are non-inferior in terms of antibody production to unadjuvanted vaccines at higher Ag doses (6).

In vaccine formulations, AS03 functions to enhance the generation of Ag-specific memory B cells and polyfunctional CD4+ T cells (7, 8), induce cross-reactive antibody responses (9). In mouse studies, AS03 also induces transient innate immune responses at the site of injection, which may increase adaptive immune responses (10).

Previously, we observed that some young children who received the *Arepanrix*™ vaccine exhibited unusual avidity profiles after vaccination (11), although in HIV-positive adults, high avidity antibodies were maintained up to 6 months after immunization (12). Given the two major changes in vaccine formulation (addition of AS03 and reduced Ag dose), we wished to determine if there were differences in the immune response after immunization with unadjuvanted full-Ag dose versus AS03-adjuvanted low-dose vaccines.

Typically, dose-sparing strategies use a fixed amount of the adjuvant with varying Ag doses to identify the best formulation. We took the same approach but with exaggerated Ag dilutions with an influenza A/H3N2 split virion model Ag (A/Uruguay/716/2007) to investigate the relationship between Ag dose and AS03. We show that varying one or the other can change the immune outcomes. High- and low-dose unadjuvanted vaccines generated similar immune responses in BALB/c mice. AS03-adjuvanted vaccines were able to generate superior humoral responses and distinct cellular immune responses compared to unadjuvanted vaccine.

## Materials and Methods

### Vaccine, Adjuvant, and Mouse Immunizations

Vaccine doses of 50 μl contained monovalent influenza A/Uruguay/716/2007 H3N2 detergent-split inactivated Ag (30 pg–3 μg HA content; GSK Vaccines, Ste-Foy, QC, Canada) and one of two variants of AS03 (GSK Vaccines, Rixensart, Belgium). AS03 is an Adjuvant System, which contains α-tocopherol and squalene in an oil-in-water emulsion. In human vaccines, $AS03_A$ contains 11.86 mg α-tocopherol and $AS03_B$ contains 5.93 mg α-tocopherol. For this publication, the quantities of the constituents in the murine doses of $AS03_A$ and $AS03_B$ were 10-fold lower than the respective human doses. Eight- to ten-week-old female BALB/c mice (Charles River Laboratories, Montreal, QC, Canada) were immunized by injection into the *gastrocnemius* muscle on days 0 and 21 (0.5 CC syringe with 28G 1/2 needle). Before each immunization, blood was collected from the lateral saphenous vein. At 3 weeks after the booster immunization, mice were sacrificed; serum and splenocytes were collected from each mouse and processed individually as described below. All procedures were carried out in accordance with guidelines of the Canadian Council on Animal Care, as approved by the Animal Care Committee of McGill University.

### Antibody Titer Measurement

Blood was collected in microtainer serum separator tubes (BD Biosciences, Mississauga, ON, Canada). Cleared serum samples were obtained following manufacturer's protocol and stored at −20°C until analysis.

Hemagglutination inhibition (HAI) and microneutralization (MN) titers were measured in serum as previously described (11, 13).

ELISA protocols were optimized (14, 15) to determine HA-specific IgG concentration and avidity. Duplicate U-bottom high-binding 96-well ELISA plates (Greiner Bio-one, Frickenhausen, Germany) were coated with recombinant HA protein from A/Brisbane/10/2007 H3N2 (0.5 μg/ml) (Immune Technology Corp., New York, NY, USA) and a standard curve of mouse IgG antibodies (Sigma, St. Louis, MO, USA) in 100 mM bicarbonate/carbonate buffer at pH 9.5 [50 μl/well, overnight (O/N) at 4°C]. The A/Uruguay/716/2007 H3N2 vaccine strain used in this study is an A/Brisbane/10/2007 H3N2-like strain and shares 100% homology with each other. Before and after each step, wells were washed with PBS. Wells were blocked with 2% bovine serum albumin (BSA; Sigma) in PBS-Tween 20 (0.05%; Fisher Scientific, Ottawa, ON, Canada) (blocking buffer) (150 μl/well, 1 h at 37°C). Serum samples were diluted 1:50 in blocking buffer and added to triplicate wells of duplicate plates (50 μl/well, 1 h at 37°C); blocking buffer was added to standard curves. Next, one plate was incubated with 6M urea in PBS for 15 min at room temperature (RT), while standard curves and the second plate were incubated with blocking buffer. After washing, plates were blocked again (150 μl/well, 1 h at 37°C) and then HRP-conjugated anti-mouse total IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:10,000 in blocking buffer was used (75 μl/well, 1 h at 37°C). Plates were detected with 3,3′,5,5′-tetramethyl benzidine (TMB) substrate (Millipore, Billerica, MA, USA) and stopped after 15 min with 0.5M $H_2SO_4$. Plates were read at 450 nm on an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The concentration of HA-specific IgG antibodies was determined using the standard curve included on each plate. The avidity index is calculated as (IgG concentration remaining after urea incubation)/(total IgG concentration) × 100%.

### Splenocyte Isolation

Spleens were excised, collected in Hank's balanced salt solution without calcium and magnesium (HBSS) (Wisent, St. Bruno, QC, Canada), and processed individually. Homogenous cell suspensions were prepared by passing organs through a 70 μm cell strainer (BD Biosciences, Mississauga, ON, Canada). Cells were treated with ACK buffer (0.15M $NH_4Cl$, 1 mM $KHCO_3$, 0.1 mM

$Na_2EDTA$; pH 7.2), and then washed with HBSS. Splenocytes were resuspended in RPMI supplemented with 10% fetal bovine serum (FBS), 1 mM penicillin/streptomycin (all from Wisent), and 0.5 mM β-mercaptoethanol (Sigma) (complete RPMI, cRPMI).

### Splenocyte Stimulation and Cell Proliferation Assay

Splenocytes were seeded in duplicate in 96-well U-bottom plates (BD Falcon, Mississauga, ON, Canada) at $10^6$ cells in 200 µl with cRPMI alone (unstimulated) or with A/Uruguay/716/2007 H3N2 split vaccine (2.5 µg/ml HA) in cRPMI. After 72 h at 37°C +5% $CO_2$, plates were spun down (300 × *g*, 10 min at RT) and supernatant was collected and stored at −80°C until analysis. Cells were pulsed with 1 µCi/well $H^3$-Thymidine (MP Biomedical, Solon, OH, USA) for an additional 18 h. After one freeze–thaw, cells were harvested on glass–fiber filters with a Tomtec harvester 96 (Tomtec Inc., Hamden, CT, USA) and $H^3$-thymidine incorporation was measured by scintillation counter (Wallac Microbeta Trilux 1450 beta-counter; Wallec, Turku, Finland). Cell proliferation values were expressed as stimulation index (SI); for each mouse SI = (average Ag-stimulated cpm)/(average unstimulated cpm).

### Quantitation of Cytokines in Supernatant

The concentrations of 16 cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-I, IFNγ, TNFα, MIP-1α, GM-CSF, and RANTES) in culture supernatants after 72 h stimulation *in vitro* were determined using Q-Plex Mouse Cytokine – Screen (16-plex) multiplex ELISA following the manufacturer's guidelines (Quansys Biosciences, Logan, UT, USA). Ag-stimulated supernatant samples for each mouse were run as singlets. Unstimulated samples were pooled for each group and run as singlets.

### ELISpot Assays

Influenza HA-specific antibody secreting cells (ASCs) were determined using the ELISpot$^{Plus}$ for Mouse IgG kit (MabTech Inc., Mariemont, OH, USA) following Protocol I using biotinylated Ag. Biotinylated HA was prepared using recombinant HA protein from A/Brisbane/10/2007 H3N2 (Immune Technology Corp.). Biotinylation was performed using the EZ-Link Micro Sulfo-NHS-LC-Biotinylation kit (Thermo Scientific, Lafayette, CO, USA) following the manufacturer's protocol. Wells were coated with mouse anti-IgG capture antibody according to the manufacturer's protocol. Splenocytes ($2.5 \times 10^5$ to $10^6$ cells in 150 µl) were added to duplicate wells and incubated for 16 h at 37°C +5% $CO_2$. Biotinylated HA protein (1 µg/ml), Streptavidin-alkaline phosphatase (ALP), and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT)-plus substrate were used to detect HA-specific IgG ASCs according to the manufacturer's protocol (MabTech Inc.). Plates were read using a CTL series 3B ImmunoSpot analyzer (CTL Analyzers LLC, Cleveland, OH, USA) with ImmunoSpot 4.0.3 software supplied by the manufacturer.

To estimate numbers of memory B cells, splenocytes were stimulated for 5 days *ex vivo* to generate memory B cell-derived plasma cells before plating onto ELISpot plates as described above. The stimulation protocol was modified from (16, 17); $4 \times 10^6$ splenocytes were stimulated with 2 µg/ml CpG Prototype ODN 2006 (Hycult Biotech, Plymouth Meeting, PA, USA) and 50 U/ml hIL-2 (kindly provided by Dr. Ciriaco A. Piccirillo, McGill University) in 2 ml cRPMI for 5 days. Cells were collected, washed, and seeded onto pre-coated ELISpot plates as described above. Splenocytes cultured without stimulation for 5 days were included as negative controls, and were at baseline (data not shown).

### Splenocyte Stimulation, Intracellular Staining, and Flow Cytometry Analysis

Protocols were modified from Moris et al. (7) to analyze influenza-specific T cell responses. Splenocytes from each mouse were seeded in 96-well U-bottom plates (BD Biosciences) ($10^6$ cells in 200 µl/well) and stimulated as singlets with anti-mouse CD28 (37.51) and CD49b (HMa2) antibodies for co-stimulation (both from eBioscience, San Diego, CA, USA) at 2 µg/ml (background) or both antibodies with A/Uruguay/716/2007 H3N2 split vaccine (10 µg HA/ml) (Ag-stimulated) in cRPMI. Following 13 h at 37°C +5% $CO_2$, Brefeldin A (eBioscience) was added according to manufacturer's protocol and incubated for 5 h. As positive controls, pooled splenocytes for each group were co-stimulated with antibodies described above, and with phorbol myristate acetate (PMA) + ionomycin (2.5 and 5 µg/ml, respectively) and Brefeldin A for 5 h. Control samples were stained and analyzed as described below (data not shown). After incubation, cells were transferred to V-bottom plates (BD Biosciences) for FC staining.

Antibodies used, the staining procedure and gating strategy are described in Figure S1 in Supplementary Material. Briefly, cells were stained for CD3, CD4, and CD8 on the surface and intracellularly for IL-2, IL-5, IFNγ, and TNFα. Results are expressed as the percentage of CD4+ and CD8+ T cells producing all combinations of the cytokines tested. Background (cells stimulated with anti-CD28 and anti-CD49d antibodies only) was subtracted from Ag-stimulated values (cells stimulated with antibodies and A/Uruguay H3N2 split vaccine).

### Statistical Analysis

For serum antibody titers and avidity, estimates of the geometric mean ratios between groups and their 95% confidence intervals (CI) were obtained using back-transformation on log10 values. All experiments were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-test to compare all possible pairs of groups. Analyses were performed using GraphPad Prism 5.0 software.

## Results

### Adjuvanted, Low-Dose Vaccines Generate Superior Antibody Responses than Unadjuvated Vaccines

Two formulations of unadjuvanted high-Ag dose vaccines (3 and 6 µg/dose) were selected based on previous studies (18). After one or two immunizations, mice produced comparable HAI titers, which were significantly higher than the control groups (**Figures 1A,B**).

Based on the *Arepanrix*™ vaccine and other mouse studies, we selected 0.75 µg as the starting point for our low Ag formulations (3, 18). After one immunization, mice vaccinated

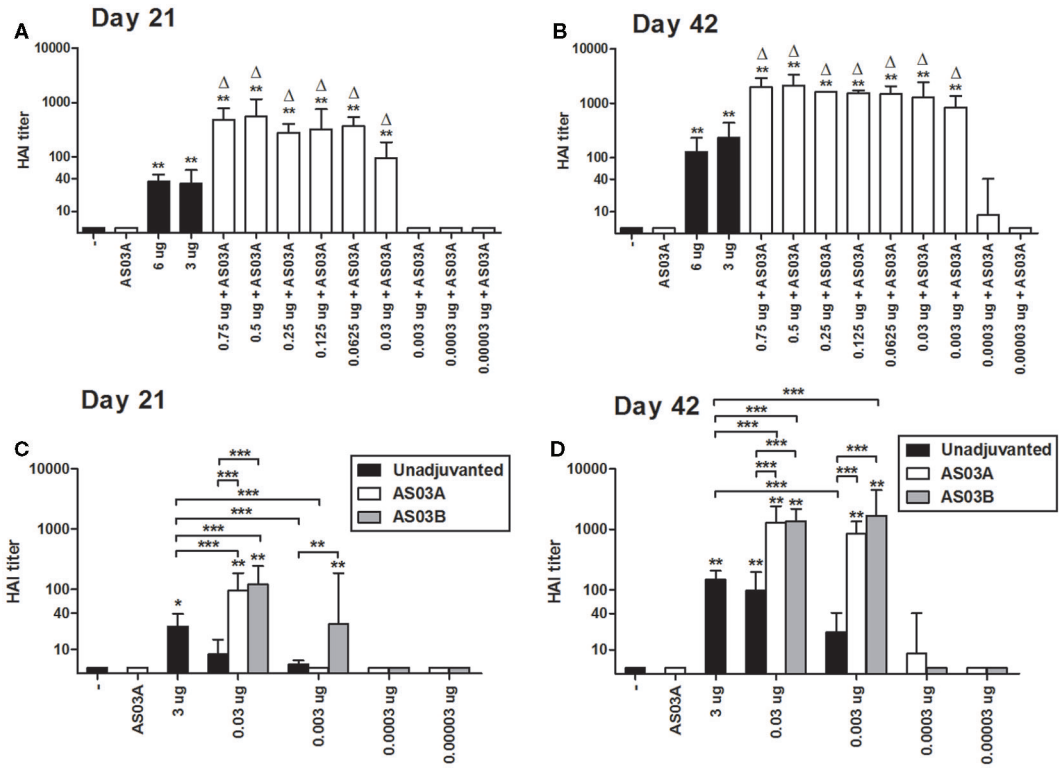


**FIGURE 1 | Hemagglutination inhibition (HAI) titers in BALB/c mice after one or two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**. Mice were immunized intramuscularly on days 0 and 21, and sera from individual mice were analyzed. HAI titers of mice immunized with $AS03_A$-adjuvanted vaccines on days 21 **(A)** and 42 **(B)**. HAI titers of mice immunized with $AS03_A$ or $AS03_B$-adjuvanted vaccines on days 21 **(C)** and 42 **(D)**. On top of bars, * indicates a significant increase ($P < 0.05$) to negative "−" group and ** indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups. For **(A,B)**, delta symbols (Δ) on top of bars indicate a significant increase ($P < 0.05$) compared to mice immunized with 3 μg vaccine only. For **(C,D)** significant differences between groups are denoted by brackets; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. For **(A,B)** data represent 4–12 individual mice per group combined from two independent studies. For **(C,D)**, data represent 4–17 or 4–25 mice per group combined from three or four independent studies, respectively. Geometric means and 95% confidence intervals are shown.

with 0.75 μg + $AS03_A$ produced significantly higher HAI titers than either high-dose unadjuvanted vaccine (3 or 6 μg/dose; **Figure 1A**). Progressively lower doses of Ag administered with $AS03_A$ were able to generate detectable HAI titers after one immunization, but titers began to fall off at doses below 0.06 μg (**Figure 1A**).

Booster immunization increased HAI titers overall (**Figure 1B**). Mice immunized with low Ag dose (0.003–0.75 μg/dose) with $AS03_A$ produced significantly higher HAI titers than 3 μg without adjuvant (**Figure 1B**). Interestingly, although no response was detected after one dose, titers increased remarkably in the 0.003 μg + $AS03_A$ group after boosting (**Figure 1B**).

We next compared $AS03_A$ with half the amount of adjuvant ($AS03_B$) to unadjuvanted formulations. After a single immunization, unadjuvanted low-dose (0.03–0.003 μg) vaccines elicited low but detectable HAI titers (**Figure 1C**). Generally, use of the adjuvant increased antibody titers. $AS03_B$ functioned as efficiently as $AS03_A$ with 0.03 μg Ag, but at the lower dose (0.003 μg), $AS03_B$ generated a better response than $AS03_A$ in terms of HAI antibodies (**Figure 1C**). After booster immunization, 0.03 or 0.003 μg Ag with $AS03_A$ or $AS03_B$ generated similar HAI titers, which were higher than unadjuvanted vaccine (**Figure 1D**). Unadjuvanted low-dose vaccines tended to generate lower titers than the unadjuvanted high-dose formulation.

We were unable to reproducibly detect HAI titers after two immunizations at Ag doses of ≤0.0003 μg with $AS03_A$ or $AS03_B$ in our mouse model (**Figures 1C,D**). These very low Ag doses also failed to generate detectable immune responses by ELISA, MN assays, ELISpot, and lymphoproliferation in restimulated splenocytes (data not shown). Therefore, we focused on low Ag doses of 0.03 and 0.003 μg in our subsequent studies.

Similar to the HAI results, two immunizations of AS03-adjuvanted, low-dose vaccines tended to induce higher concentrations of influenza HA-specific IgG by ELISA than unadjuvanted vaccine (**Figure 2A**). The strength of antibody binding (avidity) was not significantly changed in response to Ag dose or use of AS03 adjuvant (**Figure 2B**). After two immunizations with 0.03 μg + $AS03_{A/B}$ or 0.003 μg + $AS03_{A/B}$, MN titers were equivalent or superior to those observed with unadjuvanted formulations (**Figure 2C**).

These data demonstrate that strong serum antibody responses can be elicited in BALB/c mice with 100- or 1000-fold lower

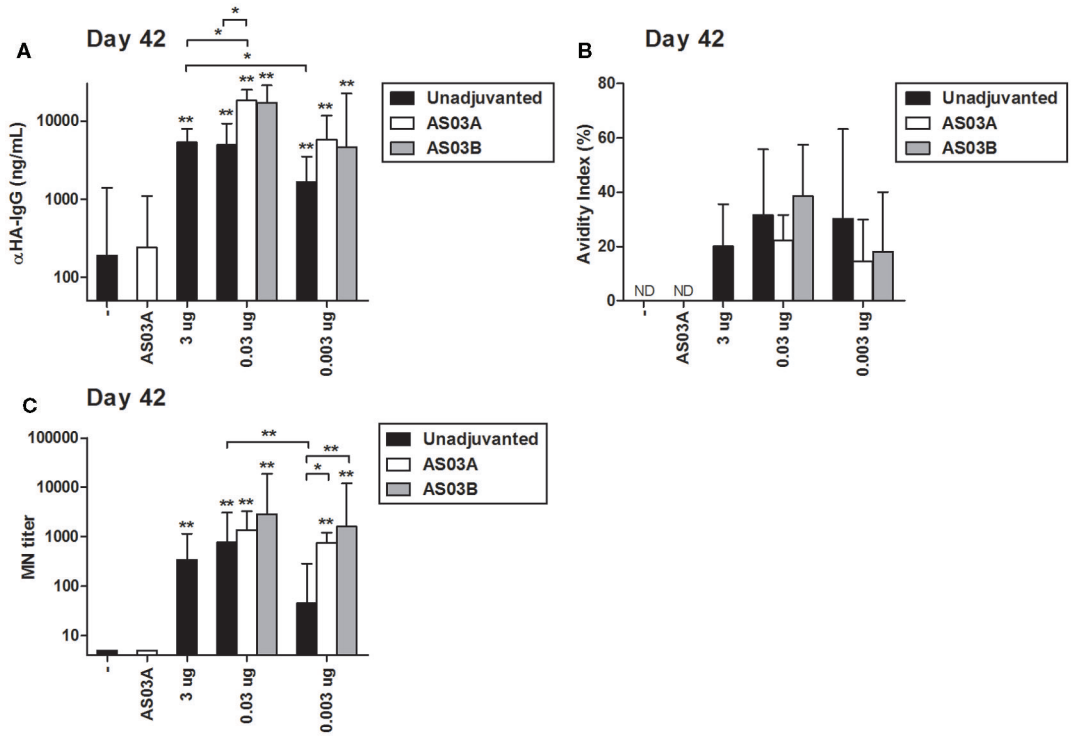


**FIGURE 2 | Influenza HA-specific IgG concentrations, antibody avidity, and microneutralization (MN) titers in BALB/c mice after one or two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**. Mice were immunized intramuscularly on days 0 and 21, and sera from individual mice were analyzed. Anti-HA IgG concentrations **(A)** and antibody avidity **(B)** measured by ELISA, and MN titers **(C)** were determined on day 42. On top of bars, * indicates a significant increase ($P < 0.05$) to negative "−" group and ** indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups. Significant differences between groups are denoted by brackets; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. For **(A,B)**, data represent 4–10 mice per group combined from three independent studies. For **(C)**, data represent 4–12 mice per group combined from three independent studies. Geometric means and 95% confidence intervals are shown. ND, not detected.

Ag than a high-Ag dose and that AS03 can increase serological responses even at very low Ag doses.

## Adjuvanted Low-Dose and Unadjuvanted Vaccines Generate Distinct Antigen-Specific Cytokine Profiles

All Ag-containing vaccines elicited detectable levels of Ag-specific lymphoproliferation in *ex vivo* stimulated splenocytes (**Figure 3A**). No statistical differences in proliferation were observed between any of the formulations so the immune microenvironment was further assessed by measuring cytokine/chemokine concentrations in culture supernatants.

Unadjuvanted vaccine at all Ag doses generated similar levels of all cytokines tested (**Figure 3**). However, adjuvanted low-dose vaccines generated very different influenza-specific cytokine milieus compared to unadjuvanted formulations. Some cytokines/chemokines, such as IL-4, IL-5, IL-10, and MCP-1/CCL2 were produced at similar levels in all groups (**Figure 3**), while others such as IL-2, IL-3, IL-6, IFNγ, RANTES/CCL5, IL-17, and GM-CSF were more strongly induced by the low-dose adjuvanted vaccines (**Figure 3**). The cytokine/chemokine responses are summarized in **Table 1**. AS03 tended to change the balance of cytokine responses by activating different arms of the immune response (Th1, Th2, Th17, and growth promoting cytokines) (**Table 1**). In comparison, all unadjuvanted groups produced similar cytokine profiles. No significant differences in the levels of IL-1α, IL-1β, TNFα, and MIP-1α were observed (data not shown).

We focused our next studies on immune responses at the cellular level. Given that no significant differences in proliferation or cytokine production were observed between unadjuvanted vaccines at different Ag doses, we focused our analysis on unadjuvanted high-dose versus AS03-adjuvanted low-dose formulations.

## Adjuvanted Low-Dose Vaccines Generate More ASCs than High-Dose Unadjuvated Vaccine

ELISpots were used to enumerate influenza-HA specific IgG ASCs in splenocytes (i.e., plasma cells), and memory B cell-derived plasma cells (i.e., memory ASCs) following *in vitro* differentiation. Low levels of ASCs and memory ASCs were detectable in mice immunized with unadjuvanted vaccine (**Figures 4A,C,E**). Mice immunized with 0.03 μg + $AS03_{A/B}$ had higher numbers of ASCs (**Figures 4A,E**) and memory ASCs (**Figure 4C**). At the lowest Ag dose (0.003 μg + $AS03_{A/B}$), the ASC (**Figures 4A,E**) and memory ASC response (**Figure 4C**) were comparable to that seen in mice receiving unadjuvanted vaccine. The mean spot size in these assays is correlated with the amount of antibody secreted. The adjuvanted low-dose groups tended to secret more antibodies per

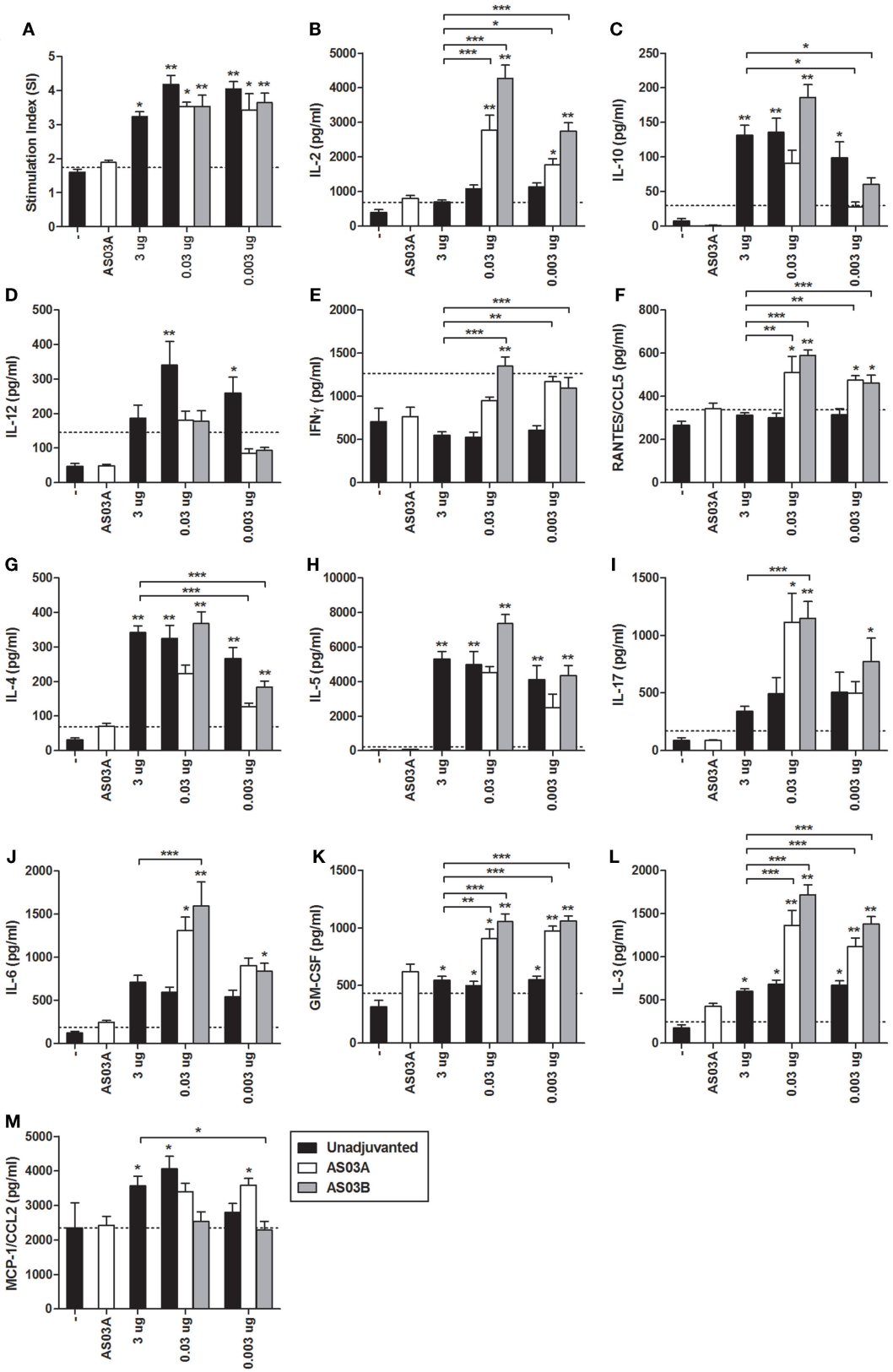


**FIGURE 3 | Influenza-specific splenocyte proliferation and cytokine production after two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**. BALB/c mice were immunized intramuscularly on days 0 and 21, and splenocytes were isolated from individual mice on day 42. *(Continued)*

**FIGURE 3 | Continued**
Splenocytes were stimulated *ex vivo* with media (unstimulated background) or with A/Uruguay H3N2 split vaccine. Culture supernatant was collected after 72 h and cells were pulsed with $H^3$-Thymidine for an additional 18 h. Cell proliferation **(A)** is shown as a stimulation index (SI). The line represents the mean +2 SDs of the negative control group. The concentrations of cytokines and chemokines in culture supernatants were determined using Q-Plex Mouse Cytokine – Screen (16-plex) multiplex ELISA and are summarized in **Table 1**: **(B)** IL-2, **(C)** IL-10, **(D)** IL-12, **(E)** IFNγ, **(F)** RANTES/CCL5, **(G)** IL-4, **(H)** IL-5, **(I)** IL-17, **(J)** IL-6, **(K)** GM-CSF, **(L)** IL-3, **(M)** MCP-1/CCL2. The line represents the mean +2 SDs of the cytokine concentration of unstimulated samples for all groups. On top of bars, * indicates a significant increase ($P < 0.05$) to negative "−" group and ** indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups. Significant differences between different groups are denoted by brackets; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent mean and SEs of 3–19 mice per group combined from three independent studies.

**TABLE 1 | Summary of influenza-specific cytokine responses in the supernatants of restimulated splenocytes after two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**.

| Category | Cytokine | High-Ag dose | Low Ag dose | |
|---|---|---|---|---|
| | | Unadjuvanted[a] 3 μg | Unadjuvanted[a,b] 0.03 or 0.003 μg | Adjuvanted[a,b] 0.03 or 0.003 μg + $AS03_{A/B}$ |
| T cell proliferation | IL-2 | | | ++ |
| Anti-inflammatory | IL-10 | ++ | ++ | ++ |
| Th1 | IL-12 | | ++ | |
| | IFNγ | | | ++ |
| | RANTES/CCL5 | | | ++ |
| Th2 | IL-4 | ++ | ++ | ++ |
| | IL-5 | ++ | ++ | ++ |
| Th17 | IL-17 | | | ++ |
| | IL-6 | | | ++ |
| Growth promoting and chemokines | GM-CSF | + | + | ++ |
| | IL-3 | + | + | ++ |
| | MCP-1/CCL3 | + | + | + |

[a] *The "+" sign indicates a significant increase ($P < 0.05$) to negative "−" group and "++" indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups.*
[b] *The "+" or "++" signs indicate a significant difference by 0.03 and/or 0.003 μg Ag dose with or without AS03.*

ASC or memory ASC than unadjuvanted vaccine (**Figures 4B,D**). These data suggest the adjuvanted low-dose formulations induced greater numbers of influenza-specific plasma cells and potentially memory B cell-derived ASCs that secreted more antibody per cell compared to the high-dose unadjuvanted vaccine.

## $AS03_B$-Adjuvanted Low-Dose Vaccines Produce More Influenza-Specific CD4+ and CD8+ T Cells

We investigated influenza-specific cytokine production in splenocytes by examining CD4+ or CD8+ T cells that produced IL-2, IL-5, TNFα, and IFNγ cytokines by flow cytometry (FC) (Figure S1 in Supplementary Material). Immunization with 0.003 μg + $AS03_B$ generated the highest percentage of Ag-specific CD4+ (**Figure 5A**; Figure S2 in Supplementary Material) and CD8+ (**Figure 6A**; Figure S3 in Supplementary Material) T cells expressing any combination of the four cytokines tested. Generally, formulations with $AS03_B$ tended to generate more cytokine-producing cells than with $AS03_A$ (**Figures 5A** and **6A**).

Ag-specific T cells can be categorized according to the number of cytokines they produced. Most CD4+ and CD8+ T cells were single positive for one of the four cytokines (**Figures 5B** and **6B**). With all formulations, over 90% of cytokine-producing CD4+ T cells secreted a single cytokine (**Figure 5B**). For CD8+ T cells, $AS03_A$-adjuvanted low-dose vaccines induced similar levels of poly-functional T cells (expressing two or more cytokines) as high-dose vaccine, which tended to be higher than that observed with $AS03_B$-adjuvanted formulations (**Figure 6B**).

Ag-specific, single positive T cells can be further categorized according to the cytokine produced. Most single positive CD4+ T cells in the high-dose unadjuvanted and $AS03_A$-adjuvanted low-dose groups expressed IL-2, whereas the $AS03_B$-adjuvanted groups produced more IFNγ-secreting cells (**Figure 5C**). In all groups except 0.003 μg + $AS03_A$, most single positive CD8+ T cells expressed IFNγ, followed by a smaller percentage of IL-2-secreting cells (**Figure 6C**). Interestingly, mice given 0.003 μg + $AS03_A$ tended to generate relatively equal proportions of IL-2 and IFNγ expressing CD8+ T cells (**Figure 6D**), although this group tended to generate fewer total responding cells (**Figure 6A**). Compared to high-dose vaccine, the 0.003 μg + $AS03_B$ recipients had significantly higher percentages of CD4+ and CD8+ T cells single positive for IFNγ (**Figures 5D** and **6D**). There were no significant differences in single positive IL-2 (**Figures 5E** and **6E**), IL-5, or TNFα (data not shown) producing cells or any combination of double or triple positive cells, and no Ag-specific, quadruple positive cells were observed (data not shown).

# Discussion

We demonstrate that use of AS03 can markedly change both the magnitude and pattern of vaccine-induced humoral and cellular immune responses in mice. Nanogram quantities of unadjuvanted vaccine were sufficient to induce Ag-specific immune responses that were, in some respects, comparable to those

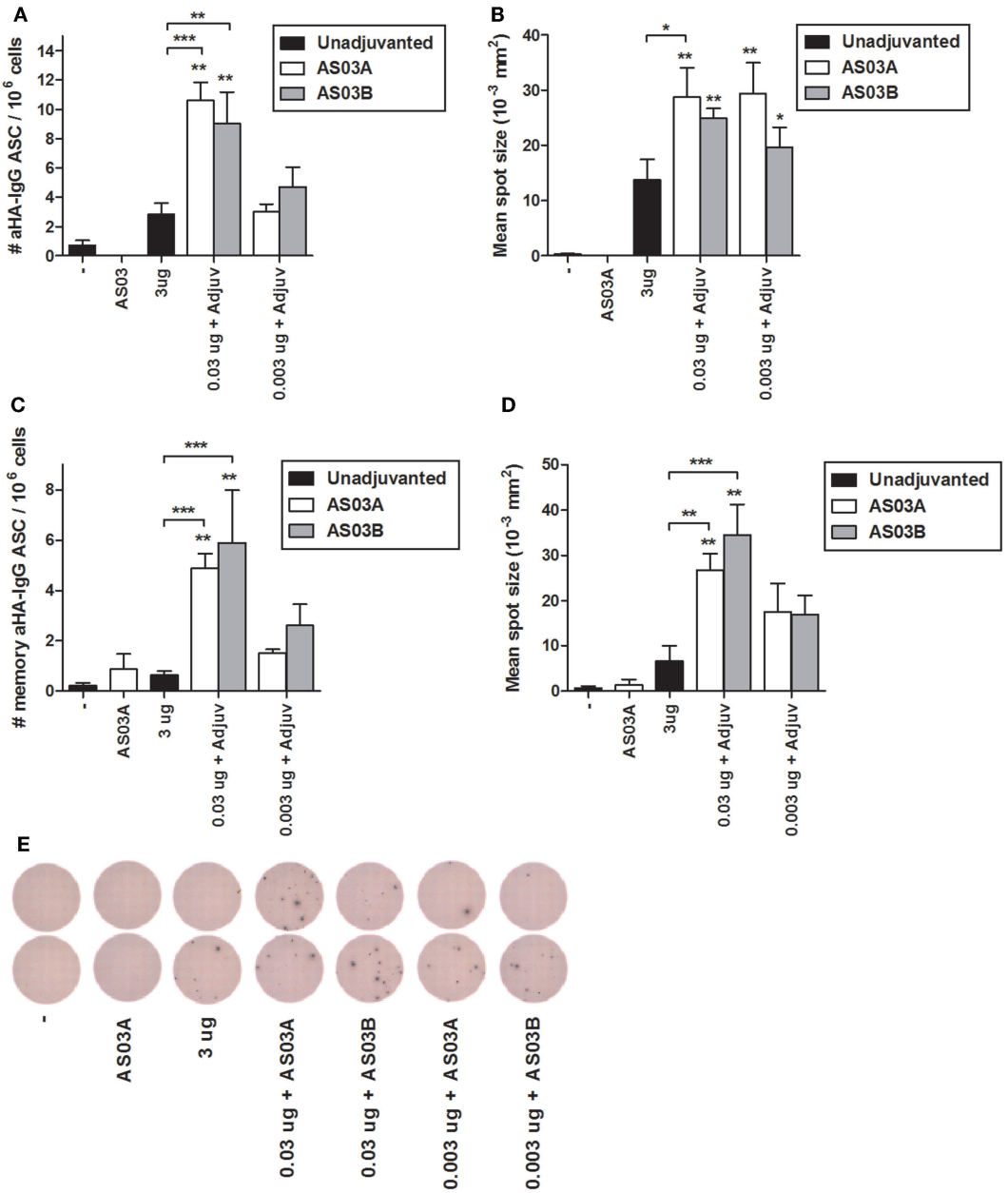


**FIGURE 4 | Generation of influenza HA-specific antibody secreting cells (ASCs) and memory B cell-derived ASCs after two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**. BALB/c mice were immunized intramuscularly on days 0 and 21, and splenocytes were isolated from individual mice on day 42. The number **(A)** and mean spot size **(B)** of HA-specific IgG antibody secreting cells (ASCs) were determined by ELISpot. Following *ex vivo* stimulation, the number **(C)** and mean spot size **(D)** of memory B cell-derived HA-specific IgG ASCs were determined by ELISpot. Representative ELISpot wells are shown in **(E)**. On top of bars, * indicates a significant increase ($P < 0.05$) to negative "−" group and ** indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups. Significant differences between groups are denoted by brackets; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent mean and SEs of 4–12 mice per group combined from two independent studies.

induced by high-dose vaccine. However, in the presence of AS03, even very low-dose (0.03–0.003 μg/dose) formulations elicited superior humoral and distinct cellular immune responses compared to unadjuvanted vaccine. Given that cell-mediated immunity is increasingly recognized to be important in protecting against influenza viral infection (19), these results suggest that over-reliance on serum antibody responses may not identify optimal vaccine formulations.

This is the first detailed pre-clinical investigation of the humoral and cellular immune responses to extreme dose-sparing with AS03. As little as 3 ng (0.003 μg) of A/Uruguay H3N2 model Ag was sufficient to induce excellent responses with or without

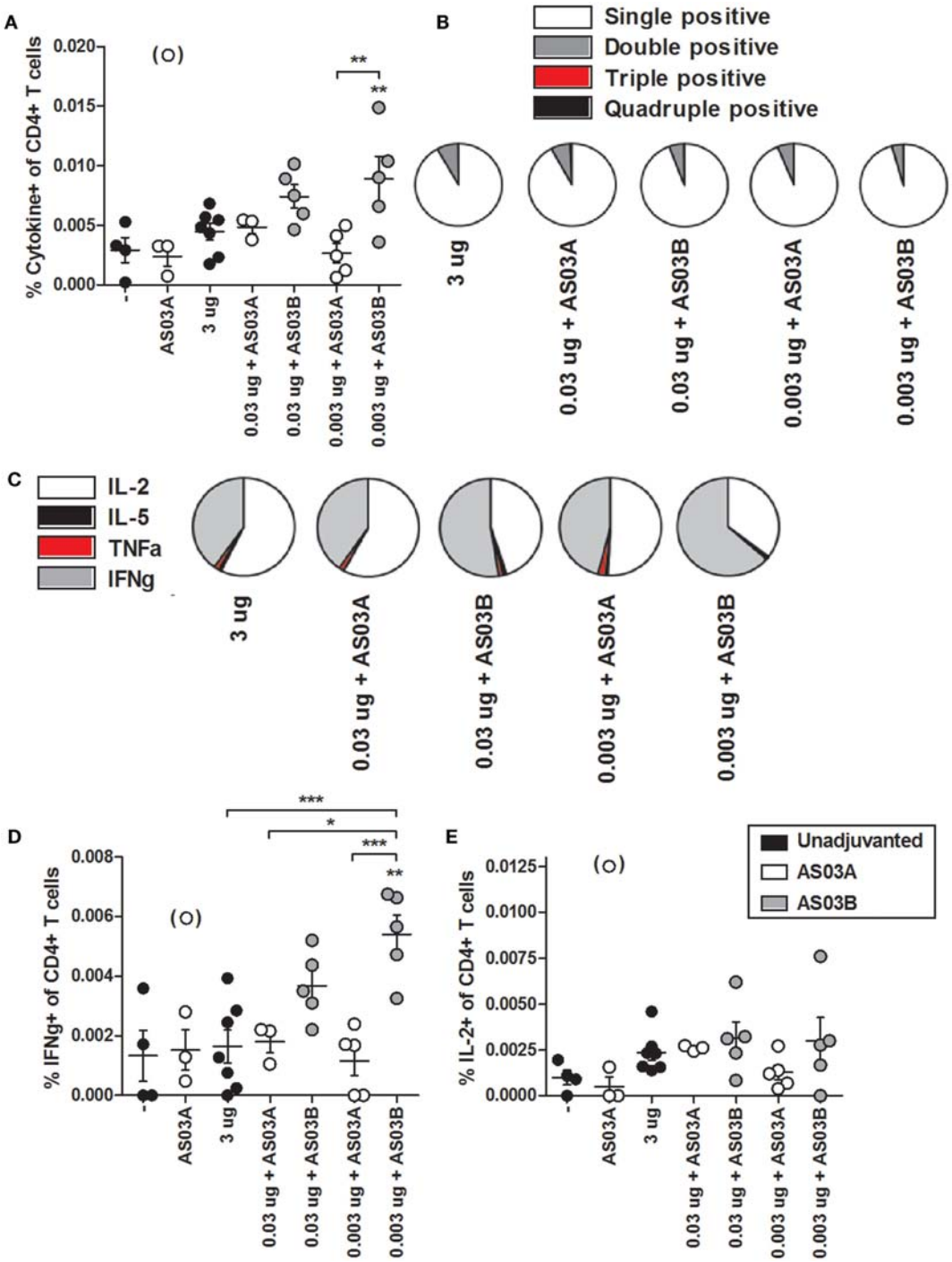


**FIGURE 5 | Influenza-specific cytokine-producing CD4+ T cells in splenocytes after two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**. BALB/c mice were immunized intramuscularly on days 0 and 21, and splenocytes were isolated from individual mice on day 42. Splenocytes were stimulated *ex vivo* with A/Uruguay H3N2 split vaccine and co-stimulatory antibodies, then analyzed by flow cytometry for CD4+ T cells that produced a combination of IL-2, IL-5, IFNγ, or TNFα cytokines. The gating strategy is described in Figure S2 in Supplementary Material and representative dot plots are shown in Figure S3 in Supplementary Material. The percentage of total responding CD4+ T cells that expressed any combination of the four cytokines **(A)**. The distribution of the number of individual cytokines produced by total responding CD4+ T cells **(B)**. The distribution of the specific cytokines produced by single-positive CD4+ T cells **(C)**. The percentage of CD4+ T cells that were single positive for IFNγ **(D)** or IL-2 **(E)**. On top of groups, ** indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups. Significant differences between groups are denoted by brackets; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data represent mean and SEs of 3–5 mice per group. An outlier in the $AS03_A$ only control group (denoted in brackets) was beyond the mean +3 SDs of the remainder of the group, and was omitted from analysis.

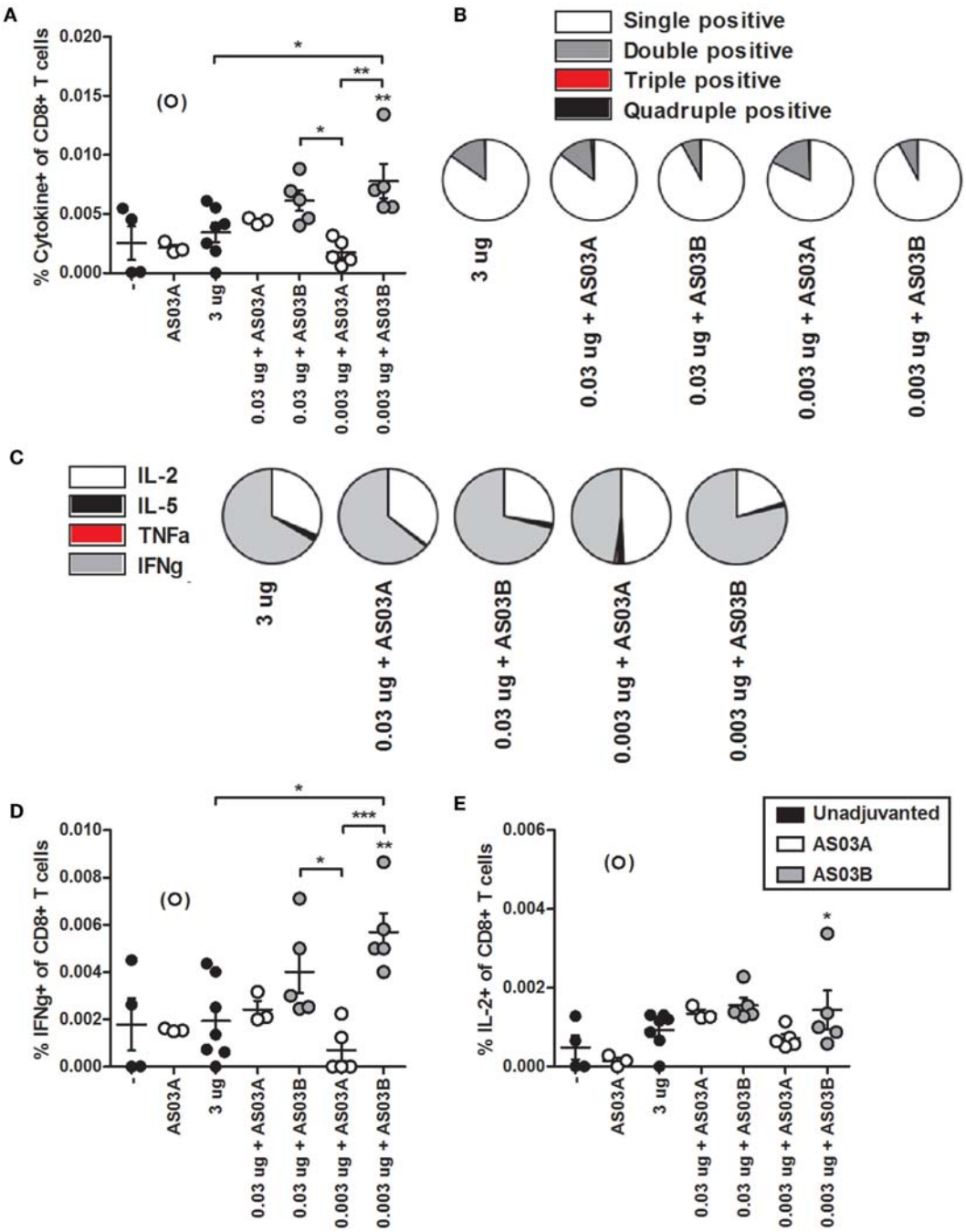


**FIGURE 6 | Influenza-specific cytokine-producing CD8+ T cells in splenocytes after two immunizations of unadjuvanted A/Uruguay/716/2007 H3N2 split vaccine or AS03-adjuvanted dose-sparing vaccines**. BALB/c mice were immunized intramuscularly on days 0 and 21, and splenocytes were isolated from individual mice on day 42. Splenocytes were stimulated *ex vivo* with A/Uruguay H3N2 split vaccine and co-stimulatory antibodies, then analyzed by flow cytometry for CD8+ T cells that produced a combination of IL-2, IL-5, IFNγ, or TNFα cytokines. The gating strategy is described in Figure S2 in Supplementary Material and representative dot plots are shown in Figure S4 in Supplementary Material. The percentage of total responding CD8+ T cells that expressed any combination of the four cytokines **(A)**. The distribution of the number of individual cytokines produced by total responding CD8+ T cells **(B)**. The distribution of the specific cytokines produced by single-positive CD8+ T cells **(C)**. The percentage of CD8+ T cells that were single positive for IFNγ **(D)** or IL-2 **(E)**. On top of groups, ** indicates a significant increase ($P < 0.05$) to both negative "−" and $AS03_A$ only control groups. Significant differences between groups are denoted by brackets; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data represent mean and SEs of 3–5 mice per group. An outlier in the $AS03_A$ only control group (denoted in brackets) was beyond the mean +3 SDs of the remainder of the group, and was omitted from analysis.

AS03. Our findings are similar to those recently reported, which found that 0.03–0.04 μg of influenza vaccines intramuscularly injected with various adjuvants could induce Ag-specific humoral and cellular immune responses, and protect against viral infection (20, 21). In contrast to these studies, we varied both Ag and adjuvant dose and performed detailed analyses of both B and T cell responses following vaccination.

The IgG avidity result is particularly surprising since the development of high avidity antibodies is thought to require both sufficient time and presence of Ag (22). Antibodies with comparable avidity were generated with high- and low-dose Ag with or without adjuvant. This was unexpected and is possibly a testament to the remarkable efficiency of the BALB/c mouse immune system or the immunogenicity of the A/Uruguay H3N2 split virion Ag used. Future studies could use different mouse strains to further investigate antibody avidity. In contrast, human trials with a similar oil-in-water adjuvant, MF59 and A/California/07/2009 H1N1 split-virus Ag in a dose-sparing formulation (7.5 μg + MF59) increased antibody avidity compared to standard unadjuvanted vaccine (15 μg) (23, 24). Another consideration is that relatively massive Ag doses are administered to mice in most pre-clinical vaccine studies. On a body-weight basis, our 0.003 μg dose in a 20 g mouse translates into an ~10 μg dose in a 70 kg human, which is close to the standard influenza vaccine dosage per strain. Our data raise the question of whether or not the doses routinely used in pre-clinical studies should be re-evaluated.

The superior performance of the adjuvanted, low-dose formulations in the induction of ASCs that secrete more antibodies per cell was intriguing. The presence of greater numbers of highly active plasma cells likely accounts for the higher serum HAI and ELISA antibody titers observed in the 0.03 μg + AS03A/B groups, although MN titers were not similarly elevated. Surprisingly, unadjuvanted and the lowest Ag dose groups (0.003 μg + AS03A/B) generated very low numbers (close to background) of ASCs and memory ASCs in the spleen, but exhibited robust serum antibody titers. It would be interesting to investigate the presence of ASCs in other compartments (e.g., bone marrow) since plasma cells and memory B cells eventually track to the bone marrow late in the immune response (22). Differences in cell trafficking might therefore account for the seeming discrepancy between the low numbers of ASCs, but high serum antibody titers. The maintenance of long-term humoral memory is influenced by several factors including the presence of sufficient Ag (25). Indeed in our study, splenocytes from the lowest dose groups (0.003 μg + $AS03_{A/B}$) had fewer ASCs and memory ASCs than groups immunized with 0.03 μg suggesting that Ag dose plays a role in our model in the generation of plasma and memory B cells. There were, however, no major differences in the level of antibody secretion per cell in the two adjuvanted low-dose groups.

Unadjuvanted and low-dose adjuvanted vaccines also induced distinct cytokine profiles in restimulated splenocytes. Compared to adjuvanted, low-dose vaccines, the unadjuvanted groups tended to produce higher levels of Th2 cytokines such as IL-4 and IL-5, as well as IL-10 that can promote B cell proliferation. However, serum antibody levels and the number of ASCs in the unadjuvanted groups were not higher than those seen in the adjuvanted, low-dose groups. Together, these observations suggest a Th2-biased response with ineffective antibody production. This apparent paradox might be explained by the activation of a broader range of cytokines in the adjuvanted vaccine groups leading to greater overall vaccine-specific responses. For example, the splenocytes of AS03-containing groups produced higher levels of IL-2 and IL-6 thereby suggesting activation of more T cells and greater differentiation of follicular helper T cells, respectively (26, 27). Furthermore, IL-6 also plays a role in the maturation of B cells into ASCs. The AS03-adjuvanted formulations also induced higher levels of IFNγ and RANTES/CCL5, the latter acting through the CCR5 receptor to promote development of IFNγ-producing Th1 cells (26). Surprisingly, the unadjuvanted low-dose vaccine tended to induce the highest level of IL-12, a Th1-promoting cytokine, although this difference did not reach statistical significance. Therefore, in our mouse model, both the unadjuvanted and adjuvanted formulations had the potential to induce cytokines/chemokines associated with Th1-type responses. Finally, only the adjuvanted formulations were found to induce Th17 type (IL-17 and IL-6) and growth promoting (GM-CSF and IL-3) cytokines. In summary, we found that the unadjuvanted vaccines could induce both Th1- and Th2-type responses, while AS03-adjuvanted vaccines induced Th1, Th2, Th17, and growth promoting cytokine/chemokine production in the restimulated splenocytes. Activation of this broad range of cytokines/chemokines by the adjuvanted vaccines likely contributed to the stronger Ag-specific immune responses generated.

Historically, little attention was paid to adjuvant dose except in the context of adverse events, with greater adjuvant doses tending to cause more reactions (28, 29). Little consideration was given to the idea that different doses of adjuvant might alter the pattern of the immune response induced. In a single clinical trial that varied the doses of both MF59 (full, half, or quarter) and trivalent inactivated influenza vaccine (15 or 30 μg) in elderly subjects, Della Cioppa et al. found that more adjuvant tended to induce higher HAI titers but had no effect on the Ag-specific CD4 T cell response (30). In our model, $AS03_A$ and $AS03_B$ (full- and half-dose, respectively) induced similar serum antibody profiles. However, the $AS03_B$-adjuvanted formulations tended to produce higher levels of most of the cytokines/chemokines measured compared to $AS03_A$. Formulations with $AS03_B$ (especially the 0.003 μg/dose) also generated more influenza-specific CD4+ and CD8+ T cells than the unadjuvanted or $AS03_A$-adjuvanted vaccines, although these cells were primarily single positive for IFNγ. However, we found that the low-dose vaccines formulated with $AS03_A$ tended to generate more influenza-specific poly-functional CD8+ T cells compared to $AS03_B$. Poly-functional T cells generally express higher levels of cytokines per cell and are considered to be functionally superior to single-cytokine-producing cells (31). Additional studies are needed to determine the functional significance of the mono-functional versus poly-functional T cells induced by these different formulations in our vaccine model.

In human studies of PBMCs isolated after AS03-adjuvanted vaccine administration, an increase in Ag-specific CD4+ T cells is usually observed in the absence of increasing CD8+ T cells responses (7, 32, 33). In contrast, we observed both CD4+ and CD8+ T cell responses similar to the findings of other mouse

studies. For example, studies from the Boivin laboratory showed that two immunizations of AS03-adjuvanted influenza vaccine (at 3 μg/dose) produced detectable Ag-specific CD4+ and CD8+ T cells, which tended to be greater than responses in control groups, although statistical differences were not observed (18, 34). We found that reductions in Ag dose with the same dose of AS03 could markedly increase T cell responses.

Clearly, we show that greater attention should be paid to the balance of Ag and adjuvant in vaccine formulations to fully understand vaccine efficacy. However, given the known differences in T cell responses in humans versus mice, our observations in the very low-dose mouse model may not be predictive of responses in humans. The majority of adult humans have been previously exposed to various strains of influenza, which is unlike the immunologically naïve mice in our studies. In human studies with AS03, vaccine-specific CD8+ T cells were detected at all timepoints including pre-immunization, but vaccination failed to significantly increase the Ag-specific CD8 responses (32, 33). In naïve mice, we observe an increase in Ag-specific CD4+ and CD8+ T cell responses after vaccination. Indeed, the two-dose vaccination schedule that we use in our study is the same as that used in immunologically naïve infants. As a result, our observations may be more relevant to this population, rather than the response in primed adults who mainly receive a single dose. Finally, AS03 is thought to function primarily through the induction of cytokines (low level inflammation) at the site of injection (10) but the mouse and human inflammatory responses can be very different as was recently demonstrated in a comprehensive transcriptomic analysis (35). Therefore, the broad activation of cytokines we observed in groups given AS03-adjuvanted formulations may also be specific to our mouse model.

The current study has additional limitations; for example, we have not yet tested whether or not the different immune response patterns correlate with protection from influenza challenge. These studies are currently underway. The A/Uruguay H3N2 split virion Ag used in this study is also relatively more immunogenic in mice than Ag prepared from other influenza strains (unpublished observations). Therefore, our results may not extrapolate to other vaccine formulations and additional studies are warranted to test this. Although Ag doses in mouse influenza vaccine studies typically range from 3 to 15 μg, we acknowledge that our selection of 3 μg as the high-dose vaccine formulation was entirely arbitrary. As noted above, our 3 μg dose does not correspond well with the 15 μg Ag dose routinely used in human vaccines on a body-weight basis. Similarly, our selection of 25 μl/12.5 μl of $AS03_{A/B}$ was based upon previous mouse studies (18, 36) but is also arbitrary and is much larger (350×) than the corresponding dose (by volume) used in humans for intramuscular injection (250 μl) on a body-weight basis (3).

In the event of an influenza pandemic, there will be great pressure to deliver the largest number of vaccine doses as quickly as possible. Our data suggest that both Ag and adjuvant-sparing strategies may make important contributions to optimization efforts from both immunologic and economic standpoints. We show that differences in both influenza Ag and adjuvant dose can significantly alter the immune response pattern following vaccination; findings that may be very relevant to the development of better vaccines. These observations also raise important questions about the use of "standard" doses of both Ag and adjuvants in pre-clinical vaccine studies in mice.

## Author Contributions

KY and BW designed the study and experiments. KY, JG, KW, EA, and AB performed experiments under the supervision of KY. KY and BW analyzed the data and wrote the paper with input from EB, CPM, and DB.

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## Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00207

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# Generation of adaptive immune responses following influenza virus challenge is not compromised by pre-treatment with the TLR-2 agonist $Pam_2Cys$

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Immunostimulatory agents provide a new category of anti-microbial agents that activate the host's innate immune system allowing control of viral and/or bacterial infections. The TLR-2 agonist PEG-$Pam_2Cys$ has been shown to mediate potent anti-viral activity against influenza viruses when administered prophylactically (1). Here, we demonstrate that the treatment of mice with PEG-$Pam_2Cys$ does not compromise their ability to generate adaptive immune responses following subsequent challenge with influenza virus. The antibody induced in mice pre-treated with $Pam_2Cys$ possessed hemagglutination-inhibiting activities and the $CD8^+$ T-cell responses that were elicited provided protection against heterologous viral challenge. In the absence of an effective influenza vaccine, an agent that provides immediate protection against the virus and does not compromise the induction of influenza-specific immunity on exposure to infectious virus provides an opportunity for population immunity to be achieved through natural exposure to virus.

**Keywords: $Pam_2Cys$, toll-like receptor-2, influenza A virus, innate immunity, adaptive immunity**

## Introduction

Vaccination is currently the most effective medical intervention available for prevention of influenza infection and disease. The efficacy of influenza vaccines is dependent on a match between the viral strains included in the vaccine and circulating influenza virus strains. Although anti-viral drugs can diminish symptoms and shorten the duration of illness (2, 3), the suggestion has been made that in the case of influenza their use can hamper the development of immunological memory (4) leaving the host susceptible to re-infection when drug treatment ceases. Although anti-influenza drugs continue to be used effectively in a clinical setting, concerns with anti-viral resistance (5–7) due to their widespread use have resulted in increased efforts to develop alternative prevention strategies.

**Abbreviations:** Ab, antibody; Influenza, influenza A virus; $Pam_2Cys$, *S*-[2,3-bis(palmitoyloxy)propyl] cysteine; TLR, toll-like receptor.

One approach is to harness the rapid anti-microbial responses of the innate immune system, particularly for pathogens that use the respiratory portal of entry. Immunostimulatory agents provide immediate non-specific protection against virulent influenza challenge (1, 8–10), and in mice, these compounds reduce the symptoms associated with influenza infection and also the viral burden reducing morbidity and promoting survival. Furthermore, by acting through the innate immune system and not directly on the pathogen, these immunostimulatory agents are unlikely to select resistant virus variants.

Many immunostimulatory agents are agonists of toll-like receptors (TLR) and mediate anti-viral and anti-bacterial activity by activating inflammatory pathways (1, 8–10). An understanding of the way in which these compounds affect acquisition of adaptive immunity and immunological memory is important because they could be employed for use during influenza pandemics where there is a high risk of re-exposure to pathogen. In the case of respiratory diseases, delivery of TLR agonists to the pulmonary tract can alter the immune environment, which could influence subsequent induction of adaptive immunity. An understanding of the way in which immunostimulatory agents, including TLR agonists, affect development of pathogen-specific immunity is therefore important.

We have developed a soluble form of the synthetic analog, *S*-[2,3-bis(palmitoyloxy)propyl] cysteine ($Pam_2Cys$), which acts as an agonist of toll-like receptor-2 (TLR-2) and provides immediate protection against challenge with a lethal dose of influenza virus A/PR/8/34 (PR8, H1N1) (1). We have also shown that although $Pam_2Cys$ treatment significantly reduces viral burden, reduces disease symptoms, and prevents death, it does not totally abrogate infection. This property provides the potential to develop immunity to influenza virus through asymptomatic natural infection.

In this study, we show that $Pam_2Cys$ prophylaxis permits development of a robust influenza virus-specific adaptive immune responses comprised $CD8^+$ T-cells, $CD4^+$ T-cells, and antibodies. We also show that this virus-independent stimulation of the innate immune system does not compromise the development of heterologous immunity. The development of an agent that can provide the host with immediate protection and does not hinder the development of pathogen-specific immunity following exposure to infectious virus provides an opportunity for population immunity to be achieved through natural exposure to the virus.

## Materials and Methods

### Synthesis of PEG-$Pam_2Cys$

$Pam_2Cys$ is hydrophobic and insoluble in physiological media; therefore, we synthesized the agonist with a polyethylene glycol molecule attached to confer solubility and allow administration by the intranasal (i.n.) route. PEG-$Pam_2Cys$ was synthesized in house using Fmoc-based chemistry as described previously (1). The product was purified by reversed-phase high-performance liquid chromatography (HPLC) using a C4 VYDAC column (10 mm × 250 mm; Alltech, NSW, Australia) installed in a Waters HPLC system (Waters Millipore, Milford, MA, USA). The purity of PEG-$Pam_2Cys$ was determined by HPLC using a VYDAC C8 column (4.6 mm × 250 mm) installed in a Waters system and was found to be >95%. The authenticity of the product was determined by mass analysis (mass value found 1,502.2 Da; expected mass 1,502.1 Da) using an Agilent 1100 Series LC/MSD ion-trap mass spectrometer (Agilent, Palo Alto, CA, USA).

### Animals

Female C57BL/6 mice (6–12 weeks old) were bred and housed in the Animal house facility at the Department of Microbiology and Immunology, The University of Melbourne. All animal experiments were conducted with approval from the University of Melbourne Animal Ethics Committee.

### Inoculations and Viral Challenge

Mice were anesthetized using isoflurane inhalation and received either 20 nmol of PEG-$Pam_2Cys$ dissolved in saline or saline alone by the i.n. route.

Mice were challenged with a virulent strain of influenza virus 3 days after treatment with PEG-$Pam_2Cys$. 200 plaque forming units (PFU) of the H1N1 influenza virus A/Puerto Rico/8/34 (PR8, Mount Sinai) were administered by the i.n. route during isoflurane anesthesia. Infection following challenge with PR8 in this way results in weight loss, labored breathing, and a hunched posture; animals were monitored daily for any signs of illness and euthanized at a pre-determined humane endpoint.

Challenge with less virulent strains of influenza virus were also carried out during isoflurane anesthesia with either (i) $10^{4.5}$ PFU of the H3N1 virus Mem/Bel (a genetic reassortant of A/Memphis/1/71 [H3N2] × A/Bellamy/42 [H1N1]) (ii) $10^{4.5}$ PFU of Mem71 A/Memphis/1/71, an H3N2 strain or (iii) $10^{4.5}$ PFU of the H3N2 virus, ×31. Each virus preparation was diluted in PBS and administered i.n. 3 days after receiving PEG-$Pam_2Cys$.

### Preparation of Lung Cells

Following $CO_2$ asphyxiation, lungs were removed and subjected to enzymatic digestion with collagenase A (2 mg/ml; Roche, Mannheim, Germany) in RPMI 1640 medium for 30 minutes 37°C. Cells were strained through a metal sieve and treated with ammonium-Tris hydrochloride (7.4% w/v ammonium chloride, 2.06% w/v Tris hydrochloride [ATC]) for 5 min at 37°C and were then washed twice with RP-10 (RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 76 mM 2-meceptoethanol, 150 U/ml penicillin, 150 mg/ml streptomycin, 150 mM non-essential amino acids [all supplements were obtained from Life Technologies]) in 7.5 mM HEPES. The number of viable cells was determined using trypan blue exclusion.

### Intracellular Cytokine Staining

Lung and spleen cells ($2–3 \times 10^6$/200 µl) were stimulated in the presence or absence of peptide $PA_{224–236}$ or $NP_{366–374}$ (1 µg/ml) with 5 µg/ml of GolgiPlug (BD Biosciences Pharmingen) 25 U/ml recombinant human IL-2 (Roche, Indianapolis, IN, USA) for 6 h at 37°C. Following stimulation, cells were stained with PercP5.5 anti-mouse CD8α (BD Biosciences Pharmingen) then fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Cells were finally stained with FITC-labeled antibody directed against interferon-γ (IFN-γ), Pe-Cy7-labeled antibodies directed against

tumor necrosis factor-α (TNF-α) and APC-labeled antibodies directed against IL-2. Samples were analyzed using a FACSCanto II and analyzed using FlowJo Software.

### Hemagglutination Inhibition Assay

Sera were prepared from blood and stored at −20°C until use. To remove any non-specific inhibitors of hemagglutination, sera were diluted $^1/_5$ with receptor destroying enzyme (RDE II, Denka Seiken Co., Ltd.) and incubated at 37°C overnight. Sodium citrate (1.6% w/v from Merck; Kilsyth, Victoria) diluted in PBS (Media Preparation Facility, Department of Microbiology and Immunology). 0.1% sodium citrate (Chem Supply) was added and samples were incubated for a further 2 h at 56°C prior to use. The hemagglutination inhibition (HI) assay was performed using either Mem/Bel or PR8 according to the method described in Ref. (11) and modified to a micro-titer format.

### *In vivo* Cytotoxic T-Cell Assay

An *in vivo* cytotoxic T-cell (CTL) assay was performed in mice that had been primed with Mem71 virus and challenged 1 month later with PR8 virus using a previously described method (12). The data generated were analyzed using FlowJo software and the percentage specific lysis of CFSE-labeled target cells in each mouse calculated using the following equation:

$$\% \text{ specific lysis} = 1 - \frac{r\,(\text{non-infected})}{r\,(\text{infected})} * 100$$

$$\text{where r} = \% \frac{\text{CFSE low}}{\text{CFSE high}}$$

### $CD4^+$ T-Cell IFN-γ Detecting ELISPOT

Membrane-based 96-well plates (MAIPS4510; Millipore, North Ryde, NSW, Australia) were coated with anti-mouse IFN-γ capture antibody (clone R4-6A2; Pharmingen) prior to addition of $5 \times 10^5$ cells to each well followed by 50 µl $NP_{311–325}$ peptide (5 µg/well). Four wells lacking peptide were included as negative controls. Cells were cultured for 18 h at 37°C 5% $CO_2$ and IFN-γ detected using biotinylated mouse anti-IFN-γ detection Ab (clone XMG1.2; Pharmingen) and streptavidin–alkaline phosphatase (Pharmingen) as described elsewhere (13). Spots formed by the deposition of enzyme substrate were counted using an ELISPOT plate reader (AID Autoimmun Diagnotika, Strassberg, Germany) and analyzed using AID software. The number of spot-forming units (SFU) was calculated by subtracting the sum of the background value plus two SD and responses considered positive when the net SFU value was >20 SFU/$10^6$ cells.

### Determination of Influenza Virus Titers

Lung viral titers were determined using an MDCK cell-based plaque assay as previously described (14).

### Characterization of the Pulmonary Cytokine Environment

The levels of IFN-γ, TNF-α, interlukin-6 (IL-6), IL-10, IL-12p70, and monocyte chemoattractant protein-1 (MCP-1) in bronchoalveolar lavage (BAL) fluid or lung tissue were analyzed using a BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions with the exception that a total of 2 µl of each capture bead was used in 50 µl of BAL sample and the PE-detection reagent was diluted 1 in 5. Samples were analyzed using a Becton Dickinson FACSCalibur flow cytometer and data analyzed using the FlowJo software package (Tree Star, Inc., Ashland, OR, USA).

### Statistical Analyses

For comparison of two data sets, a two-tailed Student's *t*-test was used. For comparison of data sets with a non-Gaussian distribution, a Mann–Whitney *t*-test was used. A $P$-value $\leq 0.05$ was considered statistically significant. Statistical analyses were carried out using the GraphPad Prism 6 software package.

## Results

### Adaptive Immune Responses Generated Following Challenge with Virulent and Non-Virulent Influenza Virus Strains

In the C57BL/6 mouse model of influenza infection, the neutralizing Ab response is directed predominantly to the viral hemagglutinin and the $CD8^+$ T-cell response is directed to the two immunodominant epitopes $PA_{224–236}$ and $NP_{366–374,}$ present on the internal proteins, acid polymerase and nucleoprotein, respectively (15).

Use of non-virulent Mem–Bel (H3N1) influenza virus allowed us to follow the adaptive immune response beyond 7 days, the time point at which mice infected with virulent PR8 (H1N1) influenza virus succumb to infection. Saline or PEG-$Pam_2$Cys was administered to mice 3 days prior to challenge with $10^{4.5}$ PFU of Mem–Bel virus (**Figure 1A**), mice were monitored daily following influenza challenge and weight loss is shown in **Figure 1B**. Animals that received PEG-$Pam_2$Cys maintained their overall bodyweight throughout the duration of infection, whereas animals treated with saline lost a small amount of weight during influenza challenge but regained weight 10 days after influenza challenge. The viral load in mice treated with PEG-$Pam_2$Cys was significantly reduced (>1 log) during the early stages of infection and by day 7, virus was cleared from the lungs of both treatment groups (**Figure 1C**).

No significant differences in titers of HI antibodies ($P = 0.4977$) were detected in the sera of saline and PEG-$Pam_2$Cys-treated animals 10 days after challenge (**Figure 1D**). Epitope-specific $CD8^+$ T-cell responses were examined 10 days postinfection, and no significant differences were detected in cytokine secreting $CD8^+$ T-cells present in the lungs or spleens regardless of the treatment received (**Figures 1E–G**). Gating strategy used to identify cytokine secreting cells is shown in Figure S1 in Supplementary Material. We also detected no significant ($P = 0.7381$) numbers of $CD4^+$ T-cells in the spleens of each treatment group (**Figure 1H**). Taken together, the results suggest that neither cell-mediated nor humoral immune responses were compromised by pre-treatment with PEG-$Pam_2$Cys.

To determine the effects of PEG-$Pam_2$Cys on infection with a more virulent influenza virus, mice were treated with saline or the

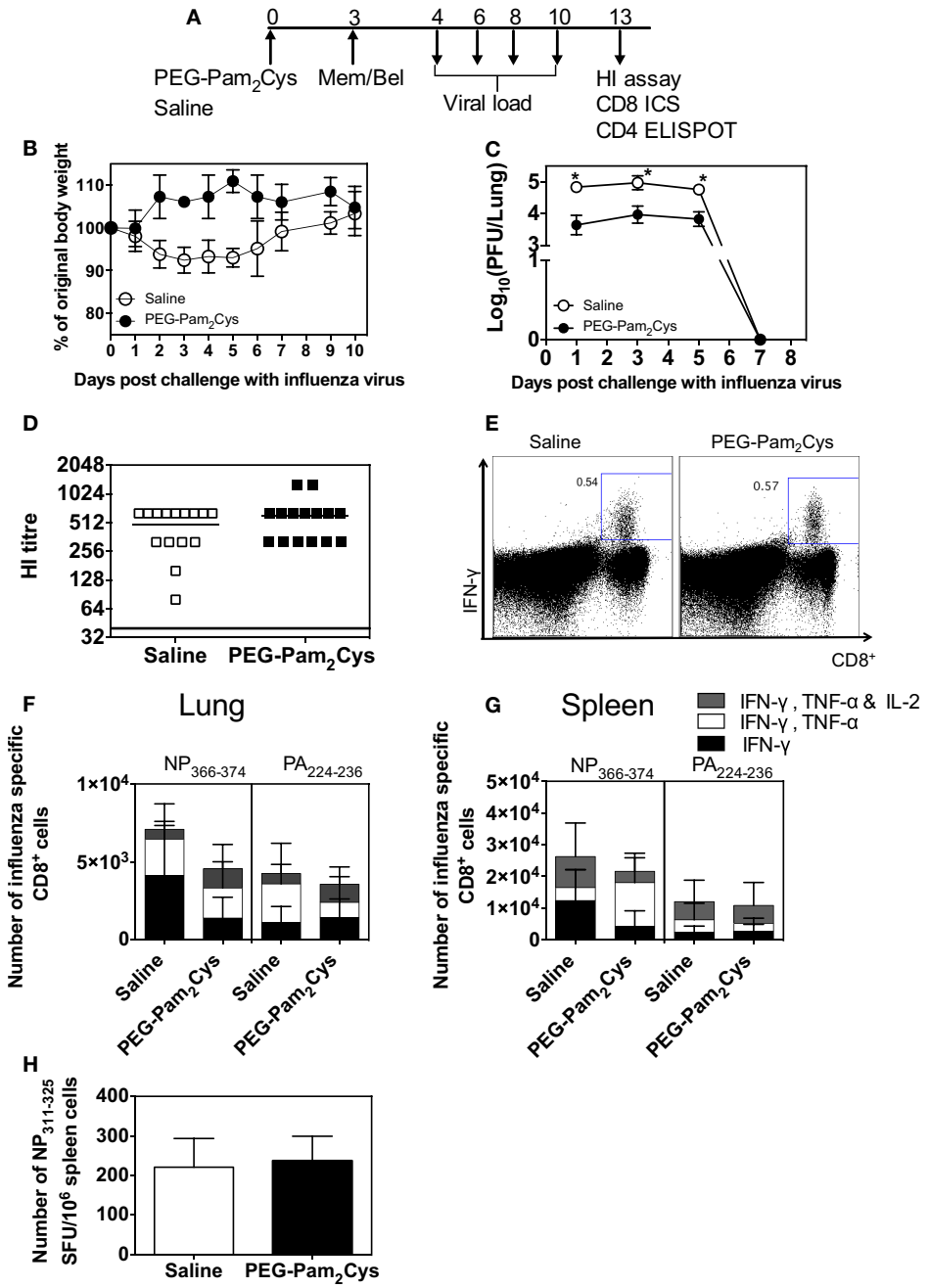


**FIGURE 1 | Antibody and cell mediated immune responses generated in PEG-Pam$_2$Cys-treated mice following infection with Mem/Bel.** **(A)** Timeline of protocols used; C57BL/6 ($n = 5$–15) mice were inoculated via the i.n. route with saline or 20 nmol of PEG-Pam$_2$Cys 3 days prior to challenge with $10^{4.5}$ PFU of Mem/Bel. At various times following challenge, mice were culled and various virological and immunological parameters measured. **(B)** Percentage weight change following influenza challenge. **(C)** Mean viral loads present in the lung throughout the course of infection. **(D)** Influenza-specific Ab levels in sera were determined using HI assays. **(E)** Representative FACS plots of the $NP_{366–374}$-specific response in the lungs of PEG-Pam$_2$Cys and saline-treated mice. Numbers of $NP_{366–374}$ and $PA_{224–236}$ specific cytokine secreting $CD8^+$ T-cells in **(F)** lungs and **(G)** spleen. **(H)** Number of IFN-γ secreting $CD4^+$ T-cells [spot-forming units (SFU) per $10^6$ cells] in spleen cells following stimulation with peptide epitope $NP_{311–325}$. Statistical significance for **(B)** was determined using an unpaired Mann–Whitney $t$-test ($^*P < 0.05$).

immunostimulant and subsequently challenged with a lethal dose of PR8 virus (**Figure 2A**). Saline control mice suffered substantial weight loss and reached the previously determined humane end point 8 days after challenge (**Figure 2B**). In contrast, mice pre-treated with PEG-Pam$_2$Cys all survived viral challenge, a result which is consistent with our previous findings (1).

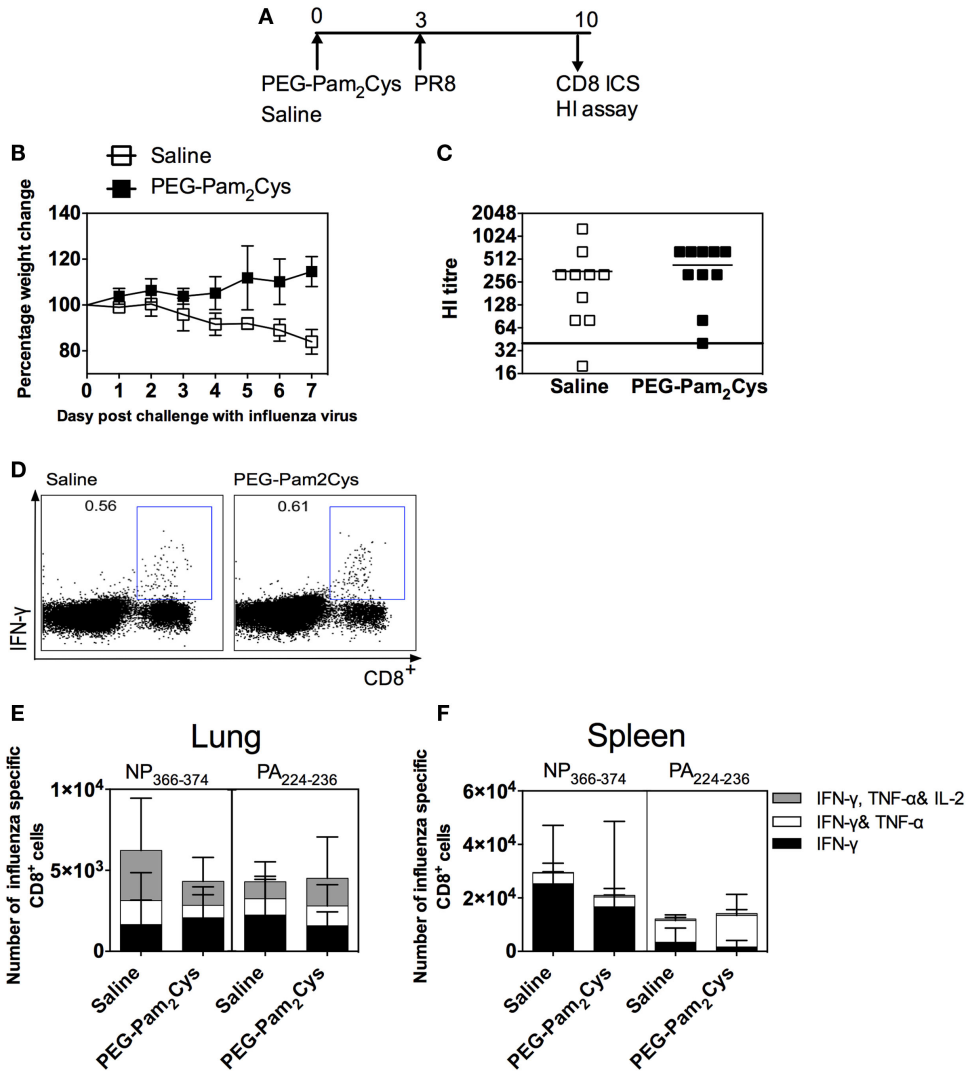


**FIGURE 2 | Antibody and cell mediated immune responses generated in PEG-Pam$_2$Cys-treated mice following challenge with 200 PFU of PR8 influenza virus**. **(A)** Timeline of protocols used; C57BL/6 ($n = 5$–10) mice were inoculated via the i.n. route with saline or 20 nmol of PEG-Pam$_2$Cys 3 days prior to challenge with 200PFU of PR8. **(B)** Mice were monitored daily following viral challenge and the percentage change in body weight recorded. **(C)** Hemagglutination inhibiting Ab levels in sera. **(D)** Representative FACS plots of the $NP_{366-374}$-specific $CD8^+$ T-cell response in lungs. Numbers of $NP_{366-374}$ or $PA_{224-236}$ specific cytokine secreting $CD8^+$ T-cells in **(E)** lungs and **(F)** spleen. Statistical significance was determined using an unpaired Student's *t*-test (*$P < 0.05$).

Another group of C57BL/6 mice treated with either saline or PEG-Pam$_2$Cys and subsequently challenged with PR8 were euthanized 7 days after viral challenge to asses the adaptive immune responses. Non-significant titers of HI antibodies ($P = 0.2607$) were detected in sera of animals 7 days after challenge with PR8 virus (**Figure 2C**) whether they had been pre-treated with saline. When the fine specificity of the $CD8^+$ T-cell response was examined, very few differences were detected in the cytokine profiles of $PA_{224-236}$ and $NP_{366-374}$-specific $CD8^+$ T-cells obtained from lungs and spleen of mice whether treated with saline or PEG-Pam$_2$Cys (**Figures 2D–F**). The results again indicate that treatment with PEG-Pam$_2$Cys has little or no deleterious effect on the development of adaptive immune responses.

## PEG-Pam$_2$Cys Treatment does not Affect the Development of Recall CD8 T-Cell Responses or the Development of Heterologous Immunity

Because $CD8^+$ T-cells target the internal, conserved epitopes of the influenza virus proteins, they are able to recognize a broad range of influenza strains (16). These cells are, however, short lived and require constant stimulation for persistence. The use of Mem'71 (H3N2) results in a resolving infection, which allowed us to track the maintenance of influenza-specific immune responses and also to determine the ability to provide protection against a second challenge with a heterologous strain of virus.

To examine the long-term functional and cross-protective capabilities of the $CD8^+$ T-cell response generated, we assessed $CD8^+$

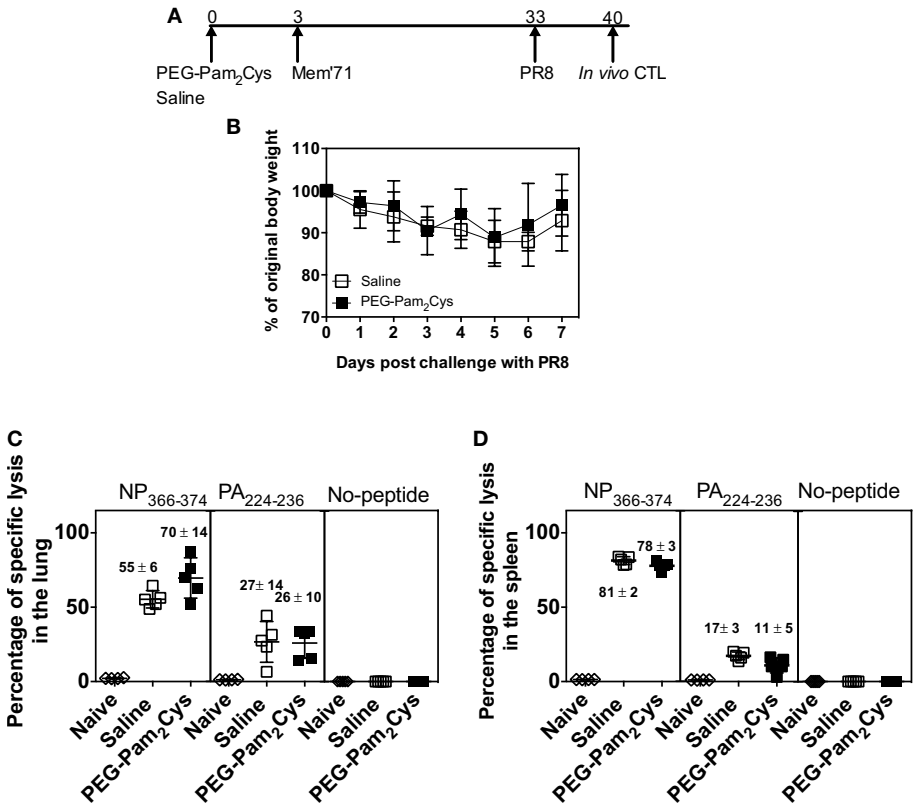


**FIGURE 3 | Influenza-specific cytotoxic CD8$^+$ T-cells persist in the lung and the spleen of PEG-Pam$_2$Cys-treated mice**. **(A)** Time line of protocol used; C57BL/6 mice ($n = 5$) received saline or PEG-Pam$_2$Cys 3 days prior to challenge with $10^{4.5}$ PFU of Mem71 influenza virus. One month later, mice were challenged with a lethal dose of PR8. **(B)** Percentage weight change after secondary influenza challenge. Seven days after challenge with PR8 naïve "donor" splenic cells were differentially labeled with CFSE and pulsed with no peptide, peptide NP$_{366–374}$, or peptide PA$_{224–236}$ before intravenous transfer via the base of tail into recipient mice. Recipient mice were killed and remaining labeled donor cells in the lungs and spleens enumerated using flow cytometry. The percentage of specific lysis observed in the lung **(C)** and spleen **(D)** are shown. Each symbol in **(C,D)** represents the percentage of specific lysis obtained by individuals and the vertical line indicates the mean of each group. Numbers above each group indicate the mean amount of specific lysis of each groups with the SD. Data are from one of the two independent experiments, which yielded similar results.

T-cell responses using an *in vivo* CTL assay. Following treatment with PEG-Pam$_2$Cys or saline and subsequent challenge with Mem'71 (H3N2) virus, mice were then challenged 4 weeks later with a lethal dose of the heterologous PR8 (H1N1) virus (**Figure 3A**). The results (**Figure 3B**) demonstrate that both groups were protected from lethal PR8 challenge, which typically causes 20% weight loss by day 7 (**Figure 2B**), indicating that treatment with Pam2Cys does not compromise the ability to elicit and maintain immunity against heterologous virus challenge.

Seven days after secondary infection splenocytes from naïve, "donor" mice were pulsed with either PA$_{224–236}$ peptide, NP$_{366–374}$ peptide or received no treatment. The cells were then differentially labeled with different concentrations of CFSE and injected intravenously via the base of tail into recipient mice. After 14 h, labeled cells present in lungs and spleen were enumerated by flow cytometry and the gating strategy is shown in Figure S2 in Supplementary Material. The difference in the number of CFSE-labeled cells in infected mice compared to uninfected mice revealed that the CD8$^+$ T-cell response generated in mice pretreated with PEG-Pam$_2$Cys or saline were equally effective at killing donor cells (**Figures 3C,D**). The results clearly demonstrate that prophylaxis with PEG-Pam$_2$Cys did not compromise the function or quality of the CD8$^+$ T-cell response generated. The results of the experiments further demonstrate that the immunostimulatory effects of PEG-Pam$_2$Cys do not affect the cytotoxic capabilities of T-cells responsible for influenza-specific immunity.

To further characterize the CD8$^+$ T-cell response, the cellular cytokine profiles were examined by ICS (**Figure 4A**) and the gating strategy is shown in Figure S3 in Supplementary Material. There were no significant differences in the numbers of PA$_{224–236}$ or NP$_{366–374}$-specific T-cells capable of secreting a combination of cytokines in the lungs and spleens of saline and PEG-Pam$_2$Cys treatment groups (**Figures 4B–D**). These results confirm our earlier findings (1) that Pam2Cys does not hinder development of influenza-specific immune responses. We now show that the influenza-specific immune response can be recalled by secondary infection with a different influenza virus and that these cells possess cytolytic function and secrete a combination of cytokines associated with protection.

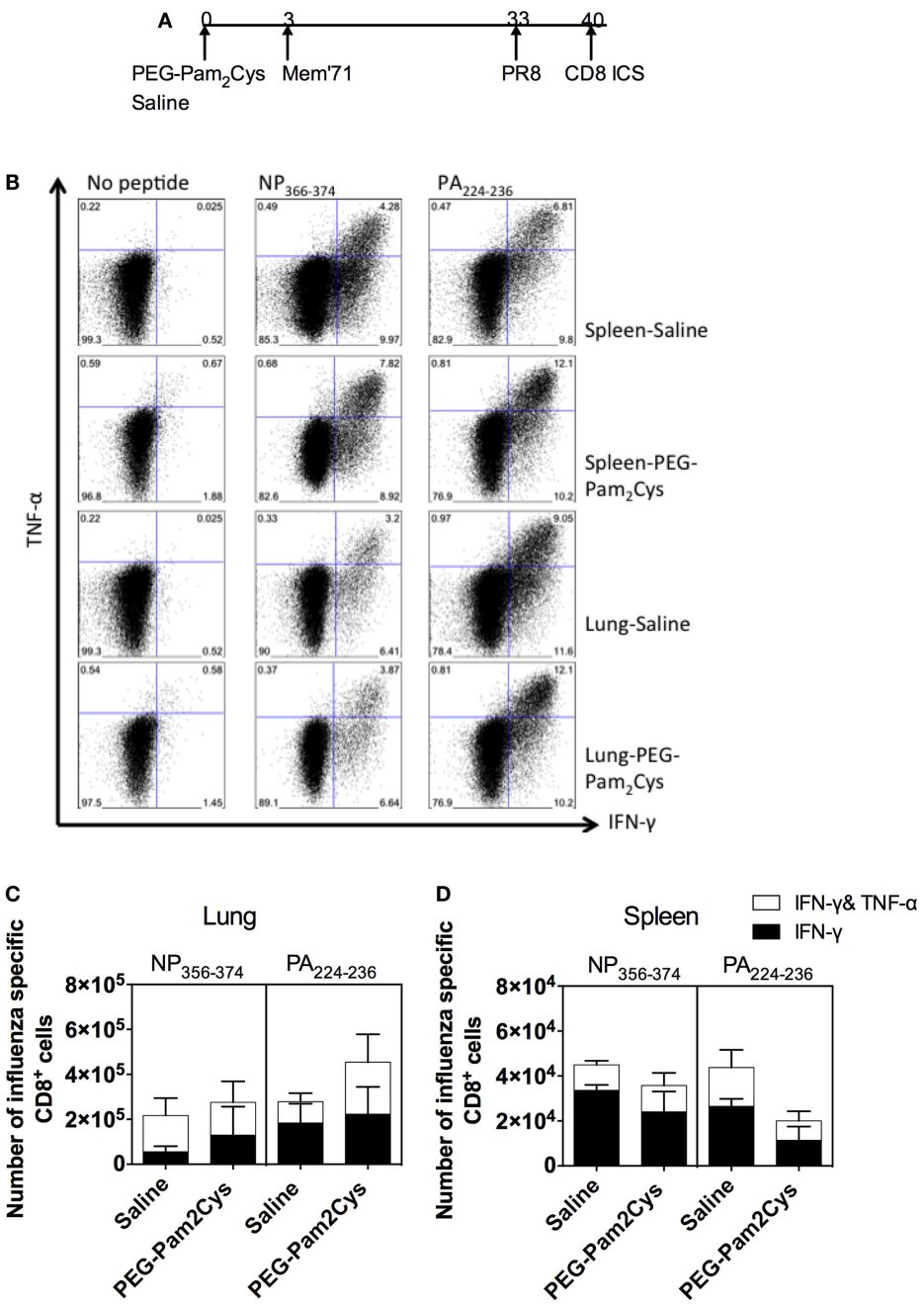


**FIGURE 4 | Influenza-specific CD8$^+$ T-cell responses persist in the spleen and lung following stimulation with PEG-Pam$_2$Cys.** **(A)** Timeline of protocol used; C57BL/6 mice ($n = 5$) received 20 nmol of PEG-Pam$_2$Cys or saline 3 days prior to challenge with $10^{4.5}$ PFU of Mem71. One month after primary challenge, mice were challenged with PR8 influenza virus, and 7 days later, an ICS assay was performed to examine the cytokine profile of influenza-specific CD8$^+$ T-cells that were generated. **(B)** Representative FACS plots show the percentage of CD8$^+$ T-cells from the spleen secreting either IFN-γ and/or TNF-α. Numbers of NP$_{366–374}$ or PA$_{224–236}$ specific CD8$^+$ T-cells secreting cytokines in lung **(C)** and spleen **(D)**. Results are expressed as the mean (±1 SD). Data are from one of the two independent experiments that yielded similar results.

## Pam$_2$Cys Treatment does not Alter Adaptive Immune Responses Generated in Immunologically Experienced Mice

Following vaccination or natural infection, human beings are no longer immunologically naïve. If immunostimulatory agents are to be used in human beings, we need to determine whether or not they affect existing antigen specific T-cells. Others (17, 18) have shown that subsequent and heterologous influenza virus infections cause an influx of CD8$^+$ T-cells into lungs. These infections, or more specifically the inflammation that they induce,

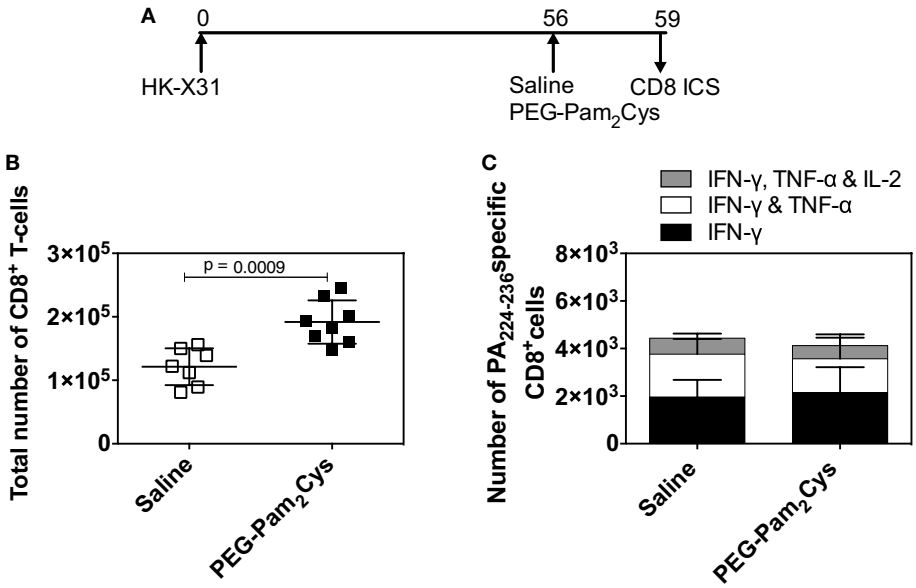


**FIGURE 5 | PEG-Pam$_2$Cys treatment alone does not activate memory CD8$^+$ T-cells**. **(A)** Timeline of protocol used; C57BL/6 mice were challenged with $10^{4.5}$ PFU of X31 virus, and 2 months later, mice received either saline or 20 nmol of PEG-Pam$_2$Cys. After 3 days, mice were euthanized and the CD8$^+$ T-cell responses assessed. **(B)** Total number of CD8$^+$ T-cells in the lung. **(C)** Numbers of PA$_{224–236}$ specific CD8$^+$ T-cells secreting IFN-γ, IFN-γ, and TNF-α or IFN-γ, TNF-α, and IL-2 in the lung. Results are expressed as the mean (±1 SD). Statistical significance is denoted * on the graph and was determined using an unpaired Student's *t*-test ($P < 0.05$).

can lead to the recruitment of cells into the lung (19). What we have previously observed following treatment of immunologically naïve animals with PEG-Pam$_2$Cys (1) is an increase in the numbers of CD8$^+$ T-cells. We therefore determined whether or not PEG-Pam$_2$Cys delivered intanasally affected resident CD8$^+$ T-cells elicited by previous infection. Immunologically experienced mice were generated by challenge with a non-lethal dose of X31 influenza virus and 2 months later, mice were treated with saline or PEG-Pam$_2$Cys (**Figure 5A**). We observed an increase in the number of CD8$^+$ T-cells in lungs of mice treated with PEG-Pam$_2$Cys (**Figure 5B**) supporting our earlier observations (1) but did not observe significant differences in the number and cytokine profiles of PA$_{224–236}$ specific CD8$^+$ T-cells (**Figure 5C**) suggesting that Pam$_2$Cys treatment does not activate memory CD8$^+$ T-cells.

We next determined whether PEG-Pam$_2$Cys altered the ability of mice to recall a previous immune state. Using the treatment regime shown in **Figure 6A,** the PA$_{224–236}$ specific CD8$^+$ T-cell response was examined 7 days after challenge with PR8 virus. Comparable numbers of CD8$^+$ T-cells secreting IFN-γ alone, IFN-γ plus TNF-α, or IFN-γ plus TNF-α plus IL-2 were detected in the lungs of mice (**Figure 6B**). Taken together, the data suggest that stimulation of the innate immune system with PEG-Pam$_2$Cys does not impact secondary recall responses.

## Discussion

In this study, we have shown that PEG-Pam$_2$Cys treatment provides the host with immediate defense against influenza by reducing viral burden, eliminating disease symptoms, and also allows the generation of adaptive immune responses that are quantitatively and qualitatively similar to those generated during natural infection.

The cellular and humoral arms of the adaptive immune system each combat influenza virus infection and both mitigate disease severity. It is therefore necessary that immunomodulatory agents developed to target influenza should not hinder the development of either arm of the adaptive immune response. Even though PEG-Pam$_2$Cys treatment causes a dramatic reduction (~90%) in antigen load compared with treatment with saline, similar numbers of CD8$^+$ T-cells were elicited. This is unexpected given previous findings (20–22) that decreased antigen loads have profound effects on resulting T-cell responses. Possible explanations for this are the enhanced proliferation of CD8$^+$ T-cells, due to their expression of TLR-2, following stimulation with TLR-2 ligands even in the absence of co-stimulation by APCs (23). Direct activation of TLR-2 has also been shown to reduce the amount of antigen required for CD8$^+$ T-cell activation even promoting proliferation of CD8$^+$ T-cells with low TCR and MHC affinity (24). It seems then that TLR-2-mediated stimulation of CD8$^+$ T-cells decreases or even obviates the need for co-stimulation by APC improving the chances for successful CD8$^+$ T-cell responses even in the presence of reduced antigen and low affinity TCR. Secondly, inflammation has been shown by many groups to play a crucial role in the contraction phase and development of memory CD8$^+$ T-cell responses (25–27). The inflammatory milieu induced by the pathogen has been found to be essential for maximal CD8$^+$ T-cell expansion and is also crucial for the development of effector functions such as cytolysis (28). Furthermore, Richer and colleagues (29) have shown that inflammatory cytokines reduce

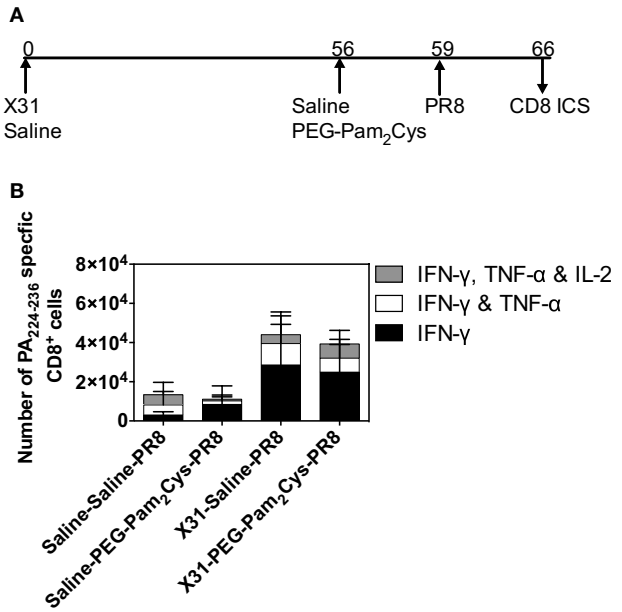


**FIGURE 6 | Pre-existing adaptive immune responses are not compromised by stimulation with $Pam_2Cys$. (A)** Timeline of infection protocol used; C57BL/6 mice ($n = 5$) were challenged with $10^{4.5}$ PFU of ×31 virus, and 2 months later, mice received either saline or 20 nmol of PEG-$Pam_2$Cys 3 days prior to challenge with a lethal dose of PR8 virus. Mice were monitored daily following challenge and the $PA_{224–236}$ specific $CD8^+$ T-cells secreting cytokine in the lung are shown in **(B)**. Results are expressed as the mean (±1 SD).

antigen sensitivity in both effector and memory $CD8^+$ T-cell responses.

The development of prophylactic agents that augment the host's innate immune system could considerably decrease the morbidity and mortality rates that are associated with influenza pandemics for which no vaccines are available or in those cases where available vaccines are ineffective, e.g., during the 2009 H1N1 influenza pandemic where the only seasonal influenza vaccine that was available failed to induce immune responses capable of protecting individuals against the emergent strain (30). Intranasal administration of PEG-$Pam_2$Cys at such times could provide the population with immediate protection and reduce transmission of virus (1). As we show in this study, individuals treated with $Pam_2$Cys and subsequently challenged with virus would develop influenza-specific adaptive immune responses providing long-term protection and removing the need for rapid vaccine production. Another feature of PEG-$Pam_2$Cys as an immunostimulatory agent is that it has the potential to be self-administered reducing the impact placed on medical staff during pandemics.

We have shown that the TLR-2 agonist PEG-$Pam_2$Cys provides mice with immediate protection against influenza virus and does not impact the induction of influenza-specific immunity following subsequent exposure to virus, which provides both homotypic and heterosubtypic protection. The data generated in this study encourages the development of immunostimulatory agents and could also alter our perception of the role that these anti-microbial agents play in long-term immunity to respiratory infections. In the absence of an effective vaccine, the use of $Pam_2$Cys can immediately reduce the impact of infectious agents and provide an individual with long-lasting immunity through natural infection.

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## Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00290/abstract

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# Hemagglutinin sequence conservation guided stem immunogen design from influenza A H3 subtype

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Seasonal epidemics caused by influenza A (H1 and H3 subtypes) and B viruses are a major global health threat. The traditional, trivalent influenza vaccines have limited efficacy because of rapid antigenic evolution of the circulating viruses. This antigenic variability mediates viral escape from the host immune responses, necessitating annual vaccine updates. Influenza vaccines elicit a protective antibody response, primarily targeting the viral surface glycoprotein hemagglutinin (HA). However, the predominant humoral response is against the hypervariable head domain of HA, thereby restricting the breadth of protection. In contrast, the conserved, subdominant stem domain of HA is a potential "universal" vaccine candidate. We designed an HA stem-fragment immunogen from the 1968 pandemic H3N2 strain (A/Hong Kong/1/68) guided by a comprehensive H3 HA sequence conservation analysis. The biophysical properties of the designed immunogen were further improved by C-terminal fusion of a trimerization motif, "isoleucine-zipper", or "foldon". These immunogens elicited cross-reactive, antiviral antibodies and conferred partial protection against a lethal, homologous HK68 virus challenge *in vivo*. Furthermore, bacterial expression of these immunogens is economical and facilitates rapid scale-up.

**Keywords: protein minimization, hemagglutinin stalk, subunit vaccine, pre-fusion conformation, antibody response, *Escherichia coli***

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## Introduction

Influenza (flu) virus infection causes respiratory illness in humans. Preventive vaccination is the best way of controlling influenza infections (1). Antiviral medications such as oseltamivir, zanamivir, and peramivir are used to treat influenza infections (2, 3). Additionally, the application of human monoclonal antibodies in therapeutic treatment of influenza infections is also being explored (4–7).

The rapidly evolving influenza viruses are diverse and have been categorized into three immunological types: A, B, and C. The influenza A viruses are further classified on the basis of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), into 18 HA and 11 NA subtypes (8). H17 and H18 HAs are putative HA-like molecules, since their hemagglutination activity has not been established. NA activity of N10 and N11 NAs has also not been shown. Antigenic relatedness within HA facilitates clustering influenza A viruses into two major phylogenetic groups: group 1 (subtypes: H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2

(subtypes: H3, H4, H7, H10, H14, and H15) (8). Currently, only influenza A (H1 and H3 subtypes) and B viruses cause seasonal epidemics in humans (9). However, the perceived threat of highly pathogenic avian influenza viruses (H5N1) and new reports of influenza strains (H7N9, H6N1, and H10N8) crossing over the species barrier and infecting humans (10–13) necessitate the development of a "universal" influenza vaccine.

Currently, there are two variants of influenza vaccine: inactivated influenza vaccine (IIV) and live attenuated influenza vaccine (LAIV). The efficacy of IIV and LAIV in children and adults has been extensively evaluated (14, 15). All commercially available influenza vaccines are manufactured by propagation of virus in embryonated chicken eggs with a production time of 6–8 months, except the trivalent recombinant influenza vaccine [RIV3] (FluBlok, Protein Sciences) and cell culture-based IIV [ccIIV3] (Flucelvax, Novartis)[1]. Therefore, manufacturing large amounts of vaccine at short notice during an epidemic/pandemic is difficult. Furthermore, preparedness against influenza infection is compromised due to the lack of a foolproof method for the annual selection of vaccine strains (16).

Hemagglutinin is highly immunogenic and its efficacy as a stand-alone vaccine candidate has been extensively investigated (1, 17). The precursor polypeptide (HA0) oligomerizes in the endoplasmic reticulum (ER) and is transported to the cell surface via the Golgi apparatus (18). HA0 is subsequently cleaved by cellular proteases into the disulfide-linked, fusion competent HA1 and HA2 subunits (19). The immunodominant, membrane distal, globular head domain of HA containing the receptor-binding site (RBS) is assembled exclusively by the HA1 subunit, while the viral membrane proximal, stem domain of HA is comprised primarily of the HA2 subunit (20).

A comprehensive analysis of H3 HA sequences revealed a high degree of conservation within the stem domain as opposed to the variable head domain, in agreement with published results (21, 22). The degree of conservation is inversely correlated with antigenic distance between influenza strains. As shown previously, the degree of overall residue conservation in HA within a subtype is significantly greater than group-specific residue conservation (1, 21), and therefore eliciting pan-influenza neutralizing antibodies has remained elusive. The dominant antibody response post-vaccination is against the variable head domain of HA, thereby limiting vaccine efficacy. However, isolation of several broadly neutralizing antibodies (bnAbs) targeting conserved epitopes in the HA stem (4, 5, 23–27) has facilitated efforts in developing stem-based vaccine candidates with the potential to confer hetero-subtypic protection (1). Nonetheless, it has been extremely challenging to activate stem-directed bnAbs in humans because of their low frequency in the influenza-specific memory B-cell pool (21). The metastable conformation of the HA2 subunit in the pre-fusion state of HA further compounds the difficulty in exploiting the conserved epitopes of the HA stem in developing a "universal" vaccine. Diverse strategies have been adopted to express the HA stem in the pre-fusion conformation (28–32). We have previously demonstrated the soluble expression of HA stem-fragments in *Escherichia coli* (*E. coli*) by maintaining the interaction network within the HA stem and introducing designed mutations. These immunogens conferred robust subtype-specific and modest hetero-subtypic protection *in vivo* against influenza A group 1 viruses (32).

Structural analysis of the HA stem reveals differences at the N-terminus of the long alpha helix (LAH) and the composition of ionizable residues proximal to the fusion peptide between influenza A phylogenetic groups 1 and 2 (33). In order to mitigate the threat of circulating influenza A viruses from these distinct structural classes (H1 from group 1 and H3 from group 2), we characterized an HA stem-fragment immunogen (H3HA10) from the H3N2 strain (A/Hong Kong/1/68), which caused the "1968 influenza pandemic." We evaluated the effect of trimerization motifs, the coiled-coil isoleucine zipper (IZ) (34) and the globular, β-rich "foldon" (35), belonging to disparate structural classes as a C-terminal fusion to H3HA10. The oligomeric derivatives of H3HA10 had improved biophysical properties and elicited cross-reactive, antiviral antibodies in mice. The elicited antibodies inhibited the entry of a heterologous H3 HA pseudotyped virus *in vitro*. These immunogens conferred partial protection against a lethal, homologous HK68 virus challenge *in vivo*. Additionally, bacterial expression of these immunogens is cost-effective and enables rapid production.

## Materials and Methods

### Sequence Analysis

All non-identical, full-length H3 HA protein sequences (3169 sequences) reported from human hosts were retrieved from the NCBI-Flu Database[2]. These H3 HA sequences were subsequently clustered at 99% homology using Cluster Database at High Identity with Tolerance (CD-HIT) (36) to filter-out 392 unique, representative sequences, which were then multiply aligned using CLUSTAL (37). The alignment file lists a quality score for every position in the protein sequence, which is a measure of residue conservation. The quality scores were then binned and mapped onto the crystal structure of H3N2 A/Hong Kong/1/68 HA [protein data bank (PDB) ID: 1HGD (38)].

### Protein Expression and Purification

The *E. coli* codon-optimized gene sequence of our designed construct H3HA10 was synthesized with a stop codon at the 3' end (GenScript, USA). The gene was cloned into the expression vector pET-28a (+) (Novagen) in-frame with the N-terminal His-tag between the *Nde*I and *Bam*HI restriction sites. The *E. coli* codon-optimized gene sequences encoding IZ and foldon were individually synthesized (Abexome, India) with *Kpn*I and *Hin*dIII restriction sites at the 5' and 3' ends respectively. In order to facilitate the cloning of a trimerization motif at the C-terminus of H3HA10 to generate H3HA10-IZ and H3HA10-Foldon, the stop codon in H3HA10 at the 3' end was mutated and a unique *Kpn*I restriction site was generated using site-directed mutagenesis. All the generated clones were confirmed by sequencing (Macrogen, South Korea).

[1]http://www.cdc.gov/

[2]http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database

The designed proteins were expressed in *E. coli* BL21(DE3) cells. H3HA10, H3HA10-IZ, and H3HA10-Foldon were all purified using a similar protocol from the soluble fraction of the cell culture lysate. Briefly, a single transformed colony of *E. coli* BL21(DE3) cells was inoculated into 50 ml of Luria-Bertani broth (HiMedia). The primary culture (50 ml) was grown overnight until saturation at 37°C. Subsequently, 2 L of Luria-Bertani broth (500 ml × 4) was inoculated with 1% of the saturated primary inoculum and grown at 37°C until an $OD_{600}$ of ~0.6–0.8 was reached. The cultures were then induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The cells were grown for another 12–16 h at 20°C post-induction. Next, the culture was spun down at 5000 × *g* for 15 min at 4°C. The pelleted cells were resuspended in 100 ml of phosphate-buffered saline (PBS, pH 7.4). The cell suspension was lysed by sonication and subsequently centrifuged at 14,000 × *g* for 45 min at 4°C. The supernatant from the cell culture lysate was incubated with buffer-equilibrated Ni-NTA resin (GE HealthCare) for 2–3 h at 4°C to facilitate binding. The protein was eluted in 2 ml fractions using an imidazole gradient (in PBS, pH 7.4). The eluted fractions were analyzed by SDS-PAGE and pooled for dialysis against PBS (pH 7.4) containing 1 mM EDTA. The dialyzed protein was concentrated to a final concentration of ~5 mg/ml and its identity was confirmed by electrospray ionization-mass spectroscopy (ESI-MS).

## Fluorescence Spectroscopy

The intrinsic fluorescence measurements for all proteins were recorded at 25°C on a Jasco FP-6300 spectrofluorometer. The protein samples (concentration of 1–3 μM) were excited at a wavelength of 280 nm, and emission was monitored between 300 and 400 nm. The spectrofluorometer slit widths for excitation and emission were set at 3 and 5 nm, respectively. The represented fluorescence signals were averaged over five consecutive scans and corrected for buffer signals. The fluorescence signal of the native protein was recorded in PBS (pH 7.4). The protein was denatured in 7M guanidine hydrochloride (GdmCl) to record the fluorescence signal in the unfolded state.

## NMR Spectroscopy

One-dimensional $^1$H NMR spectra of all the protein samples were recorded at 25°C on an Agilent 600 MHz NMR spectrometer. The spectra were recorded using a triple resonance cryo-probe fitted with a *z*-axis only pulsed field gradient accessory. External DSS was used for referencing $^1$H chemical shifts. A spectral width of 9615.4 Hz was sampled. The excitation sculpting pulse scheme was used to achieve solvent suppression (39). All the protein samples were prepared in PBS (pH 7.4) (90% $H_2O$:10% $D_2O$). A total of 1024 scans were recorded with a 1 s relaxation delay.

## Gel Filtration Chromatography

The purified proteins were analyzed by gel filtration chromatography on a Superdex-200 analytical gel filtration column (GE HealthCare) equilibrated with PBS (pH 7.4) to determine their oligomeric state under native conditions. The column was calibrated using a broad range of molecular weight markers as indicated (GE HealthCare).

## Antibody Binding Studies Using Surface Plasmon Resonance

Binding affinity of the purified proteins (H3HA10, H3HA10-IZ, and H3HA10-Foldon) and full-length recombinant HAs (rHAs) (H3N2 A/Aichi/2/68 and H1N1 A/Puerto Rico/8/34) (Sino Biological Inc., Beijing, China) to the single-chain variable fragment (scFv) of the stem-directed bnAb FI6v3 was determined by surface plasmon resonance (SPR) experiments performed with a Biacore2000 optical biosensor (Biacore, Uppsala, Sweden) at 25°C. Recombinant FI6v3-scFv was produced as described previously (32). Amine coupling chemistry was used to immobilize 750 response units (RU) of the ligand (FI6v3-scFv) to the surface of a CM5 chip (GE HealthCare, Uppsala, Sweden). A sensor channel immobilized with ovalbumin served as a negative-control for each binding interaction. A concentration series of each analyte were passed over the ligand(s) in a running buffer of PBS (pH 7.4) with 0.05% P20 surfactant at a flow rate of 30 μl/min to determine the binding kinetics. The sensor surface was regenerated with 2M $MgCl_2$ after every binding event. The kinetic data was obtained using the concentration of the monomer for H3HA10 and the concentration(s) of the trimer(s) for H3HA10-IZ, H3HA10-Foldon, and rHAs. The concentration used for fitting the kinetic data was in accordance with the oligomeric state of the protein as determined by gel filtration chromatography. The kinetic parameters of binding were obtained by a global fitting of the data to the simple 1:1 Langmuir interaction model using BIA EVALUATION 3.1 software.

## Immunization and Virus Challenge Studies

The female BALB/c mice (4–5 weeks old) (Taconic Farms, Inc., Germantown, NY, USA) used in this study were maintained at the animal facilities of Merck Research Laboratories. The animal study was approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Each group of mice ($n = 10$) were immunized intramuscularly with 20 μg of the test immunogen along with 100 μg CpG7909 adjuvant (TriLink BioTechnologies, San Diego, CA, USA) at days 0 (prime) and 28 (boost). Naïve mice were used as controls. Serum samples were obtained from all the mice 3 weeks after prime and 2 weeks after boost by tail venipuncture and collected in Microtainer serum separator tubes (BD Biosciences, Franklin Lakes, NJ, USA). About 3 weeks after the boost, mice were anesthetized with ketamine/xylazine and challenged intranasally with $2LD_{90}$ (lethal dose) of mouse-adapted H3N2 A/HK/1/68 virus in 20 μL of PBS. The mice were monitored for survival and weight change for 14 days post virus challenge.

## Determination of Serum Antibody Titers

The serum antibody-titers against test immunogens (H3HA10, H3HA10-IZ, and H3HA10-Foldon) were determined by ELISA. Briefly, 200 ng of the protein was coated on 96-well Nunc plates (Thermo Fisher Scientific, Rochester, NY, USA) and incubated overnight at 4°C. Next, the plates were washed with PBS containing 0.05%Tween-20 (PBST) and blocked with 1% bovine serum albumin (BSA) (Sigma) in PBST (PBSB) for 1 h. Antiserum raised against the test immunogen was fourfold serially diluted in PBSB and added to each well. The plates were washed with PBST after

2 h of incubation with sera at room temperature. Fifty microliters of alkaline phosphatase (ALP)-conjugated goat anti-mouse secondary antibody (Sigma) used at a predetermined dilution of 1:10000 in PBSB were added to each well and incubated at room temperature for 2 h. After washing, the plates were developed using the chromogenic substrate p-nitrophenyl phosphate (Sigma) and read at 405 nm (SPECTRAmax Plus 384, Molecular Devices, USA). Antibody titer was determined as the reciprocal of the highest serum dilution that gave an $OD_{405}$ value above the mean + 2 × SD of control wells.

### Binding of Antisera to Full-Length Recombinant HAs

The binding of antibodies elicited by the test immunogens to a panel of full-length rHA proteins was determined by ELISA. Briefly, 200 ng of mammalian-cell expressed rHA proteins (H3N2 A/Aichi/2/68, H3N2 A/Brisbane/10/07, H7N7 A/chicken/Netherlands/1/03, H1N1 A/Puerto Rico/8/34, and H1N1 A/California/04/2009 from Sino Biological Inc., Beijing, China) were coated on 96-well Nunc plates (Thermo Fisher Scientific, Rochester, NY, USA) and incubated overnight at 4°C. Ovalbumin (200 ng/well) coated wells were used as a control for non-specific binding. Plates were washed with PBST and subsequently blocked with PBSB. The rHA proteins were then incubated for 2 h with a fourfold serial dilution of the antisera (starting at a dilution of 1:100). The plates were then washed with PBST and incubated for 2 h at room temperature with ALP-conjugated goat anti-mouse secondary antibody (Sigma) used at a dilution of 1:10000. After another round of washing, the plates were developed with the chromogenic substrate p-nitrophenyl phosphate (Sigma). The antibody titer against rHA proteins was determined as the reciprocal of the highest serum dilution that gave an $OD_{405}$ (SPECTRAmax Plus 384, Molecular Devices, USA) value above the mean + 2 × SD of control wells.

### Pseudotyped Virus Particle Entry Inhibition Assay

The antisera raised against the designed immunogens were tested in a pseudotype virus particle entry inhibition assay as described previously (40, 41). Briefly, HIV gag-pol plasmid p8.91, firefly luciferase expressing plasmid pCSFLW, H3 HA (A/Wisconsin/67/2005) expressing plasmid, NA expressing plasmid (A/Udorn/307/1972 N2), and pCAGGS-HAT (42) expressing plasmid were co-transfected into human embryonic kidney (HEK 293T/17) cells using Fugene-6 transfection reagent (Promega) and incubated for 24 h. The supernatant containing the released pseudotypes was harvested 48 h post-infection, filtered through a 0.45 µm filter, and stored at −80°C until further use.

Serial dilutions of the antisera were incubated with $2 \times 10^7$ relative luminescence units (RLUs) of pseudotypes/well in 96-well flat-bottomed white plates (Nunc) in a final volume of 50 µl at 37°C for 1 h. After the incubation, $1.5 \times 10^4$ HEK293T cells were added to each well. The plates were subsequently incubated for another 48 h at 37°C. The luminescence signals were assayed using the Bright-Glo assay system (Promega). The half-maximal inhibitory concentrations ($IC_{50}$) of entry inhibition were determined using Prism v6.0 (GraphPad Software).

### Statistical Analysis

The differences in antibody/entry inhibition titers were analyzed by analysis of variance and Student's *t*-test. The fractional body weight of mice was calculated relative to their initial body weight. Differences in the mean fractional body weight of surviving mice were analyzed using analysis of variance and Student's *t*-test. Kaplan–Meier survival analysis with the log rank significance test was used to analyze the difference in survival across groups.

## Results

### Design of HA Stem-Fragment Immunogens

Current influenza vaccines elicit a robust strain-specific antibody response which neutralizes the virus and confers protection (1). The primary response is against the immunodominant antigenic sites in the head domain of HA (43). However, the virus has evolved a mechanism of "antigenic drift" wherein mutations accumulated in these antigenic sites facilitate escape from the host immune pressure. Immune selection pressures coerce influenza virus into presenting a continually "moving target". Therefore, we analyzed a large dataset of full-length H3 HA sequences (3169 sequences) reported from human hosts to identify conserved regions on HA. In agreement with previous results (1, 21, 22), the HA stem is on average more conserved than the variable head domain within a subtype (**Figure 1A**). However, there are pockets of high conservation even within the head domain of HA and monoclonal antibodies binding within the RBS have been isolated that neutralize drift variants of a HA subtype (6). The conservation within the HA stem has been ascribed to the less-than-optimal accessibility on the virion surface and the functional restraint imposed by conformational changes in the stem domain that are essential for infection. However, recent cryoEM studies of H1N1 A/California/7/2009 virus pre-incubated with the stem-directed bnAb C179 demonstrated that ~75% of HA trimers on the virion surface were in complex with the antibody (44). The study demonstrated that antibody binding to the HA stem on the virion is not severely impeded by accessibility.

A large fraction (≥90%) of the epitopes identified by the human B-cell population is conformation sensitive (45). In fact, extensive conformational rearrangement of the HA stem at low pH disrupts the epitope of HA stem-directed bnAbs. We analyzed the interaction network within H3 HA [H3N2 A/Hong Kong/1/68, PDB ID: 1HGD (38)] using PREDBURASA, as described previously (46), to identify HA stem fragments defined by stable structural breakpoints. During HA protein minimization, we performed iterative calculations to identify residue fragments: $19_1$-$46_1$, $290_1$-$321_1$, and $44_2$-$113_2$ (included in H3HA10) within the stem domain having minimalistic interactions with the rest of HA (**Figure 1B**). Residues from HA1 and HA2 subunits are distinguished by subscripts 1 or 2 respectively. The termini of HA stem-fragments in H3HA10 also had optimal Cα-Cα distances for "molecular stitching" as indicated in **Figure 1B** from our analysis of the Cα-Cα distance matrix of H3 HA. These fragments were connected with flexible, soluble linkers as described previously (47). The loss of native contacts in H3HA10 as a consequence of protein minimization exposes previously buried hydrophobic patches which can potentially aggregate. We re-surfaced these hydrophobic patches

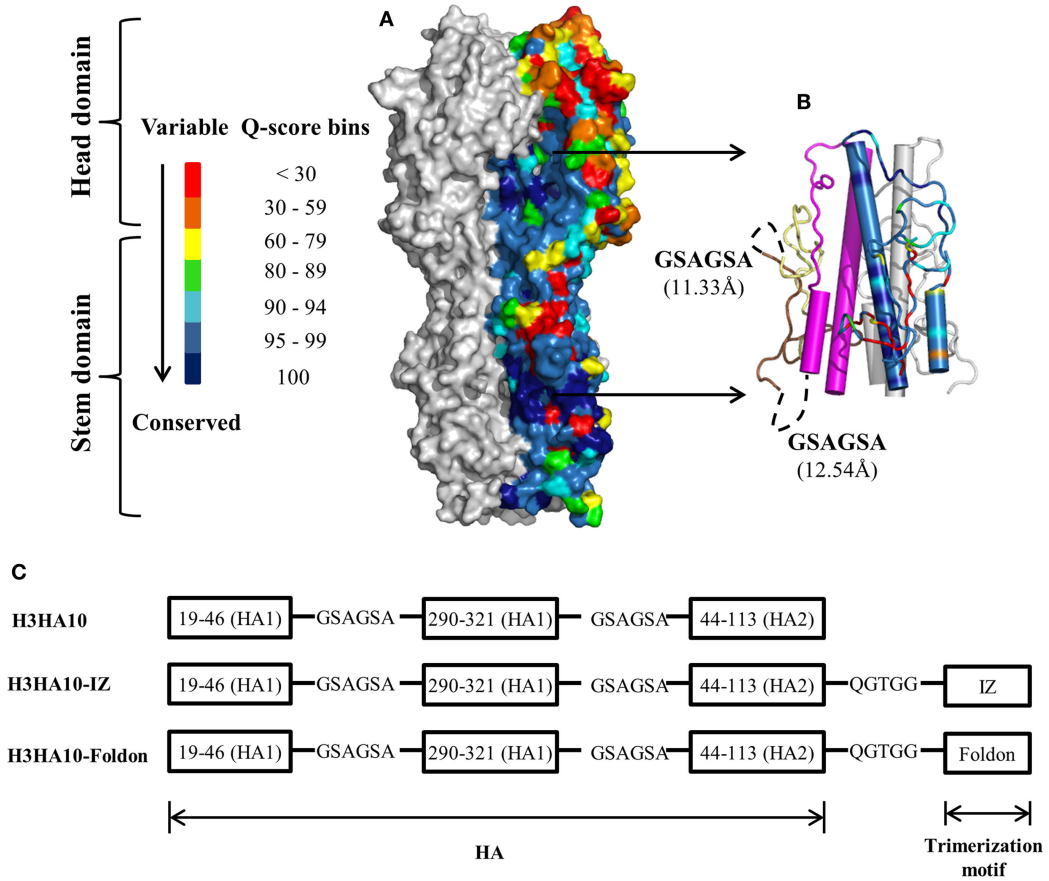


**FIGURE 1 | HA sequence conservation guided immunogen design.** **(A)** The residue conservation among H3 HA human isolates is mapped onto a surface representation of H3N2 A/Hong Kong/1/68 HA trimer [PDB ID: 1HGD (38)]. The quality score (Q-score) at every position in the protein sequence, which is a measure of residue conservation, was obtained from a multiple sequence alignment of H3 HA sequences ($n = 3169$) and binned. The HA stem is well conserved within a subtype. One monomer is colored according to the Q-score scale. Rest of HA (gray). **(B)** Conserved HA stem-fragments are "stitched" together in H3HA10 (cartoon). One monomer is colored according to the Q-score scale [indicated in **(A)**] to highlight the residue conservation in the designed immunogen. Stable structural breakpoints with optimal Cα–Cα distances (shown in another monomer) were determined by analysis of the H3 HA distance matrix. Soluble, flexible linker as indicated (black, dashed curve) was used to connect the HA1-subunit fragment $19_1$-$46_1$ (pale yellow) to $290_1$-$321_1$ (brown). A 6-residue linker (GSAGSA) connects the HA1 (brown) and HA2 (magenta) subunits in H3HA10. The third monomer is in gray. **(C)** Derivative(s) of H3HA10 were made with C-terminal trimerization motif(s), IZ, and foldon (34, 35). The figures **(A,B)** were rendered using PyMOL.

with polar amino acid substitutions as described previously (32). We have previously designed stable influenza and HIV-1 immunogens using a similar approach (28, 30, 32, 48). The following mutations were introduced in H3HA10 to resurface the hydrophobic patches: $V20_1S$, $V297_1T$, $I300_1R$, $Y302_1S$, $M320_1Q$, and $I45_2T$. In the full-length H3 HA, $Cys281_1$ and $Cys305_1$ form an intramolecular disulfide bond. Since residue $Cys281_1$ was not incorporated in H3HA10, we mutated $Cys305_1$ to Ser to prevent the formation of incorrect, intermolecular disulfide bonds in the absence of its cognate partner ($Cys281_1$). Aspartate mutations ($F63_2D$ and $V73_2D$), previously shown to destabilize the low-pH conformation of HA (28) were also incorporated in H3HA10. We have previously shown that synthetic trimerization motifs promote the oligomerization of HA stem in the absence of the trans-membrane (TM) domain (32). We made derivatives of H3HA10 with the coiled-coil IZ (H3HA10-IZ) and the globular, β-rich "foldon" (H3HA10-Foldon) (**Figure 1C**). Figure S1 in Supplementary Material lists the sequences of all the designed constructs.

## Protein Purification and Characterization

Recombinant protein expression in prokaryotic systems is economical and amenable for rapid production. However, prokaryotic expression of heterologous viral proteins in native-like conformation has been challenging. Human pathogenic viruses hijack the host protein machinery for synthesis and undergo post-translational modifications (PTMs). Influenza proteins expressed in *E. coli* lack PTMs and can potentially aggregate. Previous efforts at bacterial expression of HA resulted in inclusion bodies and entailed refolding (28, 30, 49). However, in this study, all of our designed immunogens were purified from the soluble fraction of the *E. coli* cell culture lysate. We obtained modest protein yields of ~10-15 mg/l of the culture, using unoptimized shake-flask cultures. We could achieve ≥95% purity as assayed by SDS-PAGE using a single affinity-purification step (**Figure 2A**). We did not observe any higher order impurities. The purity of the protein was also confirmed using ESI-MS over a mass range of 10–200 kDa.

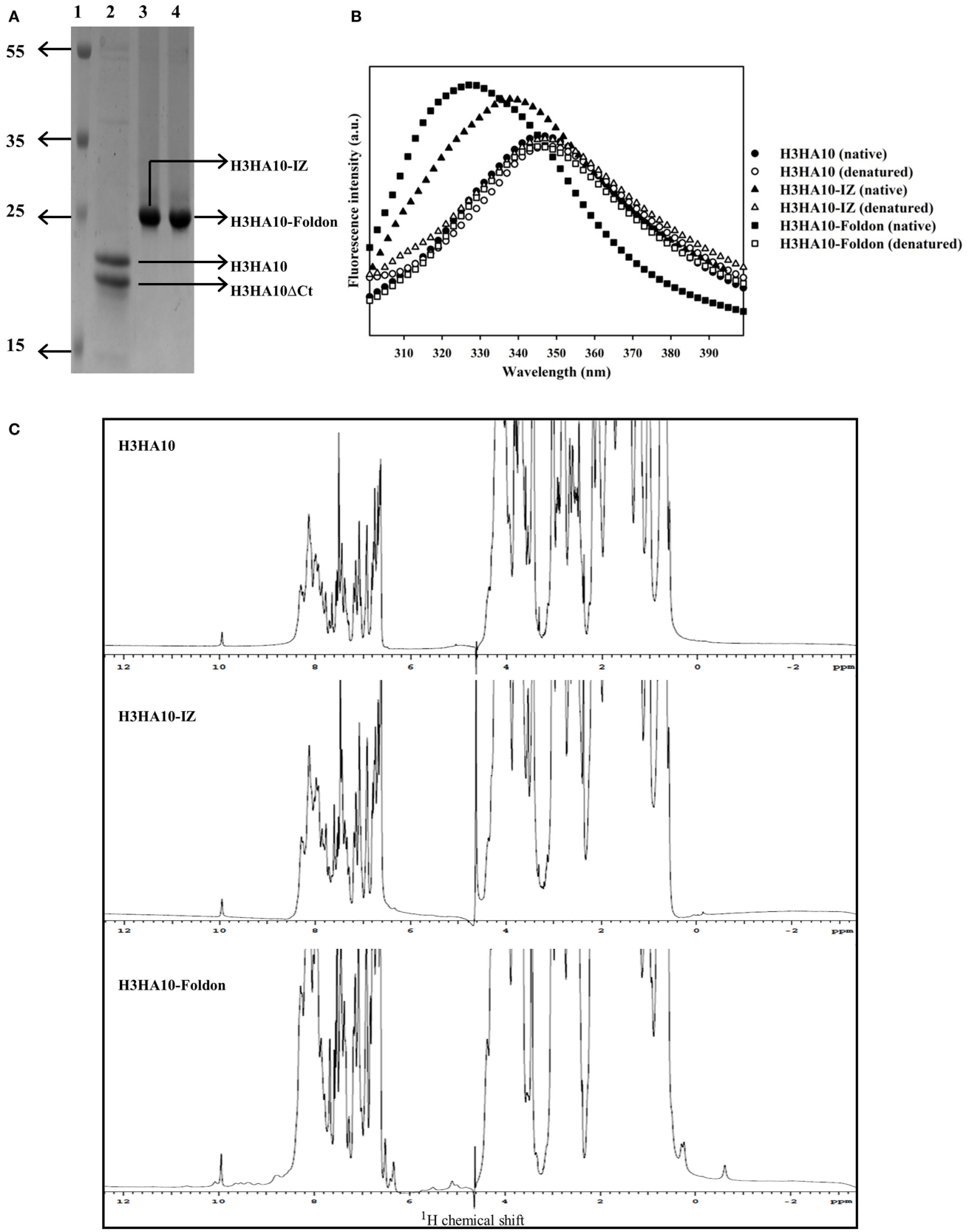


**FIGURE 2 | Protein purification and biophysical characterization of HA stem-fragment immunogens**.

*(Continued)*

**FIGURE 2 | Continued**
**(A)** SDS-PAGE profile of the purified proteins. Lane 1: PageRuler Plus prestained protein ladder (Thermo Scientific), lane 2: H3HA10, lane 3: H3HA10-IZ, and lane 4: H3HA10-Foldon. All the designed proteins were purified from the soluble fraction of *E. coli* cell culture lysate. H3HA10 was partially degraded upon purification. A C-terminal cleavage of 1367.4 Da (determined by mass spectrometry) was observed (H3HA10ΔCt = 16335.9 Da). The derivative(s) of H3HA10 with C-terminal trimerization motif(s) were resistant to *in situ* protein degradation. The migration of the purified proteins in a SDS-PAGE is marginally retarded. The SDS-PAGE gel was stained with Coomassie. **(B)** Fluorescence emission spectra of HA stem-fragment immunogens were recorded under native (PBS, pH 7.4) or denaturing conditions (7M GdmCl in PBS, pH 7.4) as indicated. Unlike H3HA10, both H3HA10-IZ and H3HA10-Foldon showed a significant red-shift in the emission maxima upon denaturation indicating a compact tertiary conformation. **(C)** 1D $^1$H NMR spectra of HA stem-fragment immunogens. The improved chemical shift dispersion in the upfield (0.5–1.0 ppm) and/or downfield (9–11 ppm) regions of the $^1$H NMR spectra of H3HA10-IZ and H3HA10-Foldon is consistent with the fluorescence data, indicating that trimerization motifs assist in the folding of the HA stem in the absence of the transmembrane (TM) domain, with H3HA10-Foldon appearing more structured than H3HA10-IZ.

Surprisingly, partial degradation of H3HA10 was observed upon purification. The addition of protease inhibitor cocktail tablet (cOmplete ULTRA Tablets, Roche) during purification did not prevent this. A C-terminal cleavage of 1367.4 Da (H3HA10ΔCt = 16335.9 Da) was confirmed by mass spectrometry. It has been previously shown that cellular proteases can degrade protein segments with extended conformations *in situ* (50). We hypothesized that C-terminal conjugation of H3HA10 with a synthetic trimerization motif might abate *in situ* protein degradation. Encouragingly, we observed that the addition of either IZ or foldon domains could completely curtail protein degradation *in situ* (**Figure 2A**).

The integrity of the protein hydrophobic core was probed by intrinsic fluorescence measurements. H3HA10 did not exhibit red-shift in the emission maximum upon denaturation with GdmCl, indicating an extended conformation which may explain the observed *in situ* protein degradation. In contrast, both H3HA10-IZ and H3HA10-Foldon showed significant red-shift in the emission maxima upon denaturation, indicating a compact tertiary conformation (**Figure 2B**). These results were further substantiated by the one-dimensional $^1$H-NMR spectrum of the designed immunogens. The C-terminal trimerization motifs assist the folding of H3HA10. Both H3HA10-IZ and H3HA10-Foldon have improved solution properties as inferred from resolved resonance lines in the upfield (0.5–1.0 ppm) and/or downfield (9–11 ppm) regions of the $^1$H-NMR spectrum (**Figure 2C**). The upfield shifted signals result from interactions between methyl protons that are spatially close to buried aromatic rings in the hydrophobic core.

The core of HA stem is assembled by three long, α-helical, parallel coiled-coils. The recapitulation of native HA contacts would promote the trimerization of HA stem mimetics. We determined the oligomeric state of the designed HA stem-fragment proteins using gel-filtration chromatography. The extended conformation of H3HA10 (inferred from fluorescence and $^1$H-NMR measurements) impedes accurate molecular weight estimation from size exclusion chromatography because of disproportionate retention along the column. However, the protein probably elutes as a monomer. The shoulder of the elution peak corresponds to H3HA10ΔCt. In contrast, H3HA10-IZ and H3HA10-Foldon predominantly elute as a homogenous oligomer (probably trimer) and do not form higher order aggregates (**Figures 3A,B**). This is consistent with previous reports which showed that trimerization motifs facilitate the oligomerization of ΔTM (transmembrane domain deleted) HA stem (32). The molecular weight (~72.5 kDa) of the oligomer estimated from gel-filtration is marginally higher, but close to the theoretical molecular weight of a trimer (~21.5 × 3 = 64.5 kDa). The discrepancy in the aforementioned molecular weight estimates of the oligomer arises

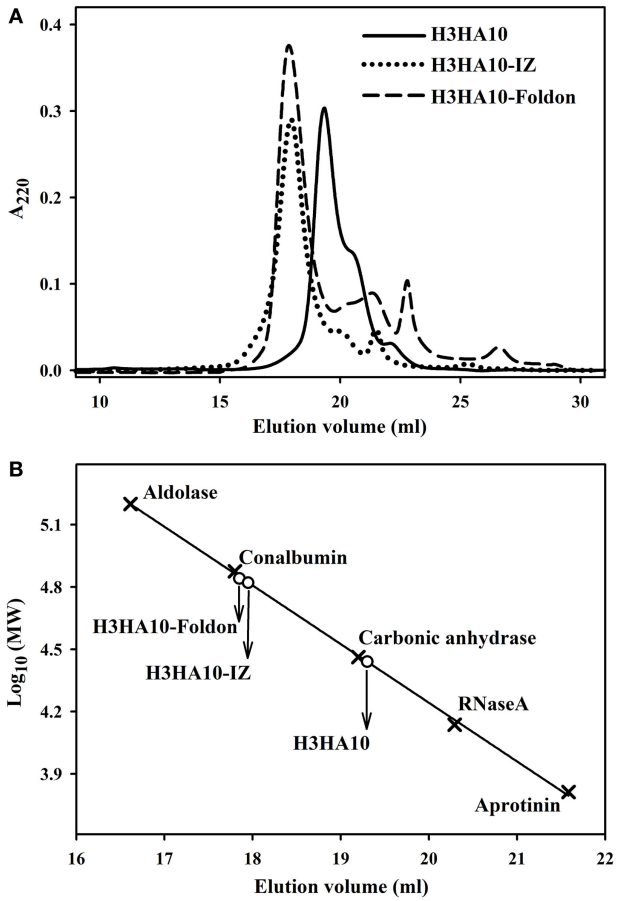


**FIGURE 3 | Oligomeric state of "headless" HA stem immunogens**. **(A)** Size-exclusion chromatography of the purified proteins was done at room temperature under non-denaturing conditions using a buffer (PBS, pH 7.4) equilibrated analytical Superdex-200 column. The disproportionate retention of H3HA10 because of an extended conformation (inferred from fluorescence and 1D $^1$H-NMR measurements) impedes accurate molecular weight estimation. The shoulder of the elution peak corresponds to H3HA10ΔCt. H3HA10-IZ and H3HA10-Foldon predominantly elute as a homogenous oligomer (probably trimer) and do not form higher order aggregates. **(B)** The column was calibrated using a broad range of molecular weight markers (x). The elution volume(s) of the designed protein(s) corresponding to $A_{220}$ signal maxima were plotted [open circles (○)] on the calibration curve to estimate the molecular weights.

**TABLE 1 | HA stem-fragment immunogens bind conformation specific bnAb.**

| Analyte | FI6v3-scFv[a] | | |
|---|---|---|---|
| | $k_{on}$ ($M^{-1}s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_D$ (nM) |
| H3HA10 | $8.11 \times 10^3$ | $3.41 \times 10^{-3}$ | $412.4 \pm 11.6$ |
| H3HA10-IZ | $1.01 \times 10^4$ | $9.03 \times 10^{-4}$ | $89.5 \pm 3.2$ |
| H3HA10-Foldon | $9.27 \times 10^3$ | $1.06 \times 10^{-3}$ | $114.3 \pm 6.8$ |
| H3 A/Aichi/2/68 rHA | $2.29 \times 10^4$ | $5.07 \times 10^{-4}$ | $22.1 \pm 2.3$ |
| H1 A/Puerto Rico/8/34 rHA | $1.93 \times 10^5$ | $2.22 \times 10^{-3}$ | $11.5 \pm 1.3$ |

[a] *750 RU of FI6v3-scFv was immobilized on the surface of a CM5-chip.*
*The kinetic parameters for binding were determined by surface plasmon resonance (SPR).*

possibly because the designed HA stem-fragment proteins are not globular.

The pan-influenza neutralizing antibody FI6 is selective in binding exclusively the pre-fusion conformation of HA. FI6 binds a conformation sensitive epitope in the HA stem that is disrupted by the structural re-arrangement of HA in the post-fusion conformation (25). Therefore, binding of the designed "headless" stem immunogens to FI6 is an infallible quality control of their conformation. The HA stem-fragment immunogen H3HA10 bound FI6v3-scFv with sub-micromolar affinity ($412.4 \pm 11.6$ nM) (**Table 1**; Figure S2 in Supplementary Material). On the other hand, full-length rHA (H3 A/Aichi/2/68) bound FI6v3-scFv with very high affinity ($22.1 \pm 2.3$ nM). There are several factors that could contribute to the weaker binding of H3HA10 to FI6v3-scFv. Primarily, the designed stem immunogen includes only a subset (~47%) of the FI6 epitope. Next, the aggregation state of H3HA10 in solution (monomer) could contribute to the slower $k_{on}$ and higher $k_{off}$ in comparison to the trimeric, full-length rHA (**Table 1**; Figures S2 in Supplementary Material). Accordingly, the oligomeric derivatives of H3HA10 had considerably tighter binding to FI6v3-scFv. H3HA10-IZ and H3HA10-Foldon had an equilibrium dissociation constant ($K_D$) of $89.5 \pm 3.2$ and $114.3 \pm 6.8$ nM, respectively (**Table 1**; Figures S2 in Supplementary Material).

## Characterization of Antigen-Specific Antibody Response

All the designed HA stem-fragment proteins were highly immunogenic in mice and elicited a robust antibody response with self-titers $\geq 1 \times 10^6$. The antibody titers against the conserved HA stem following a primary infection is lower than the titers elicited against the immunodominant, variable head domain. The predominant antibody response post-infection/vaccination is against the canonical antigenic sites in the globular head domain of HA (1). We assayed antibody binding to full-length rHAs to determine the breadth of antigen (Ag) elicited response. The homologous anti-HA titer was determined using H3N2 A/Aichi/2/68 HA that is nearly identical (99.6%) to H3N2 A/HK/1/68 HA. The anti-HK68 convalescent sera had a homologous anti-HA titer of $\sim 2.5 \times 10^4$, but extremely low/undetectable heterologous anti-HA titers suggesting a head-specific response. In contrast, the oligomeric stem-fragment immunogens elicited modest titers of cross-reactive, anti-HA antibodies, validating our design rationale (**Figures 4A–C**). We achieved a higher cross-reactive anti-HA titer by focusing the antibody response to the HA stem through successful engineering of the conserved HA stem-fragments (Table S1 in Supplementary Material).

The structural divergence in HA stem between influenza groups 1 and 2 establishes distinct group-specific antibody binding profiles. The HA stem directed bnAbs CR6261, F10, and C179 neutralize influenza A group 1 viruses exclusively (4, 24, 44). Binding of these bnAbs to group 2 HAs is abolished because of an N-linked glycan at residue $N38_1$ (HA1 subunit), which is highly conserved (6). The glycan shields the conserved eptiope on the HA stem, thereby abrogating the neutralization activity of these bnAbs against influenza A group 2 viruses. A "universal" influenza vaccine must breach this group-restricted antibody response. Extensive screening of over 13,000 monoclonal antibodies (over 90% were influenza-specific) from an individual donor led to isolation of the bnAb FI6 whose epitope overlaps with that of group 1 specific stem-directed bnAbs (25). Binding of the bnAb FI6 to H3 HA is enabled by reorientation of the N-linked glycan at residue $N38_1$ upon antibody approach (25). The proteins designed in this study were purified from *E. coli*, and hence lack PTMs like N-linked glycosylation. We therefore hypothesized that non-glycosylated HA stem immunogens mimicking the native, pre-fusion conformation of HA could elicit antibodies which bind HAs belonging to both groups 1 and 2. Encouragingly, sera elicited by H3HA10-IZ and H3HA10-Foldon had detectable antibody titers against divergent H1 HAs belonging to group 1 (**Figures 4D,E**).

The stem-directed neutralizing antibodies interfere with the establishment of viral infection by inhibiting membrane fusion. These antibodies do not prevent virus attachment to host cell receptors detected in a hemagglutinin inhibition (HI) assay (51). We measured the serum neutralization titers using a pseudotyped virus particle entry inhibition assay (40). The sera elicited by HA stem-fragment immunogens showed significant entry inhibition of the heterologous H3 A/Wisconsin/67/2005 (with A/Udorn/307/1972 N2) influenza pseudotyped virus (**Table 2**), above the background of the naïve sera (Figure S3 in Supplementary Material). The reason(s) for the high background with the naïve mice sera are not well understood.

## HA Stem-Fragment Immunogens Confer Partial Protection *In vivo*

We evaluated the *in vivo* efficacy of HA stem-fragment immunogens against lethal viral infection. Mice were challenged intranasally with a lethal dose ($2LD_{90}$) of homologous mouse-adapted H3N2 A/HK/1/68 virus 3 weeks after the secondary immunization (boost). H3HA10 conferred minimal protection (20%) against virus challenge. Although, the oligomeric derivatives of H3HA10 elicited cross-reactive, anti-HA antibodies, they conferred only partial protection (40–50%) (**Figure 5A**). However, all surviving mice showed significant weight recovery by the end of the study period after initial weight loss (**Figure 5B**).

# Discussion

The emergence of "novel" influenza virus strains with the potential to cross-over the species barrier and infect humans has raised

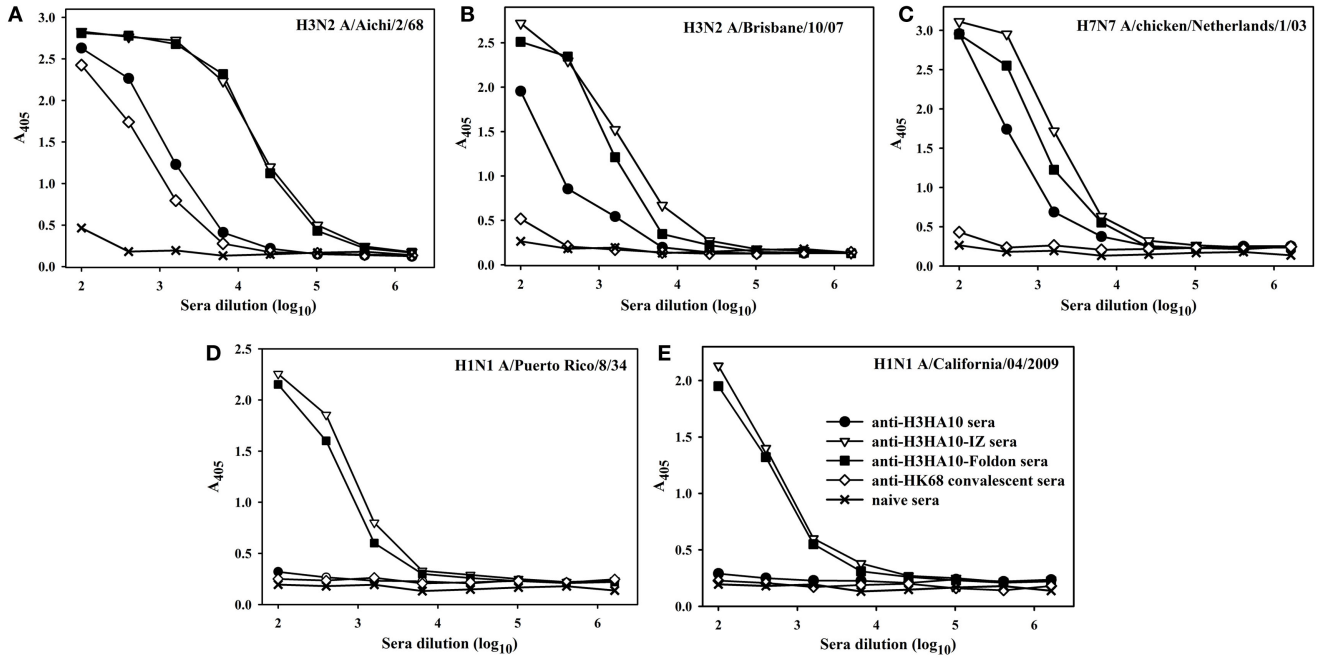


**FIGURE 4 | The designed immunogens elicit broadly cross-reactive, anti-HA antibodies in mice**. The antigen-specific antibody response of the pooled sera ($n = 10$ mice/group, collected 2 weeks after boost) was evaluated in an ELISA with full-length rHA proteins: **(A)** H3N2 A/Aichi/2/68, **(B)** H3N2 A/Brisbane/10/07, **(C)** H7N7 A/chicken/Netherlands/1/03, **(D)** H1N1 A/Puerto Rico/8/34, and **(E)** H1N1 A/California/04/2009. The oligomeric derivatives of H3HA10 induced a higher cross-reactive anti-HA antibody response in comparison to mice that received a sub-lethal, protective dose ($0.1LD_{90}$) of mouse-adapted HK68-virus (convalescent sera).

**TABLE 2 | *In vitro* pseudotyped virus particle entry inhibition with HA stem immunized mice sera**.

| Immunogen | $IC_{50}$[a] |
|---|---|
| H3HA10 | 13479 |
| H3HA10-IZ | 9082 |
| H3HA10-Foldon | 16935 |
| Naïve | 8287 |

[a] *$IC_{50}$ titer is the reciprocal of the sera dilution at which half-maximal entry inhibition was observed. Sera were collected 14 days after the boost and pooled.*
*The antibodies elicited by HA stem-fragment immunogens inhibited the entry of a heterologous H3 A/Wisconsin/67/2005 (with A/Udorn/307/1972 N2) influenza pseudotype virus.*

alarms across the global health surveillance system. The limitations of current vaccines, namely, strain-specific protection and lengthy production time, have necessitated the development of novel vaccines (1).

A primary influenza infection/vaccination results in an antibody response biased toward the immunodominant, hypervariable antigenic sites in the globular head domain of HA (43). Alternative vaccination strategies have been adopted to skew the humoral response in favor of the conserved HA stem which can potentially increase the breadth of protection. A vaccination regimen with repeated DNA and/or protein immunizations with full-length, chimeric HAs was shown to enhance the stem-directed antibody response (52). Full-length HA displayed on ferritin-nanoparticles elicited stem-directed antibodies in addition to a robust head-directed response (53). HA N-linked glycosylation has also been engineered to induce cross-strain protection against influenza infection (54, 55).

In alternate approaches, the HA stem has been stabilized in the absence of the globular head domain. We have previously demonstrated that site-specific charged (Asp) mutations can destabilize the post-fusion conformation of the HA stem and shift the equilibrium toward the metastable, pre-fusion conformation at neutral-pH (28). The bacterially expressed HA stem conferred complete protection against virus challenge in mice. However, these proteins formed inclusion body aggregates upon expression and required refolding. The refolded proteins were aggregation prone. In subsequent studies, we engineered HA stem sub-structures to elicit a stem-specific immune response (32, 56). We successfully designed "headless" HA stem-fragment immunogens from influenza A group 1 viruses which were purified from the soluble fraction in *E. coli*. These thermotolerant, trimeric immunogens conferred robust subtype-specific protection *in vivo* (32).

Structural divergence in the stem-domain of HA between groups 1 and 2 results in the group-specific neutralization profile of various stem-directed bnAbs (6). In lieu of a "universal" influenza vaccine, a composite of immunogen(s) from both groups is a practical alternative. Herein, we report the characterization of HA stem-fragment immunogens designed from the H3N2 strain (A/Hong Kong/1/68) that caused the "1968 influenza pandemic".

We intended to enhance the breadth of Ag-specific immune response by targeting the conserved regions of HA. An exhaustive

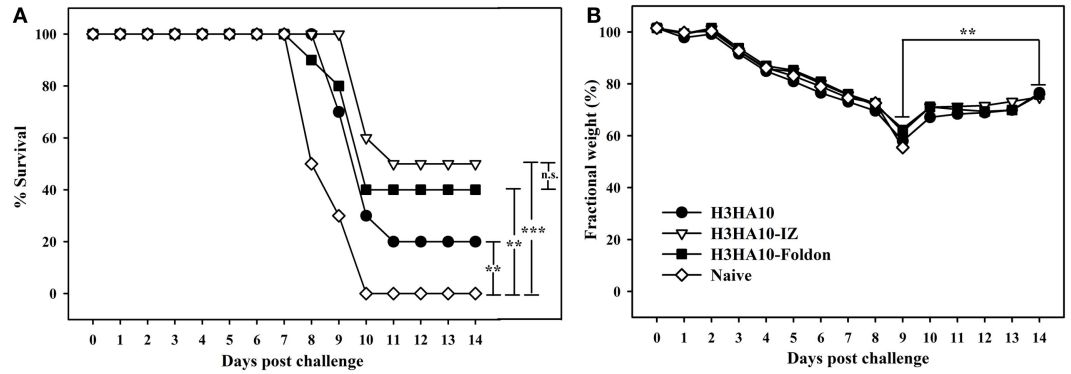


**FIGURE 5 | *In vivo* efficacy of HA stem-fragment immunogens against lethal homologous virus challenge**. Mice ($n = 10$/group) were vaccinated with the test immunogens on days 0 (prime) and 28 (boost). The immunized mice were challenged 3 weeks after boost with $2LD_{90}$ of mouse-adapted HK68 virus. **(A)** Survival and **(B)** average weight changes (of surviving mice) were monitored for 14 days post virus challenge. Naïve mice were used as controls. The oligomeric derivatives of H3HA10 conferred partial protection (40–50%). However, all the surviving mice showed significant weight recovery by end of the study period after initial weight loss. The efficacy of test immunogens was evaluated by analyzing the Kaplan–Meier survival curves with log rank significance test ($p$-values: $^{***} \leq 0.005$, $^{**} \leq 0.05$, and n.s. $\geq 0.05$). The difference(s) in fractional body weight(s) after recovery (at day 14) and maximal loss (day 9) were analyzed by Student's $t$-test ($p$-value: $^{**} \leq 0.05$).

analysis of full-length H3 HA sequences revealed multiple sub-structures within the HA stem that are conserved. These conserved sub-structures form discrete epitopes that are targeted by different bnAbs: the pan-influenza bnAb FI6 binds the epitope lined by residues of the A-helix (HA2-subunit) (25), while the viral membrane proximal β-sheet lines the epitope of group 2 specific bnAbs CR8020 and CR8043 (5, 27). Although, cryo-EM studies demonstrated that HA trimers on the virion surface could complex with a stem-directed bnAb (44), the relative accessibility of HA stem sub-structures on the crowded virion surface may influence the *in vivo* efficacy of vaccine candidates targeting these epitopes separately. The designed HA stem-fragment immunogen, H3HA10, comprises a subset (~47%) of the bnAb FI6 epitope and completely lacks the epitopes for CR8020 and CR8043. The interaction network of the HA stem was minimally perturbed to mimic the native, pre-fusion conformation in the designed immunogen. Further, the C-terminal conjugation of H3HA10 with a trimerization motif (IZ/foldon) improved the solution properties of the protein. Soluble prokaryotic (*E. coli*) expression of the designed immunogens enables rapid production. Although the oligomeric derivatives of H3HA10 elicited cross-reactive anti-HA antibodies that inhibited entry of a heterologous H3 HA pseudotyped virus *in vitro*, they conferred only partial protection (40–50%) after virus challenge in mice. There is disconnect between the high entry inhibition $IC_{50}$ values (**Table 2**) and the lack of a robust protective response (**Figure 5A**) elicited by the designed immunogens. The high entry inhibition $IC_{50}$ values are likely to be the consequence of using a highly sensitive entry inhibition assay. The lack of a strong correlation between the entry inhibition $IC_{50}$ values and survival warrants further investigation into the role of antibody-dependent effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and other Ag-specific antiviral mechanism(s). There is considerable scope to improve our current design to enhance the *in vivo* efficacy, for instance, by incorporating a larger footprint of the bnAb FI6 epitope.

Although the H3HA10 series of immunogens described here are expressed in soluble form and are not aggregation prone, they had relatively lower *in vivo* efficacy as compared to our previously designed HA stem immunogen (H3HA6). This is probably because H3HA6 includes the entire HA stem presenting distinct epitopes of multiple bnAbs to the immune system (28). The oligomeric derivatives of the designed HA stem immunogens elicited a robust antibody response against the homologous H3N2 HA. While these antibodies were cross-reactive, the titers against heterologous H3 and H7 HAs were 10-fold lower. The elicited antibodies also exhibited weak cross-group (group 1 HAs) reactivity. The antibody-HA reactivity profile correlates well with the residue conservation between the influenza strains evaluated in our assay (Table S1 in Supplementary Material). Therefore, we hypothesize that increasing the footprint of bnAb epitopes in the designed immunogen can further improve the binding profile. A comparison with the designed HA stem-fragment immunogen, H1HA10 (32), from influenza A group 1 viruses reaffirms the necessity to include a larger footprint of bnAb epitopes. H1HA10 includes ~70% of the pan-influenza neutralizing bnAb F16 epitope, while H3HA10 includes only ~47% of the epitope. Further design optimization will explore mutations to increase the strength of inter-protomer interactions. For example, a recent study demonstrated that engineered Cys mutations in the LAH of the HA2-subunit of pandemic HA (H1N1 A/California/2009) promote the formation of covalent trimers (31). Despite these lacunae, the immunogens described in this study do provide partial protection against lethal pathogenic challenge and elicit broadly cross-reactive HA stem-directed antibodies. Our studies provide a framework for the design of future influenza A group 2 HA stem-fragment immunogens.

## Author Contributions

VM, XL, and RV designed the experiments. VM, MC, FF, and NT performed the experiments. All authors analyzed the data

and assisted in manuscript preparation. VM, JF, and RV wrote the manuscript.

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## Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00329

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# Intranasal vaccination with an engineered influenza virus expressing the receptor binding subdomain of botulinum neurotoxin provides protective immunity against botulism and influenza

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Influenza virus is a negative segmented RNA virus without DNA intermediate. This makes it safer as a vaccine delivery vector than most DNA viruses that have potential to integrate their genetic elements into host genomes. In this study, we developed a universal influenza viral vector, expressing the receptor binding subdomain of botulinum neurotoxin A (BoNT/A). We tested the growth characters of the engineered influenza virus in chicken eggs and Madin–Darby canine kidney epithelial cells (MDCK), and showed that it can be produced to a titer of $5 \times 10^6$ plaque forming unites/ml in chicken eggs and MDCK cells. Subsequently, mice intranasally vaccinated with the engineered influenza virus conferred protection against challenge with lethal doses of active BoNT/A toxin and influenza virus. Our results demonstrated the feasibility to develop a dual purpose nasal vaccine against both botulism and influenza.

**Keywords: botulinum neurotoxin, influenza, intranasal vaccination, protective immunity, recombinant influenza vector**

## INTRODUCTION

Influenza A virus is a member of family orthomyxoviridae, which contains a segmented RNA genome. With the elucidation of replication mechanism of influenza virus, generation of influenza virus from DNA became feasible and extended the research of influenza dramatically. The most important application of this system is creation of live-attenuated vaccine or the generation of influenza vector to express foreign antigens (1–3). Engineered influenza viruses expressing foreign antigens have successfully induced a vigorous immune response in mice by the intranasal immunization (4–6). In early stage of influenza virus vector development, influenza virus non-structural gene (NS) segment was engineered to express foreign antigen with deletion of the nuclear export protein (NEP), formally referred to as NS2 (7–11). With the finding of 2A cleavage sequence of picornavirus, NS gene segment was modified with multi-cistronic sites. It enlarged the containment of foreign gene, kept a high growth of engineered influenza virus, and enhanced genetic stability (7, 12).

Botulinum neurotoxins (BoNTs, A–H) are the most poisonous substances known in nature. They may be used as bioterrorism agents or in biological warfare. Currently, there is no FDA licensed botulism vaccine available for public use. Natural BoNT is divided into a light chain (L) and a heavy chain (H) (13). L chain is a globular protein with $Zn^+$-metalloprotease activity (14). H chain is divided into N-terminal translocation domain and C-terminal receptor binding domain ($H_C50$), which consists of N-terminus without assigned function and C-terminus with receptor binding subdomain (rbsd) (15, 16). Previously, we developed an effective adenovirus-vectored vaccine expressing $H_C50$ (17). In this research, we created a recombinant influenza virus vector with a 2A cleavage site on NA. Subsequently, we generated an engineered influenza virus based on H1N1 PR8 virus, expressing the truncated $H_C50$-rbsd from BoNT/A. Intranasal vaccination with this engineered influenza virus was evaluated for protection against lethal challenges with BoNT/A and influenza virus.

## MATERIALS AND METHODS

### CELL CULTURE AND ANIMAL STUDIES

Madin–Darby canine kidney epithelial cells (MDCK) were cultured in modified Eagle's medium with 10% fetal calf serum. 293T cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal calf serum. BALB/c mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Animal experimental protocol (protocol number is 10020) was approved by the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center, and carried out in accordance with the US Public Health Service Guide for the Care and Use of Laboratory Animals (NRC Publication, 8th ed.) and other related federal statutes and regulations of the Animal Welfare Act. $MLD_{50}$ were determined by Reed–Muench method (18).

### GENERATION OF ENGINEERED INFLUENZA VIRUSES

First, we synthesized a gene segment coding 2A peptide followed by a multiple cloning site (mcs) (19). Then, it was linked with

185 bp of C terminal for packaging signals of NA gene segment by overlap PCR. Subsequently, the N-terminal non-coding region with ORF of NA gene segment was linked with the above gene segment by overlap PCR (**Figure 1A**). Finally, full-length PCR product was inserted into pHW-2000. New plasmid was designated pHW-NA-mcs. Synthesized human-codon-optimized gene segment of BoNT/A-$H_C$50-rbsd (amino acid 1088-1293, GenBank: CAL82360.1), was inserted into pHW-NA-mcs, and the plasmid was named pHW-NA-B/A-rbsd. Engineered influenza viruses using PR8 influenza virus as backbone were rescued by reverse genetics with eight plasmids (20). The wild type control A/Puerto Rico/8/1934(H1N1) influenza virus (PR8) was produced by similar method.

### VIRAL GROWTH AND TITRATION

About 100 pfu viruses were inoculated into allantoic cavity of a 9-day-old specific pathogen free (SPF) embryonated eggs (Charles River, CT, USA). At 72 h post-inoculation, viruses were harvested and titrated on monolayer MDCK cells by using the standard plaque assay method. To measure virus growth curve, monolayer MDCK cells were initially infected with virus at multiplicity of infection (MOI) of 0.001. After 1 h incubation, virus was removed and MDCK cells were washed with PBS supplemented with $Ca^{++}$ and $Mg^{++}$, then medium were replaced with fresh medium supplemented with 0.2% BSA and 1 μg/ml Tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin. At 24, 36, 48, 60, and 72 h post-infection, virus samples were collected, respectively, and titrated on monolayer MDCK cells in triplicate.

### RT-PCR

Viral RNA was extracted from virus samples using RNeasy kit (Qiagen, CA, USA), and subjected to the RT-PCR amplification by using SuperScript III Reverse Transcriptase kit (Invitrogen, CA, USA). RT-PCR products were analyzed by electrophoresis in 0.8% agarose gel. The images were taken by VersaDoc imager (BioRad, CA, USA).

### WESTERN-BLOTTING

Madin–Darby canine kidney epithelial cells were infected with influenza virus at MOI of 1. At 8 h post-infection, cells were harvested and lysed. Proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Then, membrane was incubated with anti-BoNT/A-$H_C$50 serum (prepared in our lab), rabbit anti-NP polyclonal antibody (Immune-tech), and β-actin antibody, respectively. After being washed, membrane was incubated with goat anti-rabbit or -mice IgG-HRP for 1 h. Finally, membrane was developed with chemiluminescence HRP substrate (Takara Bio Inc.), and images were taken by using ImageQuant Las4000 (GE Health, PA, USA).

### ANIMAL EXPERIMENTS

Forty-eight (48) 6-week female BALB/c mice were divided to 8 groups. Subsequently, the mice were intranasally vaccinated with 5 pfu influenza virus. At 4 weeks post-infection, mice were boosted with the same dose. Blood samples were collected on the days of immunization and challenge. On day 42, mice were challenged with 10× $MLD_{50}$ BoNT/A toxin (BEI Resources, cat# NR-4529, NIAID, NIH) or 100× $MLD_{50}$ PR8 influenza virus. After being challenged, mice were observed every day. In the BoNT/A toxin challenged groups, data of survived mice and dead mice were calculated. In the PR8 challenged groups, mouse weights were recorded every day. When the mouse weight loss was >25%, the mouse was euthanized and sacrificed according the guide of the Institutional Animal Care and Use Committee at Texas Tech University Health Sciences Center.

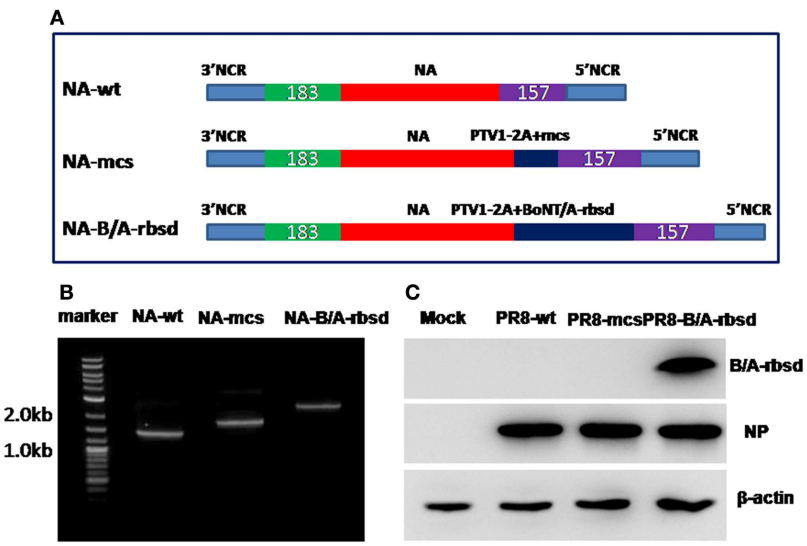


**FIGURE 1 | Design and identification of influenza virus expression of BoNT/A-rbsd**. **(A)** (Top) Organization of the original (wt) NA gene segment. (Middle) Organization of the modified NA gene segment engineered with porcine teschovirus (PTV)-1 2A cleavage site and multiple cloning site (mcs). (Bottom) Organization of the modified NA gene segment engineered with receptor binding subdomain of BoNT/A toxin. **(B)** NA gene segment amplified by RT-PCR. **(C)** Receptor binding subdomain of BoNT/A toxin is expressed in MDCK cells infected with engineered influenza virus PR8-B/A-rbsd, as determined by Western blot.

### ELISA

Briefly, microplates were coated with recombinant BoNT/A receptor binding subdomain overnight. After antigen was removed and plates were blocked, sera were diluted and added into each wells, and incubated at 4°C overnight. One hundred microliters of diluted goat anti-mouse IgG-Fc antibody conjugated to alkaline-phosphtase were added into each well and incubated for 1 h. Subsequently, plates were washed and pNPP substrate was added and incubated for 20 min. After sufficient color development, stop solution was added into each well. Finally, the plates were read in the Gene5 microplate reader (BioTek).

### STATISTICAL ANALYSIS

Comparison between virus titers was performed by using *t* test calculator (http://www.graphpad.com/quickcalcs/ttest1.cfm). *P* values $<0.05$ were considered to be significant difference. Comparisons between vaccinated groups were performed by using a non-parametric one-ways ANOVA with the Tukey multiple comparison test and Fisher's exact, and survival dates were analyzed by using the log-rank test. The analyses were performed by using GraphPad Prism version 5.0 for Windows (GrahPad Software). *P* values $<0.05$ were considered to be significant difference.

## RESULTS

### RESCUE OF ENGINEERED INFLUENZA VIRUSES

At first, NA gene segment was engineered by inserting a multi-cloning site with a 2A cleavage site. Then a universal replication-competent influenza viral vector was rescued. To demonstrate the use of this universal influenza viral vector, a 618 bp gene segment of BoNT/A $H_C$50-rbsd was inserted into engineered NA gene segment. An engineered influenza virus carrying target gene segment was rescued, purified, and propagated in embryonated chicken eggs. The rescued viruses were named PR8-wt, PR8-mcs, PR8-B/A-rbsd, respectively. Then, viruses propagated in embryonated chicken eggs were harvested, aliquoted, and stored at −80°C for subsequent use.

### CONFIRMATION OF RECOMBINANT INFLUENZA VIRUS

To confirm rescued influenza viruses carrying anticipated NA gene segment, viral RNA was extracted, and NA gene segments were amplified. Results in **Figure 1B** showed wild type NA gene or engineered NA gene segments were enclosed in the rescued influenza virus. Furthermore, expression of BoNT/A receptor binding subdomain was confirmed by western-blotting. **Figure 1C** shows the correct size of BoNT/A $H_C$50-rbsd is around 26 kDa.

### GROWTH CHARACTERISTICS OF ENGINEERED INFLUENZA VIRUSES

Plaque assay on MDCK cells was performed to analyze the growth characteristics of engineered influenza viruses. In **Figure 2A**, plaques formed by engineered influenza virus are smaller than that formed by wild type PR8. Furthermore, the growth characters were tested in MDCK cells and eggs. The results in **Figures 2B,C** showed that titers of engineered influenza viruses are lower than those of wild type. It suggested that engineering NA gene was inhibitory to replication of influenza virus. However, the engineered influenza virus propagated with reasonable titers in MDCK cells and embryonated chicken eggs. The titers could reach to $5 \times 10^6$ pfu/ml.

### HUMORAL IMMUNE RESPONSE AND PROTECTION STIMULATED BY ENGINEERED INFLUENZA VIRUS

In **Figure 3A**, results showed that intranasal vaccination with the recombinant influenza virus PR8-B/A-rbsd stimulated significant

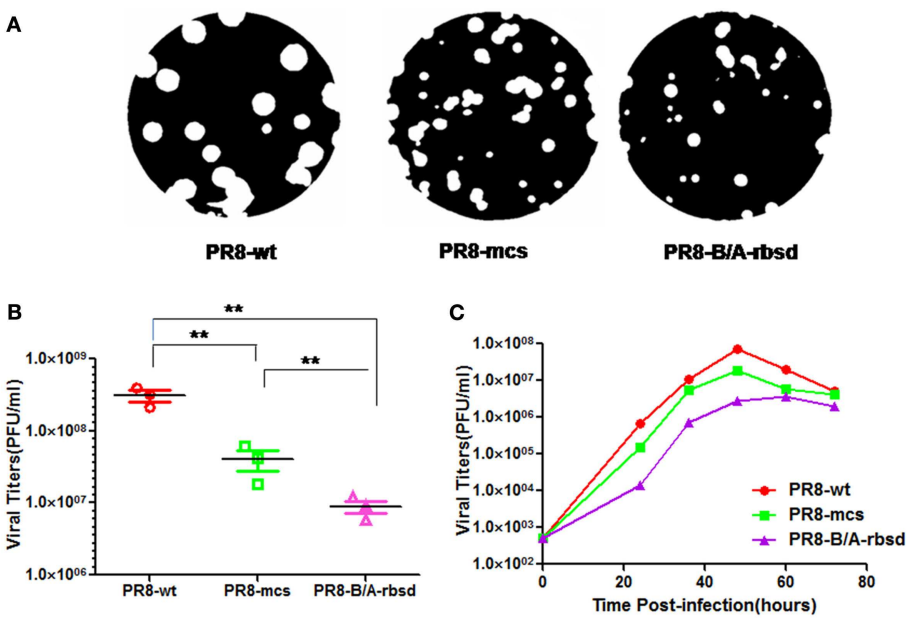


**FIGURE 2 | Growth characters of wild type and engineered PR8 influenza viruses**. **(A)** Plaque formed by infection with wild type and engineered PR8 influenza viruses on monolayer MDCK cells. **(B)** Virus growth character in embryonated chicken eggs (**$P < 0.05$). **(C)** Virus growth curves on monolayer MDCK cells.

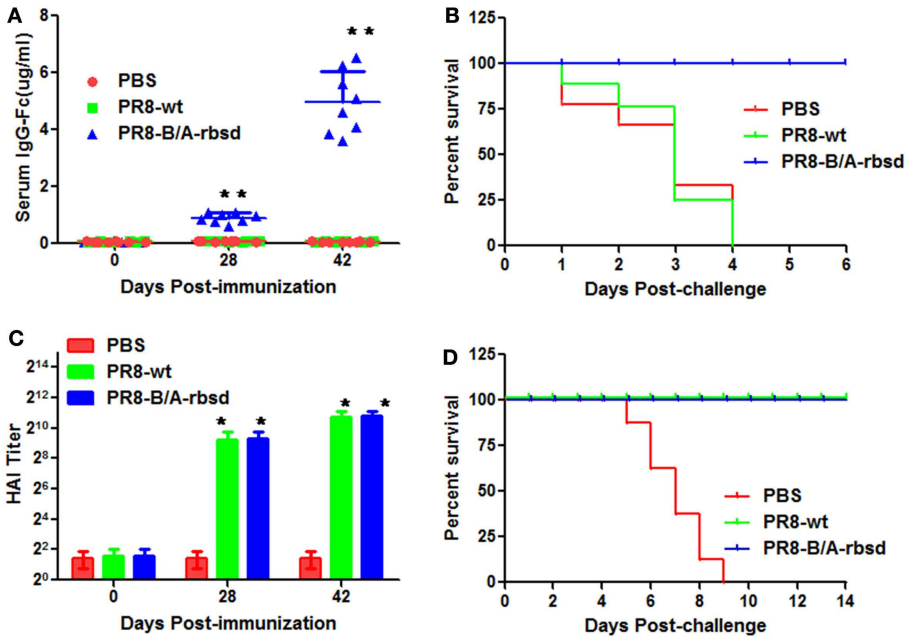


**FIGURE 3 | Intranasal vaccination with the engineered influenza virus expressing BoNT/A $H_c$50-rbsd conferred protection against BoNT/A and influenza virus infection**. Mice were intranasally inoculated with 5 pfu of PR8-B/A-rbsd virus at weeks 0 and 4, and challenged with 10× $MLD_{50}$ BoNT/A toxin or 100× $MLD_{50}$ PR8 influenza virus on day 42. **(A)** Vaccination with PR8-B/A-rbsd virus-induced antibody response against receptor binding subdomain of BoNT/A toxin (** $P < 0.05$ compared with PBS group or PR8-wt group). **(B)** Vaccination with PR8-B/A-rbsd conferred protection against lethal challenge with BoNT/A toxin. **(C)** Vaccination elicited antibody response against HA of PR8 influenza virus (* $P > 0.05$ compared with PR8-wt group). **(D)** Vaccination provided protection against lethal infection with PR influenza viruses ($n = 8$ for each group).

humoral immune response against BoNT/A toxin receptor binding subdomain compared with wild type influenza virus PR8. In **Figure 3B**, results showed that vaccination with PR8-B/A-rbsd provided protection against challenge with 10× $MLD_{50}$ active BoNT/A toxin. We also tested whether immunization with PR8-B/A-rbsd induced adaptive immunity against influenza virus infection. We tested the humoral immune response against influenza virus by using hemagglutination inhibition (HAI) method and challenged immunized mice with 100× $MLD_{50}$ PR8 influenza virus. In **Figures 3C,D**, the results showed vaccination with PR8-B/A-srbd stimulated potent humoral immune response, and resulted in high HAI titers and protection against challenge with lethal PR8 influenza virus.

## DISCUSSION

Influenza virus has plenty of advantages that make it worthy of consideration for use as a viral vector for pathogens that are problematic to vaccine development. There are well-established protocols for large-scale production of both live and inactivated influenza viruses, and live influenza vaccines have shown to elicit strong T cell immune response to stimulate mucosal and systemic responses (21, 22). Live-attenuated influenza virus may be the suitable virus vector to express foreign antigen as dual purpose vaccine. As depicted previously, there are several publications showed influenza virus could engineered to express foreign antigen; however, there is no research using influenza virus as vector to express antigen of botulism. In this research, we used mouse-adapted influenza virus as vector to express the receptor binding subdomain of BoNT/A toxin. In animal experiments, the engineered influenza viruses still have certain degree of pathogenicity in mouse ($MLD_{50}$ is 5,000 pfu, data not shown). In the future, we will use cold-adapted influenza viruses as vectors, because research has shown cold-adapted influenza virus is much safer than seasonal one (8, 23).

Botulinum neurotoxins are the most poisonous substances in the nature and potential bioterrorism agents. With the advantage of molecular technology, identification of non-toxic domains of BoNT toxin provides a useful method to develop a promising vaccine for botulism. In this research, our results showed that the smaller receptor binding subdomain of BoNT/A toxin is an effective antigen to stimulate humoral immune response. Additional research in this direction may lead to a multivalent vaccine against all types of BoNTs using the smaller antigen ($H_C$50-rbsd) instead of $H_C$50 (17) from each serotype. Data from this research demonstrated the possibility to develop a dual protective vaccine against both botulism and influenza. In this research, PR8 influenza virus is an old laboratory-adapted influenza virus. Maybe it is not suitable to be as human influenza vaccine. It just provides a research platform. In further research, cold-adapted or attenuated seasonal or pandemic influenza viruses will be recruited to provide a viable vaccine for bio-defense against BoNTs and for public health emergency against potential pandemic influenza.

## AUTHOR CONTRIBUTIONS

JL and MZ designed the research, analyzed the data, and wrote the manuscript. JL, DD-A, and YC performed experiments.

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# Polarizing T and B cell responses by APC-targeted subunit vaccines

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Current influenza vaccines mostly aim at the induction of specific neutralizing antibodies. While antibodies are important for protection against a particular virus strain, T cells can recognize epitopes that will offer broader protection against influenza. We have previously developed a DNA vaccine format by which protein antigens can be targeted specifically to receptors on antigen presenting cells (APCs). The DNA-encoded vaccine proteins are homodimers, each chain consisting of a targeting unit, a dimerization unit, and an antigen. The strategy of targeting antigen to APCs greatly enhances immune responses as compared to non-targeted controls. Furthermore, targeting of antigen to different receptors on APCs can polarize the immune response to different arms of immunity. Here, we discuss how targeting of hemagglutinin to MHC class II molecules increases Th2 and IgG1 antibody responses, whereas targeting to chemokine receptors XCR1 or CCR1/3/5 increases Th1 and IgG2a responses, in addition to $CD8^+$ T cell responses. We also discuss these results in relation to work published by others on APC-targeting. Differential targeting of APC surface molecules may allow the induction of tailor-made phenotypes of adaptive immune responses that are optimal for protection against various infectious agents, including influenza virus.

**Keywords: vaccine, APC targeting, T cells, antibody, Th1, Th2, influenza vaccines**

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## Influenza and the Need for Novel Vaccines

Annual influenza epidemics are caused by antigenic drift, whereby mutations in the major surface proteins hemagglutinin (HA) and neuraminidase (NA) alter antigenic determinants. Consequently, vaccines against seasonal influenza have to be annually updated in order to match the circulating strains. On a more sporadic basis, new virions may form from reassortment, whereby antigenically different strains combine to form a new subtype. Such an antigenic shift could result in a new global pandemic. A wide selection of influenza A viruses continuously circulate in different species, making accurate predictions of reassortments and pandemics challenging. On this backdrop, it is important to develop vaccines that can offer broad protection against influenza, and that can be rapidly manufactured.

## Correlates of Protection

### Antibodies

About 80% of the proteins that protrude from the viral influenza membrane are HAs (1, 2). During infection, HA binds sialic acid residues on host cells to initiate virus–cell interactions and entry of the viral capsid into the cytosol. The immunodominant antigenic determinants on HA are

mostly located in close proximity to the sialic acid binding receptor site, and represent mutation prone regions. Neutralizing antibodies against HA can block viral entry into host cells, and confer sterilizing immunity against influenza (3).

As induction of antibodies against HA is the focus of most current influenza vaccine strategies, several studies have shown that antibodies against NA may also be beneficial for clinical outcome (4–6). Although unable to block viral infection, antibodies against NA are thought to inhibit viral release from infected cells (7). In addition, antibodies against the extracellular domain of M2 have been shown to induce protection in animal models (8, 9). Whether anti-M2 antibodies are relevant in a human context remains unclear (10, 11).

### T Cells

In addition to antibodies, an influenza infection triggers the development of virus-specific T cells. T cells can clear influenza infection in the absence of neutralizing antibodies (12, 13), and have in the elderly population been found a good correlate of protection (14). The ability to kill infected cells is mainly attributed to $CD8^+$ T cells (15–17), and several of the $CD8^+$ T cell subsets (Tc1, Tc2, Tc17) have independently been shown capable of mediating protection (18, 19). Typically, $CD8^+$ cytotoxic T cells exert their function by secreting perforin, the polymerization of which forms a pore in the cell membrane that allows influx of serine proteases (20, 21), or by direct Fas–Fas ligand interactions (22, 23).

The main function of $CD4^+$ T cells during influenza infections is to aid the development of cytotoxic T cells and antibodies (24, 25). The Th1 subtype of $CD4^+$ T cells typically secrete interferon γ (IFNγ), and is associated with cellular immunity. However, Th1 cells can in addition help B cells, and IFNγ causes a switch to IgG2a. The hallmark cytokine of Th2 cells is interleukin 4 (IL4). Th2 cells are excellent helpers of B cells, and IL4 causes a switch to IgG1/IgE production (26). In accordance with the multiple functions of $CD4^+$ T cells, it has been shown that mice lacking functional $CD4^+$ T cells suffer more severe influenza infections, and that the development of immunological memory is impaired (27–29). In humans, pre-existing $CD4^+$ T cells have been found to be associated with lower viral shedding (30), and in mice, a subset of $CD4^+$ T cells that are able to directly lyse infected cells in a perforin-dependent manner have been described (31).

## Subunit Vaccines Against Influenza

Recently, a vaccine containing recombinant HA was licensed by the US FDA, thus laying the foundation for future vaccines containing recombinant influenza proteins (3). Subunit vaccines are considered safe, as they do not contain live viral components. However, a challenge of subunit vaccination is low immunogenicity. Several immunizations are typically needed for efficacy, and dose requirements are often high. These undesirable features have warranted the development of more potent delivery methods and adjuvants, which again could compromise the safety associated with subunit vaccination.

## Targeting of Antigen to APCs

The immunogenicity of subunit antigens can be increased by targeted delivery of antigen to antigen presenting cells (APC). In early studies, antigens were coupled to APC-specific antibodies by chemical conjugation (32–34), but genetic conjugations are now more common. Antigens can be linked directly to a Fab-fragment (35), included within loops of constant domains (36), or tail the C-terminus of the antibody heavy chain (37). In all these cases (32–37), the recombinant antibody-like molecules have APC-specific V-regions.

We have previously generated novel vaccine molecules that were designed to mimic the bivalent receptor binding capacity of an antibody, display full-length antigens, and yet remain smaller than an Ig molecule. To achieve this, a single chain variable fragment (scFv) was linked to an antigen via the $C_H3$-domain of human IgG3 (38). The $C_H3$-domains will dimerize in the ER to generate bivalent display of antigens and scFvs (**Figure 1A**). Immunization with such vaccine molecules containing scFvs directed against major histocompatibility complex MHC class II (MHC-II) molecules, and expressing HA, have recently been shown to induce complete protection against influenza in immunized mice (39).

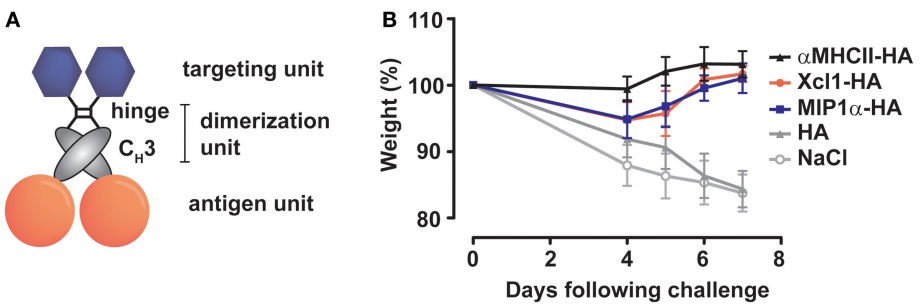


**FIGURE 1 | Immunization with APC-targeted dimeric vaccines protect mice against influenza**. **(A)** The vaccine molecules consist of targeting units (scFv or natural ligands), dimerization units (hinge region and $C_H3$ domain of human IgG3), and antigenic units [ex. influenza hemagglutinin (HA)]. **(B)** BALB/c mice were immunized with 25 μg DNA encoding the indicated vaccine molecules [HA from A/California/07/2009 (H1N1)] targeted toward MHC-II molecules (αMHCII-HA), chemokine receptors 1, 3, and 5 (MIP-1α-HA) or Xcr1 (Xcl1–HA). The mice were challenged 2 weeks after a single immunization with a lethal dose of influenza A/California/07/2009 (H1N1) and monitored for weight loss. All three APC-targeted vaccines induced protection against influenza, in contrast to vaccination with HA alone or NaCl. Modified with permission from Ref. (40).

Natural ligands such as chemokines and toll like receptor (TLR) agonists may specifically bind receptors that are preferentially expressed on APCs. Thus, genetic fusion of antigen to natural ligands has been evaluated as a method to increase immunogenicity of subunit vaccination. A fusion between a tumor antigen and chemokine CXCL10 or CCL7 has been demonstrated to increase immune responses in immunized mice, and to protect against tumor challenge (41). Similar targeting approaches have been evaluated for influenza antigens, where the targeted delivery with chemokines such as CCL3 or XCL1, or the TLR ligand flagellin, have resulted in enhanced immunogenicity and protection against influenza (40, 42, 43).

Traditionally, the main rationale behind targeting of antigens to APCs has been to enhance antigen uptake and the subsequent presentation to $CD4^+$ and $CD8^+$ T cells. Dendritic cells (DC) are capable of efficient stimulation of both $CD4^+$ and $CD8^+$ T cells, and several APC targeting approaches have therefore focused on this population of cells. DC were first described in the 1970s (44), and are now generally divided into three subclasses based on ontogeny as well as functional and transcriptional analysis (45). Plasmacytoid DC are highly efficient producers of type I interferon in response to TLR triggering, while conventional DC, cDC1 ($Xcr1^+Clec9a^+$), and cDC2 ($CD11b^+Sirp1a^+$) are the main stimulators of T cell responses. Both cDC1 and cDC2 are capable of presenting externally delivered antigen to $CD4^+$ T cells, but cDC1 is considered superior at cross-presentation to $CD8^+$ T cells (46, 47). Consequently, the specific targeting of antigen to cDC1 has gained attention as a method for induction of $CD8^+$ T cell responses.

## Polarization of Immune Responses

In a recent series of papers, we have evaluated the efficacy of a single immunization with influenza HA targeted to MHC-II molecules, chemokine receptors (CCR) 1, 3, and 5, or Xcr1 (39, 40, 42). For targeting of MHC-II molecules, HA was fused, via a dimerization domain, to a scFv specific for murine $I\text{-}E^d$ (αMHCII-HA). Similarly, targeting to CCR1/3/5 and Xcr1 was performed by fusing HA to the chemokines MIP-1α (MIP1α-HA) or Xcl1 (Xcl1–HA), respectively. MHC-II molecules are expressed on all professional APC, including B cells, macrophages (MΦ), and DC. CCR1/3/5 are expressed on MΦ, DC, eosinophils, and T cells, while Xcr1 is selectively expressed on cDC1 (48, 49). All three targeting approaches induced HA-specific immune responses, and protected mice against a lethal challenge with influenza virus (**Figure 1B**), in contrast to non-targeted controls (39, 40, 42).

While conferring protection against influenza, targeted delivery of HA to MHC-II molecules, CCR1/3/5, or Xcr1 revealed qualitative differences in induced immune responses. Targeting of HA to MHC-II molecules induced a Th2 dominant response characterized by IL4-secreting $CD4^+$ T cells, although some $IFN\gamma^+$ T cells were also observed (39, 42). MIP1α-HA induced higher numbers of IFNγ-secreting cells, and was found to polarize the immune response toward Th1 cells (42). In an assessment of T cell contribution to protection, depletion of $CD8^+$ T cells in mice previously immunized with MIP1α-HA abolished protection against influenza. By contrast, depletion of $CD8^+$ and $CD4^+$ T cells after immunization with αMHCII-HA did not diminish the induced protection (42). The importance of antibodies after immunization with αMHCII-HA was confirmed by the early presence of neutralizing antibodies in sera, and ultimately by the demonstration that transfer of sera from immunized mice could protect naïve mice against a lethal influenza challenge (39). It was also shown that the fairly low amounts of T cells induced could confer protection against influenza in the absence of relevant antibodies (39). Thus, immunization with MIP1α-HA induces $CD8^+$ T cell mediated protection, while αMHCII-HA induces neutralizing antibodies and T cells that probably act in concert.

MIP1α-HA targets several cell populations, whereas Xcl1-targeted vaccines have been demonstrated to specifically bind Xcr1 expressed on cDC1 (40). Adoptive transfer of OT-I and OT-II cells to $Xcr1^{-/-}$ knockout or wild type mice, prior to immunization with Xcl1–OVA, demonstrated that efficient proliferation was dependent upon functional targeting of antigen to Xcr1 (40). Similar observations have been made for Xcl1–OVA delivered by laser-assisted intradermal delivery (50) or for OVA directly fused to Xcl1 or to an antibody specific for Xcr1 (51). Direct conjugation of antigen to Xcl1 was required for efficacy, since delivery of unconjugated Xcl1 together with OVA failed to enhance proliferation (50). The importance of a direct conjugation has also previously been demonstrated for antigen linked to the chemokine MIP3α (52), and for a T cell epitope linked to CD40-specific V regions (53). Together, these results indicate that the observed immune responses are associated with receptor uptake, rather than a chemokine induced adjuvant effect.

Similar to MIP1α-HA, vaccination with Xcl1–HA as DNA induced a Th1 type of immunity, characterized by a marked increase of IFNγ-secreting $CD4^+$ T cells (40). Correspondingly, Xcl1–HA induced cytotoxic $CD8^+$ T cells that killed target cells presenting HA-derived peptides on MHC class I molecules. Depletion of Xcl1–HA-induced $CD8^+$ T cells before viral challenge also confirmed that these cells played a central role in mediating protection against influenza (40). Immunization with Xcl1–OVA protein also resulted in the induction of cytotoxic $CD8^+$ T cell responses when administered i.v. in combination with LPS (51). Interestingly, laser-assisted intradermal delivery of Xcl1–OVA protein induced enhanced cytotoxic $CD8^+$ T cell responses in the absence of adjuvant (50). In both these studies, targeting of OVA to the Xcr1 receptor induced protection in a murine melanoma tumor model (50, 51). Taken together, these three studies (40, 50, 51) highlight Xcr1 as a potent target for the induction of cytotoxic $CD8^+$ T cells.

Different types of immunity are associated with the induction of different IgG subclasses. When $CD4^+$ T cells provide help to B cells, they also directly influence isotype switching. IFNγ secreted by $CD4^+$ Th1 cells will promote the secretion of IgG2a, whereas IL4-secreting Th2 cells promote switching to IgG1 (54). Consequently, an assessment of IgG1 vs IgG2a could indicate the degree of induced Th1/Th2 immune polarization (**Figure 2A**). As earlier mentioned, HA targeted to MHC-II molecules induced higher levels of IL4 secreting $CD4^+$ T cells, and strong antibody responses. While such targeting increased responses for most IgG subclasses, IgG1 was indeed dominant (39) (**Figure 2A**).

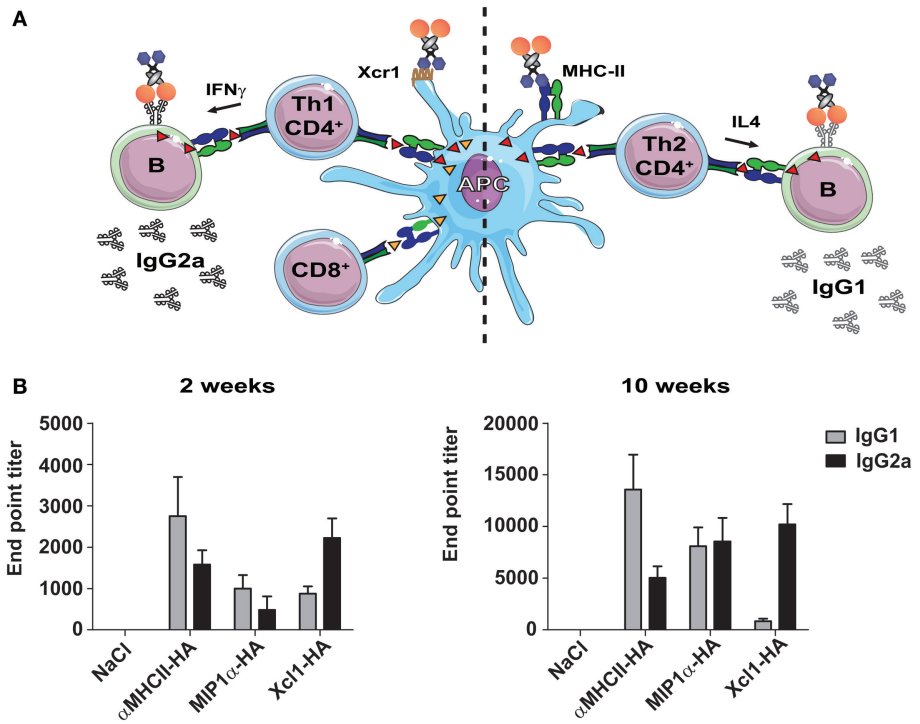


**FIGURE 2 | Targeting of selected surface receptors on APC will influence the vaccine-induced Th1/Th2 polarization and antibody subtypes.**
**(A)** Illustration of the different immune responses as induced by targeting of antigen to the chemokine receptor Xcr1 (left side) or MHC-II (right side). *Left side:* Targeting of antigen to Xcr1 induces IFNγ-secreting $CD4^+$ Th1 cells that can provide help to B cells and promote the formation of IgG2a antibodies. In addition, targeting of Xcr1 results in presentation of peptides on MHC-I molecules, and induction of strong $CD8^+$ T cell responses. *Right side:* Targeting of antigen to MHC-II molecules induces $CD4^+$ Th2 cells that secrete IL4, and that can provide help to B cells and promote the formation of IgG1 antibodies. **(B)** BALB/c mice were immunized with 25 μg DNA encoding the indicated vaccine molecules [HA from influenza A/Puerto Rico/8/1934 (H1N1)], and serums samples were harvested 2 or 10 weeks after a single immunization. Serum levels of HA-specific IgG1 and IgG2a antibodies were determined by ELISA against inactivated influenza A/Puerto Rico/8/1934 (H1N1). Modified with permission from Ref. (40).

Targeting of HA to Xcr1 also resulted in increased antibody responses as compared to non-targeted controls, but these were predominantly IgG2a (40) (**Figure 2B**). In contrast to Xcl1–HA, MIP1α-HA induced a lower and more mixed humoral response, with both IgG1 and IgG2a being present (42). It is likely that the selective targeting of cDC1 cells caused a more stringently Th1-polarized immune responses observed after vaccination with Xcl1–HA, as opposed to the more mixed responses observed after vaccination with MIP1α-HA. In summary, the three targeting approaches induce different types of humoral responses, with MHC-II-targeting promoting Th2/IgG1, CCR1,3,5-targeting giving a mixed IgG1/IgG2a response, and Xcr1-targeting polarizing responses toward Th1/IgG2a (**Figure 2B**).

Interestingly, these observations suggest that the choice of APC receptor may be used to direct immune responses toward particular antibody subclasses. This is of importance since the different IgG subclasses vary in their ability to induce antibody dependent cell-mediated cytotoxicity (ADCC) and complement activation, partly through different affinities for FcγR [reviewed in Ref. (55)]. While IgG2a binds strongly to activating FcγR, such as FcγRI, III, and IV, IgG1 have higher affinity for the inhibitory FcγRIIb receptor (56). Consequently, IgG2a antibodies induce stronger ADCC and complement activation than IgG1. Interestingly, two recent studies have suggested that broadly neutralizing antibodies against both HIV and influenza mediate their effect through FcγR binding, and that antibodies of the IgG2a subclass therefore are more efficient at this than IgG1 (57, 58). By contrast, strain-specific neutralizing antibodies against HA do not require FcγR binding, and function equally well as both IgG1 and IgG2a (58). Since IgG2a antibodies induce stronger ADCC, they might also be associated with an increased risk of induced cytopathic effects to host cells (59). Thus, in situations where FcγR binding is not required for induction of protection, it may be beneficial to induce IgG1. All this considered, our results would suggest to target antigens to MHC-II molecules for induction of specific neutralizing anti-HA antibodies, whereas targeting to Xcr1 would be more beneficial for induction of broadly neutralizing antibodies against conserved HA epitopes, such as the stem.

## Immune Polarization: A Function of Targeted Receptor or the APC that EXPRESS the Particular Receptor?

Since the targeted receptors are differently distributed on various cell types, a relevant question is whether the targeted cell type will determine the observed polarizations. Some studies have focused on antibody-mediated targeting of Clec9A for vaccinations (60–62). With its selective expression on cDC1 cells, the

Clec9A-targeted approach is comparable to Xcl1–mediated delivery of antigen to Xcr1. Targeting of antigen to Clec9A has been reported to enhance proliferation of antigen-specific $CD4^+$ and $CD8^+$ T cells, and to confer protection against melanoma (60, 63). These observations correlate with our results in that Xcl1–HA induced T cell-mediated protection against influenza. In addition, targeting of Clec9A induced strong antibody responses, with an efficient induction of T follicular helper cells ($T_{FH}$) (61, 62). Although the molecular mechanisms for how Clec9A induce antibodies are not known, $T_{FH}$ cells are presumably a key in that they are important for germinal center (GC) formation and induction of antibody secreting plasma cells (62). Interestingly, targeting of antigen to Clec9a was reported to induce more IgG1 than IgG2a (64), suggesting the induction of a more Th2 polarized $CD4^+$ T cell response. Together, this may suggest that it is the targeted receptor, rather than the targeted APC type, that determine the outcome of the immune response in these examples (40).

It should be noted that the studies targeting Clec9A and Xcr1 were done using different immunization protocols and different mouse strains, raising the possibility that other factors have influenced the results. However, experiments with targeting of Clec9A showed Th2-like responses in Th1-prone C57BL/6J mice, and targeting of antigen to Xcr1 induced Th1-polarization in both Th2-prone BALB/c mice (40) and Th1-prone C57BL/6J mice (50, 51). Similarly, the Th2 polarization observed after targeting of HA to MHC class II molecules has been confirmed in both BALB/c and Th1-prone B10.D2 mice (39).

## Receptor Expression and Endocytosis

The expression level of surface receptors and endocytosis rates could play a major role in determining the efficacy by which targeted vaccination stimulates presentation of peptides on MHC-I/II to T cells. In a recent study, comparing targeting of antigen to DEC205, Clec9a, CD11c, CD11b, and CD40, it was shown that delivery to DEC205 resulted in about 80% of surface receptors being internalized by 90 min. By contrast, delivery to CD11c or CD11b internalized surface receptors more slowly and inefficient (65). The authors concluded that endosomal trafficking of endocytosed antigen was likely to influence the efficacy of antigen presentation, a factor which has previously been suggested to influence cross-presentation to $CD8^+$ T cells (66, 67). While endocytosis is necessary for presentation of peptides from internalized antigens to MHCI/II molecules, and thus also activation of T cells, it is possible that reduced endocytosis might favor the stimulation of B cells and antibody production by allowing an extended period of time where B cells can recognize surface antigens.

## Efficient Induction of Humoral Immune Responses

Targeting of antigen to MHC-II molecules was shown early on to increase serum responses in the absence of adjuvant (68). In addition to the above mentioned studies targeting Clec9A, other groups have identified CD11c and CD180 as particular interesting receptors for induction of strong antibody responses (69–71). CD11c is predominantly expressed on DC, with more minor expression on monocytes, MΦ, neutrophils, and some B cells. CD180 is expressed on B cells, DC, and MΦ.

Ligation of CD180 on B cells has been reported to induce activation and proliferation, and may explain why targeting of antigen to CD180 could activate $CD4^+$ T cell-independent IgG responses after immunizations of CD40-KO and TCR α/δ KO mice (71–74). This is in contrast to our experiments with targeting of HA to MHC-II molecules, since immunization of thymus-deficient mice indicated that the humoral responses were T cell dependent (39). The mechanism behind the strong antibody responses induced by αMHCII-HA remains to be elucidated, but the rapid formation of IgG in sera (day 8 after a single vaccination) points toward rapid affinity maturation and GC formation. It is conceivable that the responses in this respect are aided by the vaccine molecules forming an APC-B cell synapsis (75, 76), where the bivalent vaccine molecules bridge MHC class II molecules on APCs and antigen-specific B cell receptors on B cells.

## Conclusion

We have here discussed how proper selection of target receptors on APC may polarize immune responses toward either dominant cellular or antibody responses. Furthermore, the immune responses could be tailor-made with respect to IgG isotypes and Th1/Th2 dominance. Given the importance of neutralizing antibodies in protection against influenza, targeting of antigen to MHC class II molecules should be further evaluated in larger mammals and humans. While antibodies against the influenza virus surface proteins are important, T cell responses against the conserved internal influenza antigens could offer broader protection. For eliciting strong T cell responses, use of vaccine antigens that are targeted by use of chemokines MIP-1α and Xcl1 could be important. In the future, more APC targets for vaccines should be tested for their ability to influence magnitude and polarization of immune responses. Also, a deeper understanding of the relationship between APC target specificity and immune response polarization is needed.

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**Conflict of Interest Statement:** Gunnveig Grødeland, Even Fossum, and Bjarne Bogen are inventors on patent applications filed on the vaccine molecules by the TTO offices of the University of Oslo and Oslo University Hospital, according to institutional rules. Bjarne Bogen is head of the Scientific panel in Vaccibody AS.

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# Host immunological factors enhancing mortality of young adults during the 1918 influenza pandemic

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During the 1918 influenza pandemic, healthy young adults unusually succumbed to infection and were considered more vulnerable than young children and the elderly. The pathogenesis of this pandemic in the young adult population remains poorly understood. As this population is normally the least likely to die during seasonal influenza outbreaks, thought to be due to their appropriate pre-existing and robust immune responses protecting them from infection, we sought to review existing literature for immunological reasons for excessive mortality during the 1918 pandemic. We propose the novelty of the H1N1 pandemic virus to an H1N1 naïve immune system, the virulence of this virus, and dysfunctional host inflammatory and immunological responses, shaped by past influenza infections could have each contributed to their overall susceptibility. Additionally, in the young adult population, pre-exposure to past influenza infection of different subtypes, such as a H3N8 virus, during their infancy in 1889–1892, may have shaped immunological responses and enhanced vulnerability via humoral immunity effects with cross-reactive or non-neutralizing antibodies; excessive and/or ineffective cellular immunity from memory T lymphocytes; and innate dysfunctional inflammation. Multiple mechanisms likely contributed to the increased young adult mortality in 1918 and are the focus of this review.

**Keywords: influenza, pandemic, 1918, pathogenesis, mortality**

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## Introduction

The 1918–1919 influenza pandemic caused an estimated 50 million deaths (1). In three distinct waves, the pandemic infected a third of the world's population, with the majority of the deaths occurring during the second wave in late 1918 (2, 3). Disease was characterized by unique, and to date poorly understood, epidemiological and clinical aspects. Victims died either from direct viral infection of the lung (4, 5), or most commonly from secondary bacterial pneumonia (6–8). Unusually, healthy young adults were more likely to die than young children and the elderly, two populations normally most vulnerable during influenza A virus (IAV) outbreaks (9). Fatal cases in the 1918 pandemic peaked in the 1889–92 birth cohorts, corresponding to approximately 28-year-olds (2, 10, 11), a pattern that was observed across the world (9, 12). The extraordinary mortality of young adults during the 1918 influenza pandemic is not currently understood.

Similar to the 1918 pandemic, the 2009 IAV pandemic caused more severe and fatal cases in 30- to 50-year-olds, which constituted up to one-third of patients in hospitals (13–15). Young adults had

two to four times the risk of severe outcomes from infection with this virus (H1N1pdm09) than those infected with circulating seasonal influenza (16). The majority of H1N1pdm09 infections caused a self-limited disease and the pandemic was considered the mildest on record. Until we understand the causes of enhanced illness of the young adult population during the 1918 and 2009 IAV pandemics, we are unlikely to be able to realistically estimate the impact of future pandemics. While no single explanation will be relevant to every mortality event in the young adults, we need to understand how their innate and acquired immune status may have combined with viral virulence to enhance mortality.

## Excessive Innate Host Responses Contribute to Influenza Immunopathology

Initial leukocyte infiltration into the lung parenchyma is essential for resolution of virus infection, yet dysregulation of the infiltrating effector cells be a major factor in disease (17, 18). A hallmark of highly pathogenic influenza infections is the ability for the virus to dysregulate innate inflammatory responses, leading to excessive recruitment of effector cells into the lung parenchyma causing severe pulmonary injury and diffuse alveolar damage (19, 20). To elicit cellular infiltrate into the infection site, host pattern recognition receptors (PRRs) must first recognize "danger" signals direct toward the invading IAV, causing release of pyogenic cytokines and chemokines. Excessive or dysregulated secretion can lead to a "storm" of events linked with high-mortality rates (19, 20). Young adults, with robust immune systems, may have been unusually vulnerable to the 1918 IAV due over-exuberant inflammatory responses to infection. As the elderly have less potent inflammatory responses to influenza infection compared to young adults, they may have been somewhat spared from excessive reactions and thus were less likely to succumb to infection.

The recovery of genetic fragments of the 1918 H1N1 pandemic virus and subsequent reverse engineering has enabled a complete reconstruction of the original virus (21). The 1918 pandemic virus was highly pathogenic as infection of monkeys and mice with the reconstructed 1918 H1N1 IAV resulted in acute respiratory distress and death with a pathology that matched lung tissues from victims in 1918 (21–23). Similar features occur with highly pathogenic avian H5N1 and H7N9 IAV infections (20, 24).

Virus infection followed by an extensive influx of macrophages and neutrophils can release large quantities of reactive oxygen species (ROS) contributing to the pathogenesis of lung disease. Mice infected with IAV expressing the virulence protein PB1-F2 matching that of the 1918 pandemic strain had enhanced pulmonary ROS (25), increased cellular infiltrate in alveolar spaces, and were more likely to die from secondary bacterial infections (26) compared to those infected with viruses expressing PB1-F2 proteins from seasonal IAV strains. The type-1 interferons (IFN-α and IFN-β) are the major cytokines that limit influenza replication, with TNFα, IL-1β, and IL-6 recruiting immune cells to the sites of infection and producing inflammation. Studies using mice genetically deficient in inflammatory modulators including tumor necrosis factor receptor (TNFR) and nitric oxide synthase (NOS2) exhibited reduced morbidity and mortality as well as diminished cytokine production in lung tissue following H5N1 and 1918-virus challenge compared to infected wild-type mice (27, 28). The type-1 interferons act on INF-α/β receptors to activate the antiviral signaling cascade, resulting in the production of antiviral proteins, such as MxA (Mx1 in mice). Mice genetically deficient in Mx1, interleukin-1 receptor (IL-1R), or IFNα receptor (IFNAR) exhibited increased viral load and pulmonary inflammation compared to wild-type mice (28–30). The molecular signatures of mice surviving 1918-virus infection reveal that the action of interferon via upregulation of genes involved with apoptosis, ROS production, and cell migration, together with downregulation of genes encoding cytokine and chemokine production associated with viral pathology, such as IL-6 and TNF, is critical to survival (29). As such, type-I IFNs contribute to both resolution of viral load and suppression of immunopathology caused by IAV infections. Inflammatory responses in animal infection models otherwise immunologically naïve toward IAV show that enhancement of inflammation in young adults could have been a major contributor to mortality during the 1918 influenza pandemic.

## Humoral Immunity Enhancing Susceptibility of the Young Adult Population in 1918

Influenza A virus infections during childhood typically induce B-cell memory responses that can adapt to produce antibody protecting against future infection by divergent drift strains of IAVs (31) (**Figure 1**). Such virus neutralizing antibody responses are typically directed toward epitopes on the globular head of the virus surface glycoprotein hemagglutinin (HA) and can be long-lived. This longevity was particularly evidenced by protection of the elderly against H1N1pdm09 infection, which was attributed to antibodies raised during pre-1960s exposure to a virus of the pandemic lineage (31). The elderly may have survived better than young adults during the 1918 pandemic as they may have been previously exposed to other H1 IAVs (32).

In the absence of specific-neutralizing antibodies, other antibodies that are normally immuno-subdominant can be induced and may be cross-reactive against different IAV subtypes. One such target of subdominant cross-reactive antibody is the viral ion channel protein, M2. The M2 protein is expressed on the virion surface but does not protrude to the level of other glycoproteins, making it a poor viral neutralization target. M2 is more accessible on the surface of infected cells and is thought to enable direct killing of infected cells by antibody-dependent cellular cytotoxicity (ADCC) mechanisms (33–35). Whether anti-M2 antibodies were important during the 1918 pandemic is unknown. Another target of subdominant cross-reactive antibodies are those directed toward the HA stalk (36). Antibodies to the HA-stalk employ various mechanisms of direct and indirect neutralization. By binding to the stalk domain of the HA, the antibody inhibits conformational changes of the HA in the endosome and prevents entry of IAV genomic material into the cytosol, as fusion of the endosomal and viral membranes cannot occur (37). Similar to the cross-reactive anti-M2 antibody, HA-stalk antibodies can induce ADCC (38) and complement-mediated cytotoxicity (39).

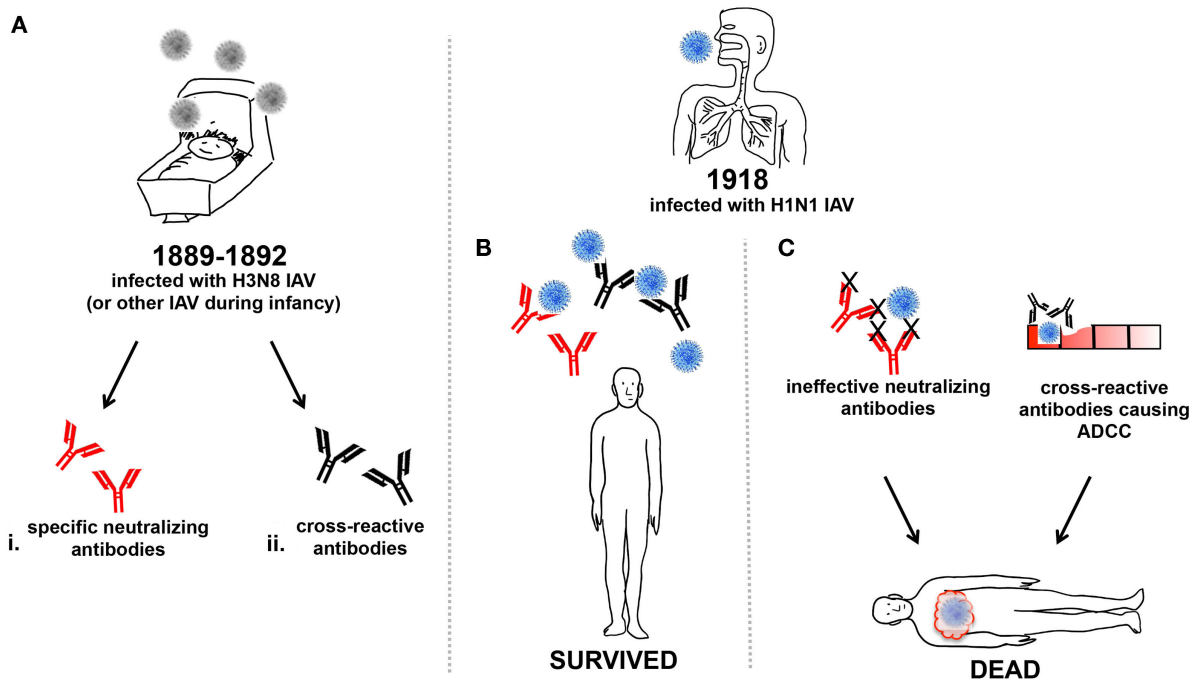


**FIGURE 1 | Humoral influence on vulnerability of young adults to 1918 IAV**. **(A)** Around 1889–92 infants were infected with the H3N8 IAV or other circulating IAV (grey) and generated either (i) neutralizing antibodies (red), or (ii) cross-reactive antibodies (black), or both. The infants from **(A)** that were young adults during the 1918 pandemic and were subsequently infected with H1N1 IAV (blue) may have produced **(B)** specific-neutralizing and/or cross-reactive antibodies enabling effective viral clearance and survival from infection. Or, **(C)** specific-neutralizing antibodies that were ineffective against the heterologous H1N1 IAV strain, and virus was unable to be cleared, resulting in death. Alternatively, the production of cross-reactive antibodies may have also caused ADCC, resulting in cellular damage and inflammatory illness, ultimately contributing to mortality.

In many animal studies, it has been shown that anti-M2 and HA-stalk antibodies induced by vaccination or passive transfer result in viral clearance and protection (35, 38, 40–45). In macaques, weakly immunogenic vaccines did not lead to robust ADCC responses and as such did not contribute to vaccination efficacy (46). Human studies have now revealed cross-reactive HA-stalk antibodies that are broadly neutralizing against divergent IAV strains (e.g., H1N1, H3N2, H5N1, and H7N9) and may protect from infection (41, 45, 47). However, it is important to note that not all individuals are capable of producing HA-stalk antibodies (48). Plasmablasts capable of secreting HA-stalk-specific antibodies have been isolated from healthy adults after H1N1pdm09 vaccination. These cells were produced from already existing memory B cells, which were presumably primed by previous IAV infections (41, 49), a scenario recapitulated by mice in sequential infections (50).

During the 1918 IAV pandemic, prior exposure to previously circulating influenzas would have shaped the memory B cell population to produce a landscape of both direct and cross-protective antibody responses (51) that may have resulted in protection from infection (**Figure 1B**). Young adults devoid of sufficient memory B cells capable of producing direct and cross-reactive antibodies, due to either their inability to mount such responses or from lack of prior IAV infections, may have fared much worse in 1918 (**Figure 1C**).

The above assumes that cross-reactive antibodies to HA-stalk or M2 would be beneficial, but evidence also exists that such antibodies may enhance disease. Enhanced respiratory disease can occur when individuals are challenged with a heterologous virus while producing cross-reactive antibodies (52). Pigs vaccinated with an inactivated swine influenza virus showed enhanced pneumonia upon challenge with H1N1pdm09. The vaccine was shown to induce high-titer cross-reactive antibodies against the more conserved HA2 stalk domain but no neutralizing antibodies to the globular head of the HA (53). The pathology associated with non-neutralizing antibodies cross-reacting with heterologous virus was characterized by severe bronchointerstitial pneumonia with necrotizing bronchiolitis and peribronchiolar lymphocytic cuffing (54), which may have resulted from excessive ADCC (**Figure 1C**).

The phenomenon of vaccine-associated enhanced respiratory disease is reminiscent of that seen in children vaccinated with inactivated RSV or measles virus following exposure to a heterotypic virus, who subsequently suffered enhanced respiratory disease or atypical measles with severe disease (55–57). Reasons for dire outcome include the quality of the antibody elicited toward the virus, the presence of large amounts of non-neutralizing antibody at the time of viral replication, and antibody-mediated activation of the classic complement cascade (56). The young adult population of the 1918 pandemic may have had prior exposure to a double-heterogenic H3N8 IAV during their childhood (32), and may have developed a cross-reactive humoral immunity. It is possible that in some young adults, the cross-reactive antibody responses produced after infection with

the 1918 virus actually enhanced subsequent pulmonary disease, for reasons similar to those observed for RSV and measles (55–57). This may be why, compared to children, the young adults were more vulnerable to the 1918 IAV infection.

Whether infection during the initial wave of the pandemic in early 1918 protected one from illness in subsequent waves of the pandemic during late 1918–1919 is not clear despite extensive study (12, 58–61). Young adults infected with the pandemic virus in early 1918 may have had a recall of the memory B cell population boosting the production of both direct and cross-protective antibody responses (51). Subsequent infection during the second or third wave of the pandemic may have resulted in further cross-reactive responses that may have induced ADCC and/or inflammatory disease. In Australian soldiers who could be followed individually, infection in early 1918 appeared to protect against death, but not illness during the subsequent wave occurring later during the 1918 pandemic (12). Additionally, recent Canadian studies showing that seasonal influenza vaccine apparently enhanced illness rates during the 2009 pandemic (62).

A recent study (32) proposes that individuals born earlier than ∼1890–1900 would have had neutralizing antibodies against the 1918 pandemic virus, induced by an emerging H1N1 virus in 1830, or an H1N8 virus in 1847 (32). Those born at the time of the 1889–92 H3N8 pandemic, or shortly thereafter, would not have such neutralizing antibodies and would be highly susceptible to 1918-virus infection. It is further postulated that an H1N8 virus re-emerged in 1900 and may have allowed the children in 1918 some degree of protection. If this did indeed occur, it would account for the troughs in the mortality curves in the young (5–15 years) and older (50–80 years) populations during the 1918 pandemic (32). Similarly, during the 2009 pandemic, about one-third of people born before 1950 had some immunity to the H1N1pdm09 virus, perhaps due to childhood exposure to an antigenically similar IAV (62, 63).

## Cross-Reactive CD8+ T Cell Immunity: Implications for Disease

Pre-existing memory CD8+ T cells established via previous IAV infections can cross-react with common epitopes presented by class I human leukocyte antigen (HLA) complexes on antigen presenting cells and promote rapid viral clearance. Animal (64, 65) and human studies (64, 66–68) have shown that CD8+ T cell-mediated immunity can be directed against highly conserved antigens among different IAV subtypes. More recently, non-conserved peptide epitopes that vary at residues other than those that anchor the peptide within the binding cleft of the HLA can still induce cross-reactive T cells (69). Memory CD8+ T cells can ameliorate infection by heterologous IAVs; however, substantial mutation in IAV peptide epitopes may lead to ineffective recruitment of cytotoxic CD8+ T cells crucial for viral clearance. Alternatively, a lack of capacity to mount any CD8+ T cell response could be equally problematic. The recruitment of cross-reactive CD8+ T cells against IAV varies across different ethnicities and has shown to be dependent upon the capability of expressing the HLA class I alleles that present conserved IAV peptides to elicit cross-protective CD8+ T cells (64).

Young adults who had survived infection by an IAV in 1890 should have had robust priming of memory CD8+ T cells that conferred some protection from lethal disease during the 1918 pandemic, provided these cells were periodically boosted by intra-pandemic IAV infection (32). Upon infection with the 1918 H1N1 IAV cross-reactive T cell responses would have been rapidly recalled in these individuals and may have protected against their death (70) (**Figure 2**). Recent studies have shown cross-reactive CD8+ T cell memory pools, generated by previous infection (s) with IAVs could provide some protection against H7N9 IAV infection (64). During 2009, the elderly population had a low infection rate compared to children and young adults, which was thought to be due to T cell immunity and neutralizing antibodies against the extremely conserved immune-dominant epitopes on viral proteins in the 2009 and 1918 H1N1 pandemic strains. Partial cross-reactivity with seasonal H1N1 IAVs that circulated in the 1930s when the elderly population would have been children may have also contributed (69, 71).

Caveats exist for the protective role described for cross-reactive CD8+ T cells. Cross-reactive CD8+ T cells cannot protect the host from initial infection; their target is an infected cell and they must be recruited to the site of infection after recall stimulation. If the heterologous IAV infecting the host presents a strong stimulus via PRRs that trigger excessive inflammatory responses and recruits an overwhelming number of cross-reactive CD8+ T cells, the resulting immunopathology may overwhelm any beneficial effects (10, 72) (**Figure 2Bii**). In addition, dysfunctional priming of CD8+ T cells may explain why the second wave of the 1918 pandemic appeared more virulent than the first. It has been postulated that the second wave of the 1918 pandemic was caused by a virus that had evolved toward a more pathogenic phenotype than the initially emerging H1N1 IAV (73). However, CD8+ T cells reactive for the immunodominant IAV nucleoprotein (NP) and matrix-1 (M1) produced during first wave of the 1918 pandemic and subsequently recalled upon infection during the second wave in late 1918 may have contributed to an over-exuberant inflammatory response enhancing disease severity (17, 58, 64, 74). As the ability to mount CD8+ T cell responses is linked to highly polymorphic HLA expression, healthy young adults infected in 1918 may have induced highly variable responses that could have been to their detriment due to recruitment of a plethora of non-cross-reactive CD8+ T cells (**Figure 2Biii**). Additionally, the robustness of the immune system in the young adult population as well as their pre-existing memory CD8+ T cell repertoire may have contributed to the vulnerability of this population over children, who may mounted a smaller repertoire of more specific CD8+ T cells toward the pandemic virus. Given a larger number of previous IAV exposures, the elderly may have mounted a more diverse cross-reactive CD8+ T cell response, but may have achieved clearance of infection without excessive cellular recruitment due to a decreased ability to recruit cells compared to the young adult population. Compared to young adults, the infected elderly CD8+ T cell response to the 1918 H1N1 virus may not have enhanced the pathophysiology of the disease and as such, may have been more effective toward clearing the viral infection.

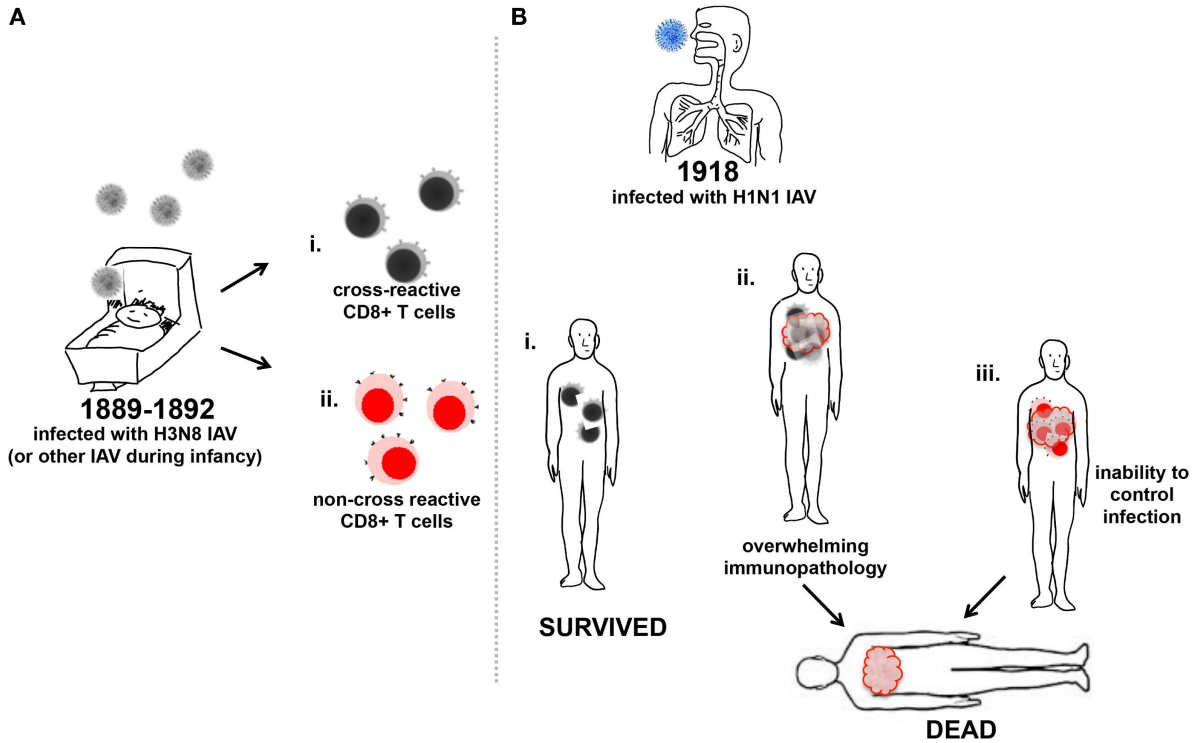


**FIGURE 2 | Memory CD8+ T cell influence on increased mortality of young adults to 1918 IAV**. **(A)** Infants were infected with the H3N8 IAV (gray) and generated either memory CD8+ T cells reactive toward (i) antigenically conserved regions of IAV (black/gray cells), or (ii) non-conserved antigenic regions of IAV (red/pink cells). **(B)** Young adults previously infected with IAV in their infancy and produced CD8+ T cells to conserved antigenic regions of the 1918 H1N1 IAV (blue) (i) survived infection as the CD8+ T cells aided viral clearance, or (ii) suffered illness due to the triggering of excessive inflammatory cellular responses to infection and recruitment of an overwhelming number of cross-reactive CD8+ T cells, which may have contributed to death. (iii) Young adults previously infected with H3N8 IAV and produced non-cross-reactive CD8+ T cells in response to heterologous 1918 H1N1 IAV (blue) were unable to control infection and may have become moribund.

## Concluding Remarks

The causes of extreme mortality in the young adult population during the 1918 pandemic are still uncertain. Childhood exposure to heterotypic IAV may have shaped humoral and adaptive immunological responses that contributed to the young adult population's enhanced disease outcomes. Ethnicity resulting in lack of appropriate immunological responses to conserved antigenic sites in the 1918 pandemic IAV may have also contributed to the mortality. PRRs may have induced over-exuberant inflammatory responses enhancing lung pathology and disease. Such mechanisms may collectively explain the increased mortality of young adults during the 1918 influenza pandemic. The enhanced illness in H1N1pdm2009 H1N1-infected young adults demonstrates that we still do not completely understand factors that enhance human vulnerability. We must continue to explore transmission models, virulence factors, and host responses to infection to better understand the pathophysiology of influenza if we are to diminish the impact of any new, highly pathogenic pandemic virus.

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# Boosted influenza-specific T cell responses after H5N1 pandemic live attenuated influenza virus vaccination

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**Background:** In a phase I clinical trial, a H5N1 pandemic live attenuated influenza virus (pLAIV) VN2004 vaccine bearing avian influenza H5N1 hemagglutinin (HA) and NA genes on the A/Ann Arbor cold-adapted vaccine backbone displayed very restricted replication. We evaluated T cell responses to H5N1 pLAIV vaccination and assessed pre-existing T cell responses to determine whether they were associated with restricted replication of the H5N1 pLAIV.

**Method:** ELISPOT assays were performed using pools of overlapping peptides spanning the entire H5N1 proteome and the HA proteins of relevant seasonal H1N1 and H3N2 viruses. We tested stored peripheral blood mononuclear cells (PBMCs) from 21 study subjects who received two doses of the H5N1 pLAIV. The PBMCs were collected 1 day before and 7 days after the first and second pLAIV vaccine doses, respectively.

**Result:** T cell responses to conserved internal proteins M and NP were significantly boosted by vaccination ($p = 0.036$). In addition, H5N1 pLAIV appeared to preferentially stimulate and boost pre-existing seasonal influenza virus HA-specific T cell responses that showed low cross-reactivity with the H5 HA. We confirmed this observation by T cell cloning and identified a novel HA-specific epitope. However, we did not find any evidence that pre-existing T cells prevented pLAIV replication and take.

**Conclusion:** We found that cross-reactive T cell responses could be boosted by pLAIV regardless of the induction of antibody. The impact of the "original antigenic sin" phenomenon in a subset of volunteers, with preferential expansion of seasonal influenza-specific but not H5N1-specific T cell responses merits further investigation.

**Keywords: influenza, H5N1, vaccine, T cells, LAIV, peptide, epitope, antigenic sin**



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## Introduction

Influenza is a global public health problem, with seasonal epidemics caused by human H1N1, H3N2, and B viruses, and sporadic disease caused by avian influenza A viruses, which can lead

to severe illness in humans (1–4). Live attenuated influenza vaccines (LAIV) that contain the A/Ann Arbor cold-adapted (AA ca) backbone (5) are immunogenic and protective and are licensed for protection against seasonal influenza (6–8). We have generated and evaluated candidate live attenuated vaccines for pandemic use (pLAIVs) bearing avian influenza A hemagglutinin (HA) and neuraminidase (NA) genes on the AA ca vaccine backbone. The H5N1 pLAIV (VN2004) bearing the HA and NA genes from the A/Vietnam/1203/2004 (H5N1) virus was evaluated in a phase I clinical trial (NCT00347672) (9). The infectivity of the H5N1 pLAIV was assessed by virus isolation and rRT-PCR amplification of vaccine virus from daily nasal washes and the immunogenicity of the vaccine was assessed by serologic methods including hemagglutination inhibition (HAI) and microneutralization and ELISA assays. The replication of the vaccine virus was highly restricted and the vaccine failed to elicit robust antibody responses (9).

Although antibody responses to inactivated influenza vaccine correlate with protection, several lines of evidence show that post-vaccination antibody titers are not the sole surrogate for vaccine efficacy, especially for LAIV (10–13). Several studies demonstrate that regardless of the presence of the antibody, influenza-specific T cell responses correlate with viral clearance (14, 15). The "Cleveland Family study" showed that protection from influenza correlated with T cell responses, and cross-reactive T cell responses might contribute to the protection (16). Therefore, as suggested by Schotsaert et al., the correlation of vaccine efficacy with alternative measures of immune function such as influenza-specific T cell responses warrants further attention (17).

In this study, we evaluated the T cell responses in peripheral blood mononuclear cells (PBMCs) from the cohort of study subjects who received two doses of the H5N1 VN 2004 ca vaccine approximately 50 days apart (9). T cell responses to overlapping peptide pools spanning the entire H5N1 proteome, as well as the HA proteins of relevant seasonal influenza viruses, were evaluated before and 7 days after each vaccination. We found that T cell responses with effector phenotypes were boosted by vaccination, regardless of vaccine infectivity or the serum HAI titer elicited. The potential role of pre-existing T cell responses in restricting the replication of H5N1 pLAIV was also evaluated.

## Materials and Methods

### Study Population

Nineteen healthy volunteers received two doses of the H5N1 VN 2004 pLAIV approximately 50 days apart, and two additional volunteers received only one dose (9). Blood samples were taken from each of the study subject at four time points: pre-vaccination, 7 days after the first dose of vaccine, 1 day prior to the second dose of vaccine, and 7 days after the second dose of vaccine. The study subjects were divided into two groups according to their infection status. Infection with the vaccine virus was inferred if the study subjects shed vaccine virus in culture, were RT-PCR positive after day 1, and/or demonstrated a fourfold or greater rise in serum antibody titer (9). Ethical approval was obtained from the Committee on Human Research Institutional Review Board (IRB) of the Johns Hopkins Bloomberg School of Public Health and the Institutional Biosafety Committee of Johns Hopkins University. Informed consent was obtained from all participating individuals prior to the study (9). The ClinicalTrials.gov identifier for this study is NCT00347672.

In order to understand the priming of the immune system induced by the H5N1 pLAIV, subjects who received two doses of the H5N1 VN 2004 pLAIV were contacted 4 years after receipt of the pLAIV and invited to participate in a follow-up study. Eleven subjects returned for this additional booster dose of 45 μg of the H5N1 inactivated unadjuvanted subvirion influenza vaccine (ISIV) (NCT01109329) (18). Antibody responses were measured after the boost dose, and compared with subjects who had received a non-H5N1 pLAIV or the ISIV alone (18).

### Synthetic Peptides for T Cell Analysis

A total of 890 15- to 18-mer peptides overlapping by 10 amino acid residues and spanning the full avian influenza H5N1 proteome and seasonal influenza H3N2/H1N1 HA proteins was synthesized by Sigma-Aldrich (Haverhill, Suffolk, UK) and used in our previous study (19). The peptides were dissolved in DMSO (Sigma-Aldrich) at 40 mg/ml and diluted with RPMI 1640 (Sigma-Aldrich) to a concentration of 2 mg/ml (long-term stock, stored at −80°C) before being individually filtered and combined into different pools: H1 HA, H3 HA, H5 HA, M, and NP (40–90 peptides/pool).

### *Ex Vivo* IFNγ ELISPOT Assay

Cryopreserved PBMCs were thawed in a 37°C water bath and re-suspended in RPMI 1640 supplemented with 2% v/v heat-inactivated fetal calf serum (FCS, Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 1% v/v (100 U/ml) penicillin streptomycin (Sigma-Aldrich) (R2 medium), and 60 μg/ml DNase solution (Type IV, Sigma-Aldrich) for 15 min at 37°C. Cells were washed and re-suspended in R10 medium (RPMI1640, 10% FCS, 2 mM L-glutamine, and 1% PenStrep) and rested overnight at a concentration of $10^6$ cells/ml. PBMCs (200,000) with 2 μg/ml the concentration of a single peptide in the pool or 400 T cells/clone with 20,000 peptide-pulsed Epstein–Barr virus transformed B cells were used in standard human IFNγ ELISPOT assays as described elsewhere (15). In brief, assays were performed in 96-well MultiScreen filter plates (Merck Millipore, Watford, Hertfordshire, UK) coated with 10 μg/ml anti-IFN-γ (1-DIK, Mabtech, Nacka Strand, Sweden). Phytohemagglutinin (5 μg/ml, PHA, final concentration 1 μg/ml; Alere, Stockport, Cheshire, UK) was used as a positive control. Plates were incubated for 16 h at 37°C and 5% $CO_2$. Spot enumeration was performed with an AID ELISPOT reader system (Autoimmun Diagnostika GmbH, Ebinger Strasse, Straßberg, Germany). To quantify antigen-specific responses, mean spots of the control wells were subtracted from the positive wells, and the results are expressed as SFU/$10^6$ PBMCs. Responses were considered positive if results were at least three times the mean of the quadruplicate negative control wells and >25 SFU/$10^6$ PBMCs. If negative control wells

had >30 SFU/$10^6$ PBMCs or positive control wells (PHA stimulation) were negative, the results were excluded from further analysis.

### Depletion of CD8$^+$ T Cells

CD8$^+$ T and CD4$^+$ T cells were depleted with M-450 Dynabeads (Invitrogen, Dynal, Oslo, Norway) according to manufacturers' instructions. This method has been validated and widely used (15). Briefly, PBMCs from the same patient were divided and incubated with anti-CD8 or anti-CD4 mAbs conjugated to ferrous beads in 0.1% FCS PBS medium at 4°C for 30 min. The CD8$^+$ and CD4$^+$ T cells were removed using a magnet stand (Invitrogen, Dynal). The efficiency of depletion was assessed using a CyAn™ ADP flow cytometer (Dako, Ely, UK) and FlowJo software (Tree Star Inc., Ashland, OR, USA). The frequency of CD8$^+$ T cells and CD4$^+$ T cells was <1% after depletion.

### Tetramer Staining and Multicolor Flow Cytometry

Cryopreserved PBMCs were thawed as described above. A total of $1 \times 10^6$ live PBMCs were labeled with tetramer-PE:HLA-A*0201 complexed with $M1_{58-66}$ peptide GILGFVFTL, produced in-house using standard methods (20), and incubated for 15 min at 37°C. Cells were then incubated with CD8-PerCP and CD4-Pacific Blue (eBiosciences, Hatfield, UK), as well as a panel of antibodies for cell activation and differentiation markers: CD28-FITC, HLA-DR-APC, CD38-PE-Cy7, and CD27-APC-H7. Cells allocated to the intracellular panels were permeabilized with Perm/fix (BD, Oxford, UK) for 15 min and washed twice with 1× perm/washing buffer (BD). Cells were then labeled with Perforin-FITC (D48, Genprobe, Manchester, UK) or GranzymeA-FITC and GranzymeB-PB (Biolegend, London, UK). Cells were subsequently washed twice with 1× perm/washing buffer and fixed in BD cellfix (BD). All antibodies were from Becton Dickinson (BD, Oxford, UK) unless otherwise stated. Cell events were acquired on a nine-color CyAn Cytometer (Dako, Ely, UK), and data files were analyzed using FlowJo software. Data were analyzed using a forward side scatter gate followed by CD8 gating, then tetramer gating within the CD8$^+$ population. These cells were then analyzed for percentage expression of a particular marker using unstained and CD8$^+$tet$^-$ populations to determine where to place the gates. Single-color samples were run for compensation, and fluorescence minus one (FMO) control samples were also applied to determine positive and negative populations, as well as channel spillover.

### T Cell Clones and EBV-Transformed B Cell Line

Cytotoxic T cell (CTL) clones specific for peptide H1 HA-56 were generated by limiting dilution from the PBMCs of study subject ID24 and maintained as described by Dong et al. (21). An autologous EBV-transformed B cell line was also generated from this subject.

### Intracellular Staining and Flow Cytometry

The following directly conjugated monoclonal antibodies were obtained from BD Biosciences (BD, Oxford, UK): IFN-γ (FITC), TNF-α (APC), CD107a (PE), CD3 (APC-H7), and CD8 (PE-Cy7). Antigen-specific CD8$^+$ T cell clones were stimulated with peptide-pulsed autologous B cells in the presence of anti-CD107a for 1 h and incubated with 0.7 μg/ml monensin (BD Biosciences) and 10 μg/ml Brefeldin A (BD Biosciences) for an additional 5 h at 37°C. Negative controls included un-stimulated cells. CD8$^+$ T cell populations producing cytokines were fixed and stained as described above and detected by flow cytometry.

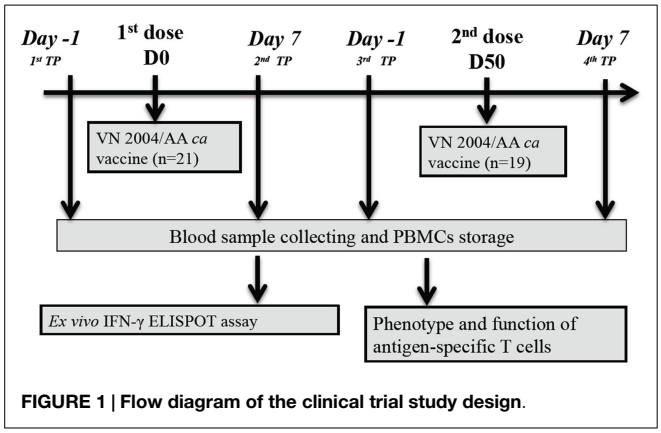



**FIGURE 1 | Flow diagram of the clinical trial study design.**

### Statistical Analysis

All statistical analyses were performed using Prism 6 (GraphPad Software). *p*-values were calculated using the Wilcoxon matched-pairs signed rank test or the Mann–Whitney test. $p < 0.05$ was regarded as statistically significant.

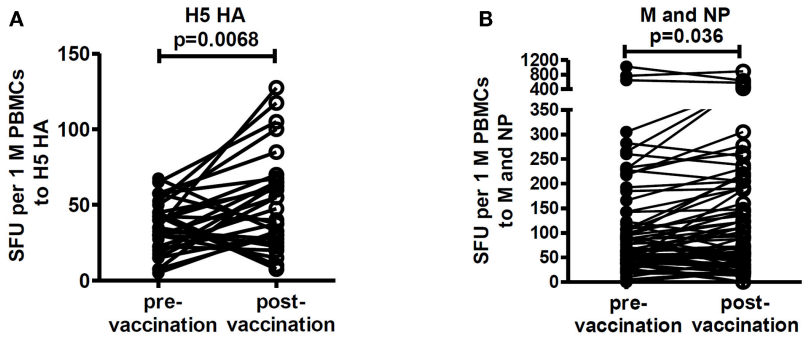
## Results

### Study Subjects and Specimens

As described by Karron et al. (4), 21 healthy volunteers were enrolled in this phase I clinical trial and received the H5N1 VN 2004 ca vaccine intranasally. With the exception of 2 study subjects (ID31 and ID41), the remaining 19 study subjects received a second dose of vaccine approximately 50 days later. As shown in **Figure 1**, blood samples were taken from each study subject at four time points (TP): pre-vaccination (first TP), 7 days after the first dose (second TP), 1 day prior to the second dose (third TP), and 7 days after the second dose (fourth TP). Stored frozen PBMCs were used for this study. Infection with vaccine virus occurred in 12 study subjects.

### H5N1 pLAIV Vaccine Boosts Influenza-Specific T Cell Responses

Significantly elevated T cell responses were observed to H5 HA ($p = 0.0068$, **Figure 2A**) after first and second dose of vaccine; elevated T cell responses to M and NP proteins were also observed (**Figure 2B**, $p = 0.036$) (**Figure 2B**). We found that 12 of 21 study subjects showed elevated T cell responses to the highly conserved M and NP proteins after the first and/or second dose of pLAIV, regardless of whether they had confirmed vaccine virus infection (**Figure 2C**). These responses did not correlate with the antibody responses following ISIV boost administered in a follow-up study (18).




C

| Protein | ID | ELISPOT data: SFU/million PBMCs | | | | Infection | Seroconversion after H5N1 ISIV[a] |
|---|---|---|---|---|---|---|---|
| | | 1st TP | 2nd TP | 3rd TP | 4th TP | | |
| **M** | 22 | 23 | **90*** | **105*** | **192*** | N[b] | P[c] |
| | 26 | 90 | 27 | 25 | **75*** | N | n/d |
| | 30 | 3 | n/a[d] | **38*** | 0 | N | P |
| | 28 | 42 | n/a | 10 | **45*** | P (1st/2nd) | n/d |
| | 37 | 55 | 23 | 28 | **73*** | P (2nd) | n/d |
| **NP** | 22 | 45 | 57.5 | **100*** | **145*** | N | n/d |
| | 23 | 87.5 | 92.5 | 37.5 | **205*** | N | n/d |
| | 24 | 230 | **502*** | 232 | 263 | N | n/d |
| | 26 | 93 | 53 | n/a | **140*** | N | n/d |
| | 33 | 20 | 18 | **83*** | **110*** | N | P |
| | 35 | 75 | 143 | 45 | **113*** | N | N |
| | 36 | 108 | **215*** | 143 | 145 | P (1st/2nd) | N |
| | 39 | 53 | **125*** | 38 | 58 | P (2nd) | P |
| | 40 | 93 | **220*** | 88 | 123 | P (1st) | P |

[a] ISIV = inactivated H5N1 subvirion influenza vaccine. [b] N = negative. [c] P = positive.
[d] n/d: Not done. Subject did not receive ISIV. [e] n/a: Not available due to limited cell number.
*≥2 fold increment after first or second dose of pLAIV

**FIGURE 2 | Elevated T cell responses after each vaccination**. T cell responses at all four time points (TP) were screened by *ex vivo* IFN-γ ELISPOT using overlapping peptides fromH1 HA, H3 HA, H5 HA, and H5N1 VN 2004 Matrix proteins (M1 and M2) and Nucleoprotein. $n = 2$ replicates. **(A)** Comparison of T cell responses targeting H5 HA peptides pre- and post-first and second vaccination ($n = 21$). **(B)** Comparison of T cell responses targeting internal proteins M ($n = 20$) and NP ($n = 19$) pre- and post- first and second vaccination. *p*-values were calculated using the Wilcoxon matched-pairs signed rank test. **(C)** Study subjects who showed >2-fold elevated T cell responses to M and NP peptides after vaccination.

## The H5N1 pLAIV is able to Stimulate Cross-Reactive T Cell Responses with an Effector Phenotype, Specific to Internal Viral Proteins

We next evaluated the phenotype of antigen-specific $CD8^+$ T cells by staining PBMCs with an MHC class I tetramer specific to an HLA-A0201-restricted M1 protein (58–66) epitope and a panel of antibodies specific for cell activation and cytotoxicity markers. **Figure 3A** displays the gating strategy used in flow cytometry. T cells from two study subjects (ID36 and ID42) who were infected with the vaccine virus were positively stained with this tetramer. **Figure 3B** clearly demonstrates that the proportion of $CD8^+$ $tetramer^+$ T cells increased after vaccination. In study subject ID36, the $CD8^+$ $tetramer^+$ T cells expanded from 0.038 to 0.067% after the first dose of vaccine. Although the size of the antigen-specific T cell population shrank slightly thereafter, from 0.067% 7 days after the first dose of vaccine to 0.057% 7 days following the second dose of vaccine, it was still greater than the baseline level. In study subject ID42, the $CD8^+$ $tetramer^+$ T cells were boosted after each dose of vaccine, with an approximately 0.05% increase post-vaccination. The number of antigen-specific T cells increased, and there was an enhancement in expression levels of cell activation and cytotoxicity molecules, such as CD38, HLA-DR, and perforin, on the T cells (**Figure 3C**), indicating that the H5N1 pLAIV could boost $CD8^+$ T cells specific to internal viral proteins with effector functions. Moreover, we also observed stronger systematic activation of $CD8^+$ T cells from study subjects who were infected with the vaccine virus. As shown in **Figure 3D**, the expression level of CD38 on the surface of $CD8^+$ T cells was higher after each dose of vaccine compared to pre-vaccination levels.

## Elevated HA-Specific T Cell Responses to Seasonal Influenza Viruses with Low Cross-Reactivity to H5N1 HA Peptides

We also observed elevated T cell-specific responses to HA proteins of seasonal influenza viruses (H1 and H3) in 6 out of 21 subjects following receipt of the H5 pLAIV vaccine (**Figure 4A**). The

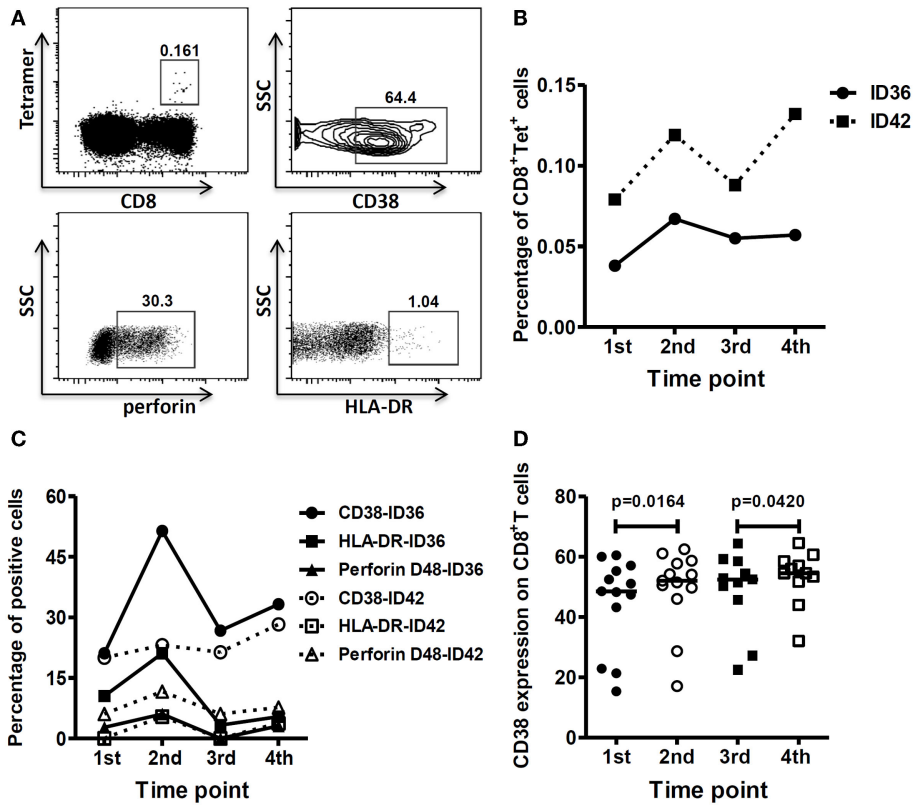



**FIGURE 3 | HLA-A*0201 $M1_{58–66}$-specific T cells were boosted and showed better effector function after each vaccine dose**. **(A)** Gating strategy. Gating of $CD8^+$ $tetramer^+$ cells, $CD8^+$ $CD38^+$ cells, $CD8^+$ $perforin^+$ cells, and $CD8^+$ $HLA\text{-}DR^+$ cells. The gating of $CD38^+$, $Perforin^+$, and the $HLA\text{-}DR^+$ population on antigen-specific T cells is same as the gating on $CD8^+$ T cells. The percentage of $tetramer^+$ cells shown is within $CD8^+$ T cells. **(B)** Frequency of HLA-A*0201 $M1_{58–66}$ tetramer positive cells before and after each vaccine dose. **(C)** Expression of activation markers (CD38 and HLA-DR) and cytolytic marker (Perforin D48) on HLA-A*0201 $M1_{58–66}$ tetramer positive cells before and after each vaccine dose. Lines represent the percentage of tetramer positive cells with the noted markers. **(D)** Comparison of CD38 expression on $CD8^+$ T cells before and after each vaccine dose in the vaccine virus-infected group (first vaccination: $n = 13$; second vaccination: $n = 11$). *p*-Values were calculated using the Wilcoxon matched-pairs signed rank test. For the scatter dot plots, the line represents the median value.

enhanced responses to seasonal influenza virus HAs, particularly H1 HA, were higher than the responses to the H5 HA protein (**Figure 4B**), indicating that the H5N1 pLAIV preferentially boosted T cell responses to seasonal influenza HA proteins rather than H5N1 HA in some individuals.

As illustrated in **Figure 5A**, both study subjects ID23 and ID24 displayed responses to peptide HA1-56 from the H1 HA protein, and the responses were mainly elicited by $CD8^+$ T cells. However, our *ex vivo* ELISPOT data showed that the T cells did not recognize the corresponding peptide from the H5 HA, known as HA5-59 (**Figure 5B**). Subsequently, we generated $CD8^+$ T cell clones specific to the H1 HA1-56 peptide from study subject ID24 and tested their cross-reactivity to the H5 HA5-59 peptide. As shown in **Figures 5C,D** and Figure S1 in Supplementary Material, all three T cell clones were capable of degranulation and producing IFN-γ and TNF-α when stimulated by peptide HA1-56. However, they did not show any responses to peptide HA5-59, even at a high peptide concentration. These data suggest that T cell responses to seasonal influenza HA proteins had low-level cross-reactivity to the H5 HA, an example of original antigenic sin (OAS) for T cells.

## High Pre-Existing Cross-Reactive Responses to Internal Influenza Virus Proteins do not Restrict Infectivity of the Vaccine Virus

On screening for T cell responses to internal influenza virus proteins, we observed strong cross-reactive responses to internal proteins of the H5N1 virus, especially M and NP, in some infected study subjects prior to immunization. For example, as illustrated in **Figure 6A**, study subjects ID27 and ID32 who were infected with the H5N1 pLAIV vaccine, showed strong T cell responses to the M protein before vaccination, with a level of IFN-γ production $>600$ SFU/$10^6$ PBMCs. Among the study subjects infected with the pLAIV, pre-existing T cell responses targeting the NP protein were also detected in ID27, ID32, and ID34, with a magnitude $>250$ SFU/$10^6$ PBMCs. There was no significant difference in the pre-existing T cell responses targeting internal viral proteins M and NP between the pLAIV-infected and un-infected groups (**Figures 6B,C**, and data not shown). These data indicate that high pre-existing cross-reactive responses to internal influenza viral proteins are unlikely to have played a role in restricting the infectivity of the vaccine virus.

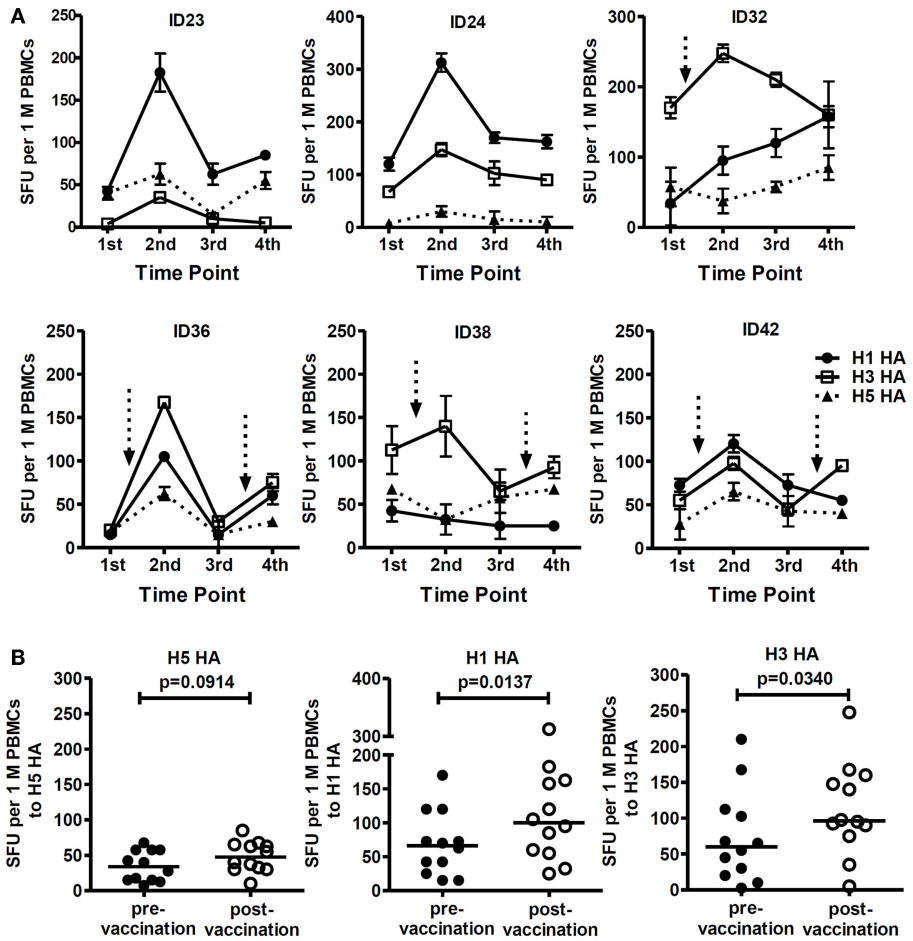



**FIGURE 4 | T cell responses to seasonal influenza HA peptides were elevated after the first and second dose of the pLAIV**. **(A)** T cell responses to H1 HA, H3 HA, and H5 HA peptides at all four time-points in six study subjects. $n = 2$ replicates; Arrows represent documented vaccine virus infection. **(B)** Comparison of T cell responses targeting H5 HA, H1 HA, and H3 HA pre- and post- first and second vaccination, $n = 6$. $p$-Values were calculated using the Wilcoxon matched-pairs signed rank test. For the scatter dot plots, the line represents the median value.

## Discussion

Vaccination with the H5N1 pLAIV stimulated modest influenza-specific T cell responses in most vaccine recipients. The responses were in both $CD4^+$ and $CD8^+$ T cells and the T cells showed evidence of cytolytic function. There was no relationship between the T cell responses and other evidence that of pLAIV vaccine infection, either by PCR-detected shedding or a fourfold rise antibody titer. Although the H5N1 pLAIV was highly restricted in replication and was poorly immunogenic in the phase I clinical trial, we recently showed that the H5N1 pLAIV induced long-term immune memory (18). We detected a high titer, rapid antibody response in most of the study subjects following the administration of a single dose of an H5N1 inactivated subunit influenza vaccine (ISIV) almost 5 years after the initial H5N1 pLAIV (18). Interestingly, pLAIV priming of these antibody responses occurred even in the absence of significant vaccine virus shedding and immunogenicity measured by traditional end points in the initial phase I clinical trials of the H5N1 pLAIV (18). In the current study, indications of antigen exposure by significantly elevated T cell responses were observed after the first and/or second dose of pLAIV in most volunteers; these responses did not correlate either positively or negatively with the antibody responses following ISIV boost. In a separate study in Vietnam, we have detected H5N1-specific T cell responses in a village cohort with H5N1 virus exposure, regardless of the detection of antibodies (22). Thus, it is likely that exposure to infectious influenza virus can sometimes stimulate $CD8^+$ T cell responses without inducing antibody responses or infecting sufficient cells in the respiratory tract to be detectable by PCR or virus culture. Detection of influenza virus-specific T cell responses may serve as an additional marker for subclinical H5N1 virus infection in humans.

T cell immune responses were detected targeting internal viral proteins, which are highly conserved between different influenza virus strains. These highly cross-reactive T cells are likely to confer broader or potentially "universal" protection against a wide range of influenza viruses (19). T cell responses, especially cross-reactive T cell responses, correlate with protection in several studies, including our own (14, 15). Although very low antibody

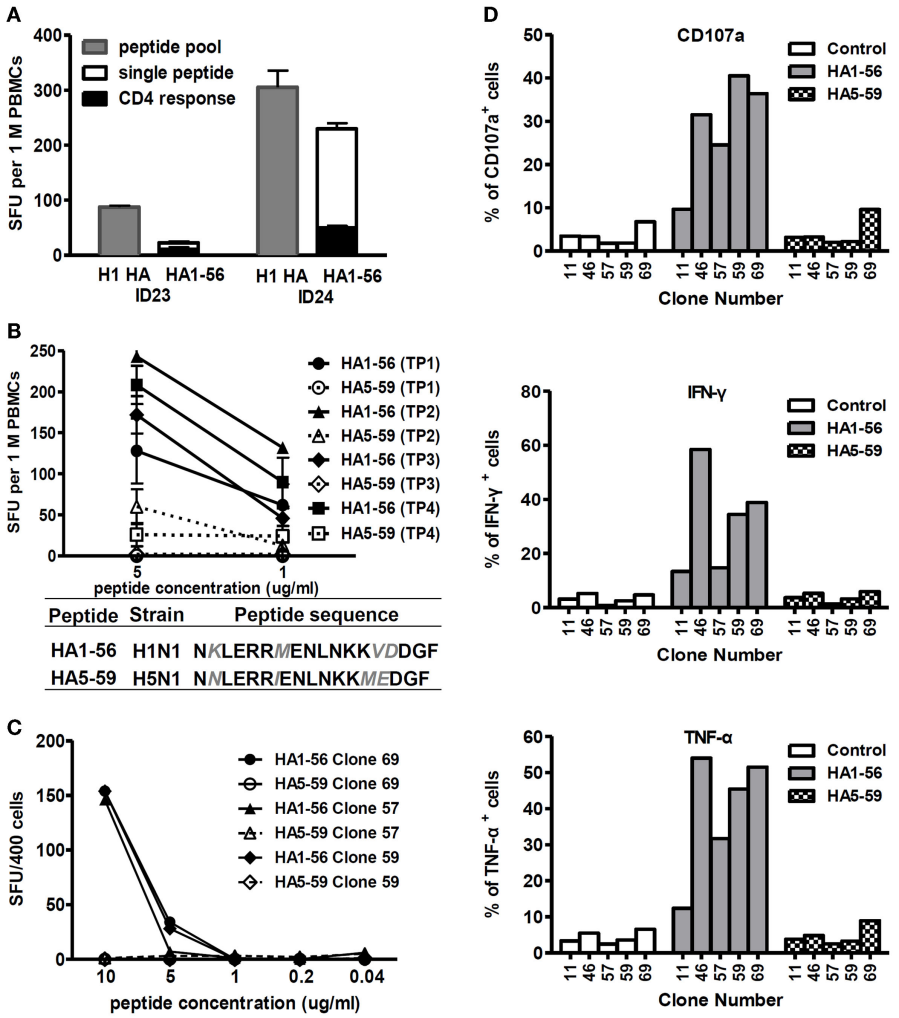



**FIGURE 5 | T cell responses to seasonal influenza HA proteins showed a low level of cross-reactivity to H5 HA**. **(A)** T cell responses to peptide pools, single peptide, and responses to single peptide elicited by $CD4^+$ T cells from study subjects ID23 at time point 2 and ID24 at time point 4. $n = 3$ replicates. **(B)** Cross-recognition of peptides HA1-56 and HA5-59 by T cells from study subject ID24 at all four time points. $n = 3$ replicates. Amino acids differing between HA1-56 and HA5-59 are shown in gray italics. **(C,D)** Cross-recognition of peptides HA1-56 and HA5-59 by T cell clones generated from study subject ID24. The cross-reactivity of T cell clones was assessed by IFN-γ ELISPOT using titrated peptides **(C)**, degranulation (CD107a expression), and intracellular TNFα and IFN-γ production **(D)**.

responses were detected in pLAIV study subjects (9), our results showed elevated T cell responses in >60% of study subjects in response to at least one influenza virus internal protein (mostly to M and NP protein, >2-fold increase) after the first and/or second dose of vaccine. However, some increased responses were seen 50 days after the first vaccination but not at 7 days, suggesting that further optimization of the timing of the T cell assays after LAIV administration might be needed. Taken together, the advantages and potential of evaluating T cell responses against internal viral proteins, especially M and NP, along with neutralization and HAI antibody responses might be considered for future evaluation of vaccine immunogenicity.

Elevated HA-specific T cell responses to seasonal H1 and H3 influenza viruses, with low cross-reactivity to H5N1 HA peptides were detected in study subjects after the first or second dose of H5N1 pLAIV, suggesting an "original antigenic sin" phenomenon. Original antigenic sin in T cells in humans was first described for dengue viruses by Mongkolsapaya et al. (23) and implies that the response to a secondary infection by a dengue virus is dominated by the proliferation of cross-reacting memory T cells induced by primary infection with a different viral strain, which is of lower affinity for the secondary viral antigen. However, whether this will be to the benefit or the detriment of the host remains unanswered.

Finally, the presence of high level pre-immunization T cell responses in three volunteers did not prevent boosting of T cell responses. Therefore, this does not appear to be the reason why the pLAIV did not infect all the study subjects. However, as discussed above, the vaccine boosted T cell responses in the absence of detectable virus shedding or a rise in antibody titer. It is likely therefore that low-level infection by the attenuated pLAIV rather variably stimulates both T and B cell responses.

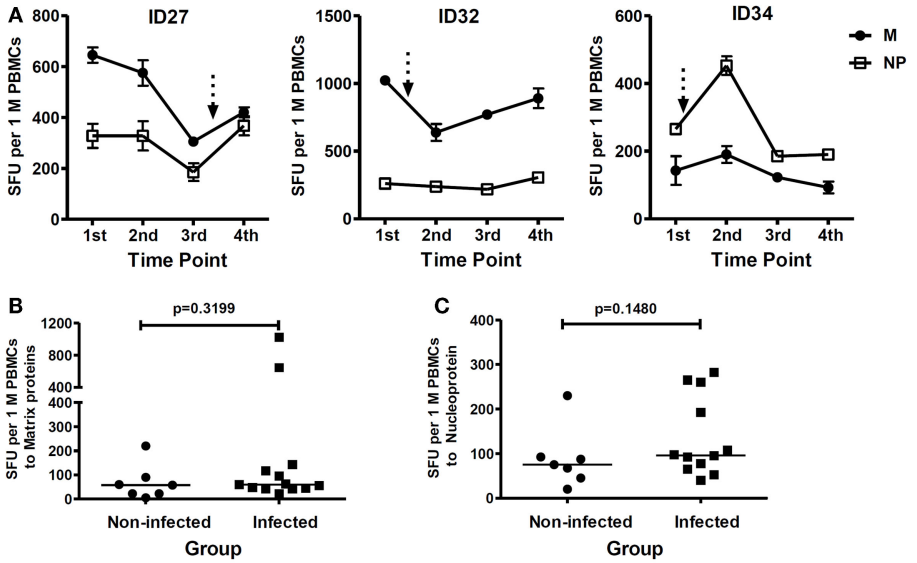



**FIGURE 6 | T cell responses targeting H5N1 internal viral proteins.** **(A)** Examples of the study subjects from the vaccine virus-infected group (ID27, ID32, and ID34) who showed high pre-existing cross-reactive T cell responses. $n = 2$ replicates. Arrows represent documented vaccine virus infection. **(B)** Comparison of pre-existing cross-reactive T cell responses targeting the viral M proteins between the vaccine virus-infected ($n = 13$) and un-infected ($n = 7$) study subjects. **(C)** Comparison of pre-existing cross-reactive T cell responses targeting NP protein between the vaccine virus-infected ($n = 12$) and un-infected ($n = 7$) study subjects. Study subject *ID29* was excluded because of limited cell numbers. *p*-values were calculated using the Mann–Whitney test. For the scatter dot plots **(B,C)**, the line represents the median value.

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## Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00287/abstract

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# Immunogenicity and clinical efficacy of influenza vaccination in pregnancy

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Pregnant women are at high risk from influenza due to disproportionate morbidity, mortality, and adverse pregnancy outcomes following infection. As such, they are classified as a high-priority group for vaccination. However, changes in the maternal immune system required to accommodate the allogeneic fetus may alter the immunogenicity of influenza vaccines. A large number of studies have evaluated the safety of the influenza vaccine. Here, we will review available studies on the immunogenicity and efficacy of the influenza vaccine during pregnancy, focusing on both humoral and cellular immunity.

**Keywords: influenza, pregnancy, inactivated influenza vaccine, immunogenicity, antibody**

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## Introduction

Pregnant women are at increased risk of severe disease secondary to influenza infection, particularly during influenza pandemics. In the 1918 influenza pandemic, maternal mortality was 27% (1), and in one report from the1957 pandemic, half of the fatal cases in reproductive-aged women were among those that were pregnant (2). Rates of hospitalization were higher among pregnant than non-pregnant reproductive-aged women during the pandemics of 1918, 1957, and 2009 (1–3). Though the increased rate of hospitalization in pregnant women is substantially less pronounced during non-pandemic years, pregnant women are still at increased risk from seasonal influenza (4, 5), particularly during the third trimester (6).

As early as 1962, the U.S. Public Health Service identified pregnant women as a priority group for influenza vaccination. However, questions then and now have been raised about how pregnancy alters the quality of the immune response to influenza vaccination. It is generally believed that alterations in immune function contribute to increased influenza severity during pregnancy. Logically, it has also been hypothesized that vaccination during pregnancy may result in a less favorable immunologic response. Immunomodulation during pregnancy has been the subject of several recent reviews (7–10). Here, we will focus, instead, on reviewing the history and evidence on the immunogenicity of the influenza vaccine during pregnancy and its clinical efficacy.

## Assessment of Immunogenicity

To place the existing studies in context, a brief review of the methods used to assess the immune response to vaccination, or immunogenicity, is warranted. The "gold standard" method is the hemagglutinin inhibition (HI) titer, which measures the concentration of antibody required to prevent influenza from agglutinating red blood cells. Thus, the HI titer is a measure of the total amount of antibodies to the hemagglutinin (HA) protein. The WHO defines a "protective" titer as 1:40, based on a 50% reduction in disease, and thus the term seroprotection refers to those individuals with a titer of 1:40 or better (11). Seroconversion is defined as an increase in HI titer following vaccination of fourfold or greater. Virus microneutralization (VMN) assays measure the

ability of antibodies in serum to prevent a specific strain of influenza from infecting a cell line, typically Madin–Darby canine kidney epithelial cells. This assay therefore measures the functional capability of antibodies at a specific dilution, rather than just the total quantity. In settings of impaired host immunity, such as HIV infection, the VMN titer is more sensitive than the HI titer (12); VMN is also better for detecting antibodies to avian influenza viruses (13). Multiple studies have demonstrated good correlation between HI titers and VMN titers in pregnant women following monovalent H1N1 vaccination (14, 15), and now seasonal influenza vaccination (16).

## Immunogenicity of Influenza Vaccine in Pregnant Women

In 1962, when a resurgence of the 1957 A2 pandemic influenza strain was anticipated, the U.S. Public Health Service identified pregnant women as a priority group for vaccination based on their historically poor outcomes. However, due to concerns that the same immune alterations that led to increased morbidity could compromise the immune response to the vaccine during pregnancy, Hulka compared the immunogenicity of the vaccine between pregnant and non-pregnant women (17). This placebo-controlled clinical trial compared immune responses after two doses of inactive polyvalent influenza vaccine containing 200 U of A2 antigen and placebo. Overall, in those receiving the vaccine, pooled and individual complement fixation titers were similar between pregnant and non-pregnant women. Further, pooled titers in the non-vaccinated groups were also similar, though they rose at a later time point following circulation of influenza virus in the community. Interestingly, in this study, there was only a marginal, and not-statistically significant, decrease in the rates of influenza-like illness in those receiving the vaccine, whether pregnant or not. While these results indicate that pregnancy did not appear to compromise the immunogenicity of the vaccine, Hulka also did not observe increased disease severity in pregnant women during the 1962 season (17). Consistent with these results, there was no evidence of increased morbidity or mortality in pregnant women from 1958 to 1962 (18). There was also no evidence of increased morbidity and mortality during the 1968 pandemic, which had variable global penetration (19). Thus, the risks posed to pregnant women by influenza may differ according to the circulating strain in a given year. This is an important consideration in evaluating immunogenicity, because it remains possible that differences in the immune response to vaccination could also differ based on vaccine strain.

Without clear evidence for increased morbidity and mortality among pregnant women in 1960s, influenza vaccination of pregnant women during seasonal epidemics was deemphasized as a public health approach until 1976–1977, when a novel influenza strain with pandemic potential emerged at Fort Dix, NJ, USA. The influenza outbreak was ultimately confined to the military base, but in preparation for its spread, approximately 25% of the U.S. population was vaccinated with a novel monovalent A/New Jersey/8/76 (Hsw1N1) vaccine. Vaccine responses to this novel monovalent vaccination were compared between pregnant women and non-pregnant women by HI titers and by using 2-mercaptoethanol treatment to assess the amount of IgM antibody (20). As with the prior study by Hulka, no significant differences between pregnant and non-pregnant women were observed in the geometric mean titers (GMT) following vaccination, nor was there a significant difference in the mercaptoethanol IgM reduction indicative of antigen-specific IgM. Together, these two studies suggested that pregnant women had vaccine responses on par with those of non-pregnant women.

In 1990s and early 2000s, increased attention was given to vaccination of pregnant women. In 1997, the American Committee on Immunization Practice recommended seasonal influenza vaccination for pregnant women in the second and third trimesters, and in 2004, this recommendation was modified to include all pregnant women. In 2008, a large randomized-controlled trial of vaccination in pregnant women in Bangladesh demonstrated that influenza vaccination was clinically efficacious in preventing influenza in pregnant women and their infants (21). The immunogenicity data from this trial were subsequently released in 2010 (22). In this study, there was no non-pregnant control group, but the pregnant women had significant increases in their GMTs to the H1N1, H3N2, and influenza B strains following vaccination, and seroconversion rates of 83.6% for H1N1, 69.2% for H3N2, and 39.7% for B influenza strains (22), which are similar to those seen among healthy adults receiving seasonal influenza vaccination.

Early reports that pregnant women were experiencing disproportionate morbidity and mortality during the 2009 H1N1 pandemic prompted renewed interest in the immunogenicity of the inactivated influenza vaccine (IIV) in pregnant women (3). Multiple studies evaluated immune responses to the monovalent pH1N1 vaccination during pregnancy. Ohfuji et al. enrolled 150 pregnant women receiving the thimerosal-free monovalent pH1N1 (15 μg) vaccination during pregnancy (23). Immune responses were tested by HI titers 3 weeks after the first dose and 4 weeks after the second dose, controlling for body mass index, age, and the receipt of the 2009 seasonal influenza vaccination. Robust responses were noted to the initial vaccination, with an average HI antibody increase of more than 10-fold and a seroconversion rate of 91%. The second vaccination conferred little additional benefit. Importantly, it was noted that receipt of seasonal influenza vaccination <19 days prior to pH1N1 vaccination significantly reduced the fold increase in titer ($p = 0.021$).

A similar study by Tsatsaris et al. enrolled 110 women equally divided between the second and third trimester of pregnancy who received a single dose of monovalent pH1N1 containing 15 μg of HA (15). Subjects were evaluated with HI and VMN assays pre-vaccine and at 21 and 42 days after vaccination. Infant cord-blood titers were also assessed. Pregnant women responded robustly in this study as well: 21 days post-vaccination 98% of women had HI titers of >1:40 (seroprotection) and 93% had seroconverted. It was again noted that women with prior seasonal influenza vaccination had lower fold increases in GMT. In this study, a lower HI GMT was observed in women with twin pregnancy ($p = 0.006$), although it is unclear whether this is secondary to decreased production or increased placental transfer of antibody. Maternal and cord-blood titers were highly correlated ($r = 0.86$). Lastly, VMN titers correlated significantly with HI titers ($r = 0.96$).

Jackson et al. also evaluated responses to the monovalent H1N1 vaccine in pregnant women, investigating whether pregnant women responded differently to a vaccination containing 49 μg HA as compared to 25 μg HA (14). HI and VMN assays were again used to evaluate vaccine responses. Following the first vaccination, 93% of women had titers of >1:40 (seroprotection). The second vaccination did not significantly increase antibody titers, and there was no benefit to using the higher antigen content vaccine. As with Tsatsaris et al., VMN and HI responses were significantly correlated ($r = 0.81$) following vaccination. However, unlike the other two studies, prior receipt of seasonal influenza vaccination was not associated with decreased responses to the monovalent vaccine. Zuccotti et al. published a study of adjuvanted pH1N1 in pregnant women that resulted in titers consistent with seroprotection in 100% of pregnant women, but did not include a pre-vaccine timepoint (24). Overall, though none of these studies included non-pregnant women, the percentage of pregnant women seroconverting and achieving protective titers was similar to studies of the pH1N1 monovalent vaccine performed on non-pregnant men and women (25, 26). However, comparisons with cohorts including men and women are limited by the fact that non-pregnant women respond more robustly than men to influenza vaccination (27, 28). Despite this caveat, as a whole, these studies did not reveal any immunologic deficits, as measured by HI and VMN, to the monovalent pH1N1 vaccination in pregnant women.

Several studies have evaluated vaccine responses to modern seasonal IIV in pregnant women, although only two have included non-pregnant women as a control group. Sperling et al. performed a large multiyear study of 239 pregnant or postpartum women vaccinated with the seasonal influenza vaccine between October 2006 and January 2010, in addition to monovalent H1N1 vaccination in 2009–2010 (29). Overall, the timing of vaccination during pregnancy did not significantly alter HI GMT responses, although there was a trend toward lower responses in the first trimester and 6 weeks postpartum. Seroprotection for H3N2 ranged from 65 to 95% and for H1N1 from 75 to 98%, with higher baseline titers and receipt of vaccination in the prior year associated with lower rates of seroconversion. They found that antibody responses were dominated by IgG1 regardless of trimester.

Along similar lines, Madhi et al. evaluated clinical efficacy and immunogenicity of seasonal trivalent IIV in 2011 and 2012 in South African pregnant women between 20 and 36 weeks gestational age compared to placebo. HIV-uninfected pregnant women responded robustly to IIV with high rates of seroprotection to all vaccine strains (30), though responses were not compared to those of non-pregnant women. In the HIV-infected cohort, the percentage of women with seroprotection was lower; however, HI titers in this group may have underestimated vaccine efficacy, which was 70.6% to confirmed influenza infection. VMN assays were not performed but given prior data on HI titers in HIV infection (12), it is not surprising that HI titers may have underestimated efficacy in this group. Importantly, there was no increase in HIV viral load following maternal vaccination.

Schlaudecker et al. compared HI responses to the 2011–2012 seasonal IIV3 between pregnant women ($n = 29$) and non-pregnant women ($n = 22$) of similar ages (31). They found that while pregnant and control women achieved seroconversion and seroprotection at similar rates, pregnant women had lower post-vaccination GMTs to A/California (H1N1) ($p = 0.027$) and A/Perth (H3N2) ($p = 0.037$). This cohort was unique with respect to prior vaccination history in that 97% of pregnant women and 96% of non-pregnant women had received the influenza vaccination in the previous year. This may suggest that pregnant women are less able to mount serologic responses to previously encountered influenza antigens. However, this would seem to contradict epidemiological data suggesting only subtle differences in disease severity following seasonal influenza infection during pregnancy.

Kay et al. evaluated responses to the 2012–2013 seasonal IIV3 in pregnant women ($n = 20$) and non-pregnant women ($n = 18$) of similar ages. The cohorts were matched by age, but the non-pregnant women were more likely to have received prior influenza vaccinations and had higher baseline HI titers. In contrast to the findings of Schlaudecker et al., pregnant women in this study had equivalent post-vaccination HI titers to those of non-pregnant women for pH1N1 and B/Wisconsin and higher HI fold-change, even after controlling for baseline titers, for the pH1N1 and B/Wisconsin strains ($p = 0.016$ and $p = 0.014$, respectively). This study included VMN titers, which significantly correlated with HI titers across all strains tested. Unlike the HI titers, there were no significant differences in VMN response between pregnant and control women after controlling for baseline VMN titer. Further, pregnant women had higher total IgG levels before immunization ($p = 0.042$) but not following vaccination. Thus, no deficit in the quantity or quality of the antibody response to seasonal IIV was noted in pregnant women in this study.

Kay et al. also assessed the induction of plasmablasts, antibody-secreting, activated B cells, in pregnant and non-pregnant women, relying on data collected in the 2010–2011, 2011–2012, and 2012–2013 seasons. Pregnant women had a significantly greater induction of plasmablasts following vaccination than did non-pregnant controls ($p = 0.03$), though this difference was no longer significant when comparing a small number of pregnant and control women from the 2012 to 2013 influenza season alone. In this study, pregnancy remained predictive of increased plasmablast induction after controlling for baseline average HI titer, suggesting an enhanced induction of antibody-secreting B cells during pregnancy.

Overall, these data suggest that the immunogenicity of IIV, based on the induction of antibodies, is similar in pregnant and non-pregnant women. However, differences in vaccine responses to different influenza strains may complicate our ability to assess subtle changes in responses among pregnant women. Further, responses vary based on prior vaccination or exposure, and pregnant women may mount less robust antibody responses to a secondary challenge. The comprehensive data from pH1N1 monovalent vaccination suggests that pregnant women mount robust antibody responses to a novel influenza vaccine.

## Induction of Cellular Responses

Most studies of influenza vaccine immunogenicity have focused on the induction of antibodies as the correlate of protection.

However, several recent studies have evaluated cellular immune responses within peripheral blood mononuclear cells (PBMCs). Forbes et al. compared the induction of cytokines by ELISA, cytometric bead array, and mRNA levels between pregnant and non-pregnant women whose PBMCs were cultured for 48 h with pH1N1 (32). Production of interferon protein and mRNA was reduced in pregnant women who were unvaccinated ($N = 12$) compared with healthy controls, suggesting a deficit in interferon induction during pregnancy. However, interferon production normalized in pregnant women that had undergone pH1N1 vaccination during the prior 12 months, suggesting that vaccination could rescue this defect. Supporting the idea that pregnant women had decreased interferon production, expression of the mRNA encoding protein kinase R, an early interferon stimulating gene, was reduced in pregnant women (32). There were no differences between pregnant and non-pregnant women in the expression of genes encoding the toll-like receptor-3 (TLR3), TLR7, and TLR 9, nor was there a difference in the ability of PBMCs from pregnant or non-pregnant women to support viral replication (32). Subsequently, Vanders et al. found that the percentage of plasmacytoid DCs was reduced in pregnant women and that PDL-1, CD86, and HLA-DR are upregulated on plasmacytoid DCs in pregnancy (33). Blocking antibodies to PD1/PDL1 in pH1N1 PBMC cultures from pregnant women resulted in increased production of IFN-α and IFN-γ, suggesting that deficits in interferon production during pregnancy could be rescued by blocking these inhibitory pathways.

Recently, Kay et al. evaluated NK and T cell responses of pregnant women ($n = 21$) and controls ($n = 29$) to pH1N1 and H3N2 infection of PBMCs *ex vivo* for 7 h (34). Consistent with earlier data, pregnant women had lower IFN-γ production, as measured by intracellular cytokine staining, than did non-pregnant women following stimulation of PBMCs with phorbol 12-myristate 13-acetate and ionomycin. However, in response to influenza infection, the NK and T cells from pregnant women had significantly increased MIP-1β production and enhanced polyfunctional NK and T cell responses compared to non-pregnant women. In this study, vaccination did not significantly affect T or NK cell cytokine and chemokine responses in pregnant women or controls. The assay performed in this study was of shorter duration and used a higher multiplicity of infection than did the assay described by Forbes et al. and Vanders et al. In addition, it is likely that both the pregnant and control women in this study could have been either previously infected by or vaccinated against pH1N1.

In addition to intracellular cytokines, researchers have also evaluated the impact of pregnancy on serum cytokines before and after IIV. Christian et al. compared serum levels of IL-6, TNF-α, IL-8, IL-1β, and migration inhibitory factor (MIF) in 28 pregnant women (average weeks gestational age = 28.4) and 28 non-pregnant healthy women immediately prior to IIV and 1, 2, and 3 days following vaccination (35). Baseline levels of IL-8 and MIF were significantly higher in non-pregnant women. There was no difference in pregnant vs. non-pregnant women in IL-6, TNF-α, or IL-1β responses to vaccination. Pregnant women experienced an increase in MIF levels and no change in IL-8 levels, while non-pregnant women had decreases in both post-vaccination. This group also evaluated HI responses pre- and post-vaccination and found no difference in seroconversion or seroprotection between groups.

Overall, additional study of cellular responses is needed to understand how pregnancy modifies these responses, as a clear picture has not yet emerged. Some data would suggest a deficiency in interferon production, yet other inflammatory pathways may be elevated in response to influenza infection and vaccination during pregnancy. These differences could well be a result of the specific cell types being assessed, kinetic variations in the immune response, or disparities in prior exposure to the influenza strains studied.

## Vertical Antibody Transfer

Vertical transfer of maternal antibodies to the fetus is of equal importance when evaluating influenza vaccine immunogenicity in pregnant women. To this end, Sumaya et al. investigated the immunogenicity of the 1976 monovalent A/New Jersey/8/76 (Hsw1N1) influenza vaccine in 26 maternal serum and cord-blood pairs at the time of delivery. A titer of ≥20 by HI was considered protective against influenza in this study. The GMT of newborn cord bloods was 23.6 and 54% of specimens had protective titers. The GMT of maternal serum was 35.8 and 73% had protective titers. Newborn titers were not significantly affected by the trimester of maternal vaccination (second vs. third). However, the antibodies waned in the infants by 3 months following delivery, when only 12% of infants but 92% of mothers had protective titers. The magnitude of the maternal antibody response correlated strongly with the infant's antibody titer at 3 months of age ($r = 0.77$, $p < 0.01$). Thus, the authors concluded that passive transfer of antibody did occur, though it appeared to be relatively short-lived. A second study by Englund et al. evaluated placental transfer of maternal antibody to tetanus toxoid and seasonal IIV (36). The 13 women vaccinated with seasonal IIV had robust antibody responses to all three strains as measured by ELISA. The infants had comparable levels of antibody at birth with infant/mother antibody ratios of between 94 and 99% for all three strains (36).

In 2009, Tsatsaris et al. evaluated cord-blood titers in addition to maternal immunogenicity and found that maternal and cord-blood titers correlated ($r = 0.86$). Infant titers of 1:40 or greater were observed in 95% [confidence interval (CI) 89–99%] of the 88 cord-blood samples tested, and cord-blood titers were significantly higher than maternal blood titers. Neither gestational age at vaccination nor delivery significantly affected the neonatal seroprotection rates. Similar results were obtained by Jackson et al. (14). Cord-blood HI GMTs were higher than maternal titers at both vaccine doses and significantly so for the 49 μg dose group ($p = 0.002$). In this study, there was a trend toward lower cord-blood titers with longer intervals between the time of vaccination and delivery. In both of these studies, the cord-blood titers were higher than maternal titers, suggesting active transfer of antibodies across the placenta.

Further confirmation of vertical antibody transfer has come from the randomized-controlled trials of influenza vaccine efficacy in pregnant women. In the study performed in Bangladesh, there was no difference in maternal and infant cord-blood HI

**TABLE 1 | Studies evaluating IIV and monovalent pH1N1 vaccine responses during pregnancy**.

| Reference | Vaccine, years | Study participants | Immune assays and outcomes | Vaccine response | Summary |
|---|---|---|---|---|---|
| **IMMUNOGENICITY OF MODERN SEASONAL INACTIVATED INFLUENZA VACCINE (IIV) IN PREGNANT WOMEN** | | | | | |
| Steinhoff et al. (22), Zaman et al. (21) | Seasonal IIV 2004 | 340 pregnant Bangladeshi women in the third trimester | HI titers pre- and post-vaccination. Influenza disease endpoints | Seroprotection for H1N1 88%, H3N2 98%, and B 45%. Ratio of maternal to infant titers at delivery ranged from 0.7 to 1.7 | High rates of seroconversion and seroprotection following IIV in pregnant women. Reduction in febrile respiratory illness in mothers. Reduction in laboratory-confirmed influenza in infants |
| Sperling et al. (29) | Seasonal IIV 2006–2010 and H1N1 vaccination 2009–2010 | 239 pregnant women (73 first, 183 second, 142 third Trimester, 73 immediately postpartum, 36 6 weeks postpartum | HI Titers pre- and post-vaccination to influenza A strains IgG subtyping pre- and post-vaccination | Seroprotection for H3N2 varied from 65 to 95% and between 75 and 98% for H1N1 strains. | No significant difference in seroprotection or seroconversion by trimester or postpartum. No differences in IgG subtype production in pregnancy vs. postpartum |
| Christian et al. (35) | Seasonal IIV, 2011–2012 | 28 pregnant women (average gestational age 28.4 weeks) and 28 non-pregnant women | Serum cytokines (prior to, 1, 2, and 3 days post-vaccination) and HI titers pre- and 1 month post-vaccination | Seroprotection rates in pregnant vs. control for H1N1 (89 vs. 85%), H3N2 (81 vs. 93%), and B (83 vs. 100%) were not-statistically different. There were also no significant differences in seroconversion | High rates of seroprotection and seroconversion were observed in both groups. There was not a significant effect observed secondary to vaccination in the prior year. See text for a review of the cytokine responses |
| Schlaudecker et al. (31) | Seasonal IIV, 2011–2012 | 29 pregnant women, all trimesters, 22 non-pregnant women Greater than 96% of participants received vaccine in the prior year | HI titers pre- and post-vaccination | Seroprotection H1N1 93–100%, H3N2 100%, B 58.6–68.2%. Post-vaccination H1N1 GMT 152.53 pregnant vs. 300.46 control, H3N2 GMT 142.0 pregnant vs. 241.0 control | No difference between pregnant and control groups in seroprotection, seroconversion, or fold increase. Significantly increased post-vaccination titers to H1N1 and H3N2 in control women |
| Madhi et al. (30) | Seasonal IIV, 2011–2012, 2012–2013 | 2116 pregnant women were enrolled, 1062 received IIV, and 1054 received placebo. All trimesters included. An HIV positive subset was included | HI titers pre-vaccination and 28–35 days post-vaccination. Multiple influenza disease endpoints evaluated | Seroprotection to H1N1 93.3%, H3N2 78.0%, and B 96%. Overall vaccine efficacy of preventing confirmed influenza 54.4%. Seroprotection in HIV-infected was 48.6% H3N2 and 68.6% H1N1, but vaccine efficacy was 70.6% in this subset | High levels of seroprotection for HIV-uninfected women post-vaccination. Decreased seroprotection in HIV-infected women but increased vaccine efficacy. Protection from laboratory-confirmed influenza in pregnant women and their infants |
| Kay et al. (16) | Seasonal IIV, 2012–2013 | 20 pregnant women, second and third trimesters, 18 non-pregnant women. Significantly fewer pregnant women had received the vaccination in the prior year | HI titers, VMN titers, and plasmablast identification pre- and post-vaccination | Seroprotection H1N1 100%, H3N2 94.4–100%, B 77.8–90.0%. HI and VMN titers were strongly correlated for each strain. Plasmablasts 1.32% pregnant vs. 0.46% control 7 days post-vaccination ($p = 0.03$) | No difference in post-vaccination titers in pregnant vs. control women. Increased fold-changes and decreased pre-vaccine titers in pregnant women. Possibly increased plasmablast induction in pregnant women |
| **Reference** | **Vaccine, dose** | **Study participants** | **Immune assays** | **Vaccine response** | **Summary** |
| **IMMUNOGENICITY OF MONOVALENT PH1N1 VACCINE IN PREGNANT WOMEN** | | | | | |
| Zuccotti et al. (24) | MF59 adjuvented pH1N1 monovalent vaccine (7.5 μg) | 75 pregnant women (third trimester). Infants were also enrolled through 5 months | HI titers were collected at delivery, 2 months and 5 months post-delivery. No pre-vaccination titer | Seroprotection in 100% of pregnant women at delivery, 2 and 5 months. Seroprotection in 95.6% of infants at delivery and 2 months and 81.2% at 5 months. Infant/maternal antibody ratio of 0.55 at delivery | High rates of seroprotection in pregnant women and their infants following adjuvanted pH1N1 vaccination. Persistent protective antibody levels through 5 months in infants and their mothers |

*(Continued)*

**TABLE 1 | Continued**

| Reference | Vaccine, dose | Study participants | Immune assays | Vaccine response | Summary |
|---|---|---|---|---|---|
| Ohfuji et al. (23) | Two doses of monovalent pH1N1 vaccination (15 μg) | 150 pregnant women, all trimesters | HI titers before the first vaccine, 3 weeks after the first vaccine and 4 weeks after the second dose | Seroprotection was observed in 91% after the first dose. No significant change was noted after the second dose | High rates of seroprotection were seen after one dose of the vaccine. Receipt of seasonal influenza vaccination <19 days prior to receipt of the monovalent pandemic vaccination was associated with decreased HI responses |
| Tsatsaris et al. (15) | Single dose of monovalent pH1N1 vaccine (15 μg) | 110 women equally divided between the second and third trimesters. Infant cord bloods collected | HI and VMN titers pre- and post-vaccination | Seroprotection was observed in 98% post-vaccination and 93% of women seroconverted. HI and VMN titers were highly correlated ($r = 0.96$). Maternal and infant cord-blood titers were also correlated ($r = 0.86$) | High rates of seroprotection after a single dose. Women with twins had significantly lower post-vaccination titers. Prior seasonal influenza vaccination was associated with lower fold increase. Significantly higher titers in cord blood suggesting active transport of antibody generated by IIV |
| Jackson et al. (14) | Two doses of monovalent pH1N1 vaccine at different doses (25, 49 μg) | 120 women in the second or third trimester, 60 received the 25 μg and 60 received the 49 μg vaccine. Infant cord bloods collected | HI and VMN pre and post-vaccinations | 93% of women met criteria for seroprotection after a single dose. No significant benefit to two doses or the vaccine with increased antigen content. HI and VMN correlation ($r = 0.81$). GMR of cord-blood titer/maternal titer was 1.81 in the 25 μg group and 2.96 in the 49 μg group | High rates of seroprotection after a single dose. No association with vaccine response and prior receipt of seasonal influenza. Significantly higher titers in cord blood suggesting active transport of antibody generated by IIV |

*HI, hemagglutinin inhibition assay; VMN, viral microneutralization assay; GMT, geometric mean titer; GMR, geometric mean ratio; Seroprotection, HI titer ≥1:40; Seroconversion, fourfold or greater change in HI titer post-vaccination; IIV, inactivated influenza vaccine.*

titers at the time of delivery (22). The infants whose mothers were vaccinated continued to have elevated titers when compared to infants of mothers vaccinated with pneumococcus vaccine through 20–26 weeks of life. The South African study by Madhi et al. also evaluated cord-blood titers compared to maternal titers 1 month after vaccination (30). The ratio of cord blood to maternal titers was 0.7 (0.6–0.8) for pH1N1 and for H3N2 and 0.8 (0.7–0.9) for B/Victoria. Zuccotti et al. also evaluated cord-blood titers following maternal vaccination with an MF-59 adjuvanted pH1N1 monovalent vaccine and found GMT HI titers of 141.8 at birth vs. 257.9 in the mothers, 106.5 at 2 months and 38.3 at 6 months in the infants.

In summary, IIV during pregnancy results in efficient transplacental transfer of the generated antibodies. While, most studies have demonstrated equivalent titers in mothers at delivery and cord-blood samples, some have shown elevated titers in cord blood consistent with active antibody transport (37). While some studies have hinted at lower cord-blood titers among pregnant women vaccinated in the first trimester, it is unclear if this is clinically significant. It has been demonstrated that a 2-week window prior to delivery from the time of influenza vaccination is necessary for placental antibody transfer (38). The maternal antibodies are still present at 3 months post-delivery, and wane slowly thereafter. While maternal antibodies may suppress infant responses to influenza vaccination at 1–2 months of life (39), there is no evidence to date suggesting a decreased response to influenza vaccination at 6 months of life.

## Clinical Efficacy: Maternal

There is clinical data to evaluate the efficacy of influenza vaccination in reducing the incidence of influenza during pregnancy. Two randomized-controlled trials have evaluated both maternal and infant outcomes. In the study by Zaman et al., women vaccinated with IIV during pregnancy had a 35.8% (CI 3.7–57.2) reduction in febrile respiratory illness when compared to pregnant women vaccinated with pneumococcus during pregnancy. In the study by Madhi et al., of 2116 HIV-negative South African pregnant women undergoing seasonal IIV vs. placebo, there was a 50.4% (CI 14.5–71.2) reduction in laboratory-confirmed influenza. This effect was also observed in HIV-infected pregnant women ($p = 0.03$). However, in this trial, there was not a significant difference in the more non-specific diagnosis of influenza-like-illness.

The question of clinical efficacy has also been approached through the use of large health databases. The efficacy of the monovalent pH1N1 vaccine in pregnant women was evaluated through an analysis of Norwegian health registries (40). Approximately 54% of over 113,000 eligible pregnant women received the monovalent pH1N1 vaccination. The risk of receiving a clinical diagnosis of influenza was significantly reduced in this group (adjusted hazard ratio, 0.30; 95% CI 0.25–0.34), suggesting that the monovalent pH1N1 vaccination was efficacious in pregnant women. The efficacy of seasonal influenza vaccination has also been evaluated through analysis of data from a large health plan in the U.S. (41).

This was a case–control study over two influenza seasons using a test negative design that estimated a vaccine efficacy of 44% (CI, 5–67%). This was well within the range of efficacy estimates for healthy adults in the same influenza seasons. Thus, both randomized-controlled trials and database analyses suggest that vaccination is efficacious in reducing maternal influenza.

## Clinical Efficacy: Infants

There is even more substantial evidence suggesting that maternal influenza vaccination reduces laboratory-confirmed influenza and influenza related hospitalization in infants of vaccinated mothers. This is critically important because infants <1 year of age, and particularly those <6 months of age, are at very high risk for hospitalization from influenza infection (42). Further, infants <6 months are not vaccinated themselves because IIV does not produce an adequate immune response in this age group, possibly a result of the preexisting maternal antibody (39). Fortunately, two randomized-controlled trials have revealed a significant reduction in clinical and laboratory-confirmed influenza in the infants of vaccinated mothers (21, 30). The study by Zaman et al. of 340 mothers and their infants found that maternal vaccination with IIV was associated with a vaccine efficacy of 63% (95% CI 5–85%) in preventing laboratory-confirmed influenza (21). In addition, they found a significant reduction in respiratory illness with fever, with an associated vaccine efficacy of 29% (95% CI, 7–46). Finally, maternal influenza vaccination was associated with a 42% (95% CI 18.2–58.8) reduction in infant clinic visits. Madhi et al. found that there was a vaccine efficacy rate of 48.8% (95% CI, 11.6–70.4) in preventing RT-PCR-confirmed influenza. However, there was not a difference in infants presenting with influenza-like illness or with any respiratory illness (30).

In addition to the randomized-controlled trials, several other studies suggest a benefit to the infant from maternal influenza immunization. Benowitz et al. performed a matched case–control study with case patients defined as infants under 12 months that were admitted to the hospital due to laboratory-confirmed influenza between October 2000 and April of 2009 (43). For each case, one to two infants who tested negative for influenza were also enrolled. Only 2.2% of the mothers of 91 case subjects aged <6 months had received the influenza vaccination during pregnancy as compared to 19.9% of 156 controls. The adjusted vaccine efficacy was 91.5% (95% CI, 61.7–98.1%; $p = 0.001$). There was not a significant benefit in infants older than 6 months of age, potentially secondary to the waning of maternal antibodies, as infants that were vaccinated were excluded from the study. Poehling et al. performed a similar analysis through use of the New Vaccine Surveillance Network that monitored admission due to influenza among infants in three U.S. counties (44). The study included data from multiple years before the 2009 pandemic and found that infants of vaccinated mothers <6 months of age were 45–48% less likely to be hospitalized for influenza than infants of unvaccinated mothers.

Another group approached this in a prospective fashion evaluating 1169 mother–infant pairs delivering in three consecutive influenza seasons between December 2002 and March 2005 (45). In this study, influenza vaccination did not have an impact of the number of outpatient visits attributable to influenza-like illness. However, it did significantly reduce hospitalizations secondary to influenza-like illness and laboratory-confirmed influenza virus infection by 39 and 41%, respectively. Infants of vaccinated mothers also had significantly higher HI titers to all vaccine strains compared with infants of unvaccinated mothers. Earlier studies by Puck and Reuman had demonstrated that infants with increased cord blood neutralizing antibodies to influenza had delayed infection with influenza, also suggesting a role for maternal antibodies in the prevention of influenza disease in infants (46, 47).

## Conclusion

Overall, the data indicate that pregnant women mount adequate and effective responses to influenza vaccination. There is strong evidence that pregnant women respond at a level that is comparable with other healthy adults. Further, there is also good evidence that protective antibodies are transferred to infants, with the majority of studies indicating that cord-blood titers are equivalent to maternal titers at the time of delivery. Although both second and third trimester vaccinations appear to be equally efficacious, there is less data on first trimester vaccination. Data are mixed relative to whether first trimester vaccination results in diminished cord-blood titers. In some cases, especially following monovalent H1N1 vaccination, cord-blood titers have been consistently higher than maternal titers. The data mirror those of other vaccines that elicit a primarily IgG1 vaccine response, as active placental transfer of antibody through placental Fc receptors primarily occurs with this class of IgG (48). The nature of cellular responses to influenza vaccinations is less well defined; a deficit in interferon production *in vitro* has been observed in pregnant women; however, this effect was rescued by vaccination. Pregnant women have equal and perhaps increased plasmablast induction compared to non-pregnant women following IIV.

The robust immunogenicity of influenza vaccination in pregnancy correlates with clinical efficacy. The vaccine is effective at reducing clinical illness in pregnant women at a level on par with that observed for non-pregnant healthy subjects. In addition, there is a clear benefit to infants of vaccinated mothers up through 6 months of age, presumably through active transport of maternal antibody. While not addressed in this review, there is substantial evidence that influenza vaccination is safe for pregnant women and the fetus with no evidence that immunization increases the risk of preterm delivery or other adverse pregnancy outcomes (19, 49–52). In summary, influenza vaccination is both highly immunogenic and clinically beneficial for pregnant women and their infants.

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# Antibody-based strategies to prevent and treat influenza

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Passive immunization using antibodies is a promising alternative to other antiviral treatment options. The potential for seasonal protection arising from a single injection of antibodies is appealing and has been pursued for a number of infectious agents. However, until recently, antibody-based strategies to combat infectious agents have been hampered due to the fact that most antibodies have been found to be strain specific, with the virus evolving resistance in many cases. The discovery of broadly neutralizing antibodies (bNAbs) in influenza, dengue virus, and HIV, which bind to multiple, structurally diverse strains, has provided renewed interest in this area. This review will focus on new technologies that enable the discovery of bNAbs, the challenges and opportunities of immunotherapies as an important addition to existing antiviral therapy, and the role of antibody discovery in informing rational vaccine discovery – with agents targeting influenza specifically addressed. Multiple candidates have entered the clinic and raise the possibility that a single antibody or small combination of antibodies can effectively neutralize a wide variety of strains. However, challenges remain – including combating escape variants, pharmacodynamics of antibody distribution, and development of efficacy biomarkers beyond virologic endpoints.

**Keywords: influenza A virus, monoclonal antibodies, mutations, therapeutics, hemagglutinins, viral**

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## Introduction

New alternative countermeasures for influenza are urgently needed. Vaccines to seasonal and pandemic influenza are foundational to provide widespread herd immunity to influenza. However, most inactivated and live-attenuated vaccine technologies are strain specific – requiring constant updating of the strains used in yearly multivalent vaccine preparations. In addition, severe influenza disease occurs each season in many high risk groups, to whom the vaccine provides limited or no protection, such as young children, the elderly, patients that are immunocompromised or who have pulmonary conditions, inflammatory conditions, or malignancies, as well as pregnant women (1, 2). In addition to vaccines, current treatment and prophylaxis of influenza is limited to the neuraminidase inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza). Despite the availability of these treatments, 10–44% of hospitalized patients require intensive care and 25–50% of these patients die. In the United States, it is estimated that over 200,000 patients are hospitalized with influenza, with up to 48,000 deaths, per year (3). A comparison of annual mortality rates from infectious disease in the United States further demonstrates the lack of effective interventions against this deadly disease (Table S1 in Supplementary Material).

Furthermore, certain strains of influenza have resulted in infections with high mortality rates: the 1918 H1N1 Spanish Flu strain resulted in deaths of 1–3% of the world's population, compared to the 1968 pandemic strain that resulted in the death of 0.03% of the world's population (4). More recently, Avian H5 strains have had documented mortality rates up to 60% despite the use of currently licensed anti-viral treatments (5). Finally, the continued emergence of resistance to current anti-viral drugs increases the need for new therapeutics (6).

## Passive Immunotherapy has a Long History

Prior to the advent of antibiotics, convalescent serum was the only antidote available for bacterial toxins, such as diphtheria and tetanus (7). Eventually, it was discovered that the protective properties within the serum were predominantly due to neutralizing antibodies. The therapeutic use of passive antibodies has been well established for several viral infections. There are licensed polyclonal antibody products for several viruses, including hepatitis B (HBIG), varicella (VZIG), cytomegalovirus (Cytogam), rabies (HRIG), and respiratory syncytial virus (RSV) (Respigam). More recently, monoclonal antibodies (mAbs) for viral infections have been developed, including licensure of Synagis for prevention of RSV infection. mAbs for the prevention and treatment of a number of other viral targets, including Hepatitis C, Rabies, and West Nile Virus, are in clinical development (8–11). Historically, these antibody products have primarily focused on the prevention of viral disease, although application to treatment of infection has been investigated for RSV (12). Of note, no monoclonal antibody-based solution has been approved for the *treatment* of active infection.

In the absence of development of a universal, broadly protective vaccine, passive immunization using antibodies potentially offers several benefits. First, passive immunity provides the opportunity to protect at-risk individuals from infection. At-risk segments of the population include first responders to a relatively novel strain as well as those who do not mount an immune response to vaccines including the immunocompromised, those in poor health, pregnant women, and critically ill patients. Indeed, recent modeling analyses completed by us and our collaborators (M. Boni, Oxford University Clinical Research Unit) indicate that for a sufficiently potent and long-lasting antibody ($t_{1/2} = \sim$18 days, protective period = 3 half-lives), population coverage of only 4–6% would be required to significantly reduce hospitalization rates. Notably, given the current state-of-the-art in the production of antibodies, it is possible to readily generate enough monoclonal antibody to protect the population at the epicenter of an epidemic outbreak in a much shorter time scale than that of vaccine production (>6 months) (13). The potential for long-lasting protection, covering an entire exposure period (i.e., an entire season for influenza), arising from a single injection of antibodies is appealing and has been pursued for a number of infectious agents. For example, in the case of hepatitis A, prophylactic administration of immunoglobulins can protect against viral exposure. Additionally, post-exposure prophylaxis with immunoglobulin is >85% effective in preventing hepatitis A if administered within 2 weeks after viral exposure, and efficacy is even higher when administered early in the incubation period (14).

The use of antibody therapy to treat influenza has recently received more attention, with some clinical experience to support efficacy. A meta-analysis of studies conducted during the 1918 pandemic using blood products strongly supports a benefit for treated patients (15). Overall, the six studies documented a 21% reduced risk of mortality in treatment groups (16 vs. 37% mortality in controls, 95% of risk difference, CI: 15–27). Furthermore, a recent study evaluated the use of convalescent plasma in 93 patients with H1N1 2009 influenza in Hong Kong (16). In this prospective multi-center case-control study, patients with severe influenza, who were hospitalized and required intensive care unit support, were recruited and offered convalescent plasma containing influenza neutralizing antibody in addition to the standard of care with either oseltamivir or zanamivir. Mortality was significantly lower in the treatment groups who received convalescent plasma compared to the controls (20.0 vs. 54.8%, $p = 0.01$).

Given the overall promise (and current limitations) of passive immunization approaches, as well as the overarching goal of accounting for viral mutations, it is highly desirable to identify and/or engineer antibodies that bind with high affinity and that neutralize many or all strains that are capable of infecting humans. To this end, there is intense focus on antibodies that bind to influenza hemagglutinin (HA) since antibodies to HA are known to be protective in animal models and in humans. The challenge is that HA is a highly diverse protein; there are 18 subtypes categorized into two groups – 1 and 2. As such, the ability to identify an antibody or small collection of antibodies that can bind to and neutralize all clinically relevant strains is a substantial challenge. The ability to rapidly identify broadly neutralizing antibodies (bNAbs) has been enhanced by the development of several new high-throughput technologies that now promise to enable comprehensive tracking of all of the immunological cell subsets, extending even to the level of the individual clones of B cells that carry out adaptive responses (17, 18). Improvements in the toolkit for human immunological studies are continually evolving and are likely to increase our understanding/discovery of antibodies for therapeutic use.

Characterization of the overall B-cell response to infection or vaccination has provided potentially important insights into lasting immunity, including the heterogeneous nature of individualistic responses to vaccination/infection. However, with next-generation deep sequencing data, it has become clear that, in different individuals, expansion of B-cell clones in response to infection arising from similar or "convergent" antibody gene rearrangements can be detected. For example, tracking of B-cell clones following pandemic single antigen H1N1 vaccination revealed a strong clonal signature dominated by antibodies using the IGHV3-7V gene, and correspondence of highly similar CDR3 sequences in different humans. Convergent monoclonal antibody sequences display HA inhibition activity against H1N1 and other influenza strains (19). This raises the possibility of a so-called "universal" vaccine strategy-through selection of the appropriate immunogen(s) to elicit the most effective immune

response. Other recent work using regions of or chimeric proteins derived from the stem region of HA seem to elicit a broadly protective response against multiple subtypes of influenza (20, 21). Furthermore, studies in the area of HIV have suggested that a broadly protective response may, in principle, be completed through eliciting specific B-cell responses (i.e., "training the immune system") using temporally spaced immunization with different antigens (22).

In addition to the use of the above tools to study the adaptive immune response, there has recently been a concerted effort to identify, engineer, and characterize antibodies that bind to a number of influenza subtypes (so-called "broadly" neutralizing antibodies). Several of these antibodies are listed in **Table 1**. These broadly neutralizing mAbs are a new, promising modality for treatment of influenza, potentially across all strains of the virus. Such antibodies have been identified through panning the B-cell repertoire of vaccinated or infected individuals (23, 24) and are estimated to be ~0.001–0.01% of the total antibody response (23). Identification of such antibodies has generated interest for several reasons, including (i) mapping of the epitopes to which these antibodies bind provides insights into epitopes that can be targeted for vaccine development and (ii) the antibodies by themselves are useful products to provide passive immunization or therapeutic efficacy against a wide variety of influenza subtypes.

## Study of bNAbs has led to Insight on Universal Vaccine Targets

In the context of vaccine efforts, identification of bNAbs to infectious agents provides a basis for the design of more protective vaccine strategies (25). Recent work on the evolution of bNAbs containing the $V_h$1-69 heavy chain demonstrates that somatic mutations to the germline, which result in recognition of a hydrophobic patch on the HA stem results in the antibodies becoming more hydrophobic and binding influenza HA with higher avidity (26). Another recent study characterized ~200 anti-stem antibodies and identified two key elements that are required for the initial development of most $V_H$1-69 antibodies: a polymorphic germline-encoded phenylalanine at position 54 and a conserved tyrosine at position 98 in the third complementary determining region of the heavy chain (27). By tracing the development of such antibodies, these studies have demonstrated that it may be possible to develop an immunofocusing strategy to promote the production of bNAbs containing $V_h$1-69.

## Properties of bNAbs

Antibodies to two major surface antigens, the M ion channel and HA, have been studied as potential passive immunotherapies (**Table 1**). Antibodies that target the highly conserved M2 protein possess the requisite breadth of binding across group 1 and group 2 (i.e., all subtypes of influenza A). However, such antibodies are non-neutralizing; the predominant mechanism of action for M2-specific antibody is indirect through ADCC-mediated killing of infected cells. This leads to incomplete protection. For example, in a lethal influenza mouse model; an M2-targeted antibody product required three injections with M2-specific antibodies at 24, 72, and 120 h post-infection and still provided only a partial (~60%) protective response (28).

**TABLE 1 | Recent discoveries in broadly neutralizing antibodies to influenza.**

| Antibody | Target | Breadth | Development |
|---|---|---|---|
| CR6261 | Stem region/HA | Group 1 | Phase II |
| CR8020 | Stem region/HA | Group 2 | Phase II |
| CR9114 | Stem region/HA | Group 1/group 2 | Pre-clinical |
| F10 | Stem region/HA | Group 1 | Pre-clinical |
| F16 | Stem region/HA | Group 1/group 2 | Pre-clinical |
| TCN-032 | M2 | Group 1/group 2 | Phase II |
| MHAA4549A | Stem region/HA | Group 1/group 2 | Phase II |
| CH65 | Receptor binding site/HA | H1 | Pre-clinical |
| VIS410 | Stem region/HA | Group 1/group 2 | Phase II |

In contrast, antibodies to HA can clearly neutralize influenza virus *in vitro*, provide complete protection after a single administration *in vivo*, and protect against multiple strains of influenza (24, 29, 30). Additionally, use of such antibodies likely also mitigates bacterial secondary infection, since rapid reduction in viral titers prevents bacterial adherence (31). These data are supported by the fact that the efficacy of current vaccine approaches to influenza (especially inactivated virus strategies) is measured by the HA neutralizing titer. However, through the processes of antigenic drift and shift, the HA of influenza virus can develop resistance to antibodies that target HA. Such has been the case with, for example, CR6261 (32), CR8020 (24), and F10 (33), where several mutations are known to lower the binding affinity of the antibody to HA and confer resistance. The fact that HA is subject to mutation and the virus can undergo reassortment questions whether an immunotherapy strategy can be adequately developed due to facile development of escape mutants.

## Strategies to Design Effective Immunotherapy

There are at least two points that need to be considered with regard to an ideal immunotherapeutic strategy, particularly when considering a variable system like HA. The first is the epitope targeted by the antibody. Many of the bNAbs in **Table 1** target the relatively conserved stem region of HA. While certain stem-binding-antibodies target epitopes that can mutate under selective pressure with apparently little or no fitness cost, other epitopes are less amenable to mutation and are more likely to engender a fitness cost (34). A structure-based network approach (35) can be used to provide insights into the tolerance to mutability of an amino acid in a protein system. This approach is based on analysis of sequences across different viral surface proteins that reveal amino acids that are highly networked (higher weighted contacts with neighboring amino acids), and therefore are more constrained in their ability to mutate (**Figure 1A**). By targeting these amino acids, it is possible to generate an antibody-based solution that is more refractory to resistance development while still maintaining binding to a potent and broadly neutralizing epitope. Additionally, in the context of therapy, it is likely that any antibody can be used in combination with a neuraminidase inhibitor, where there appears to be synergistic

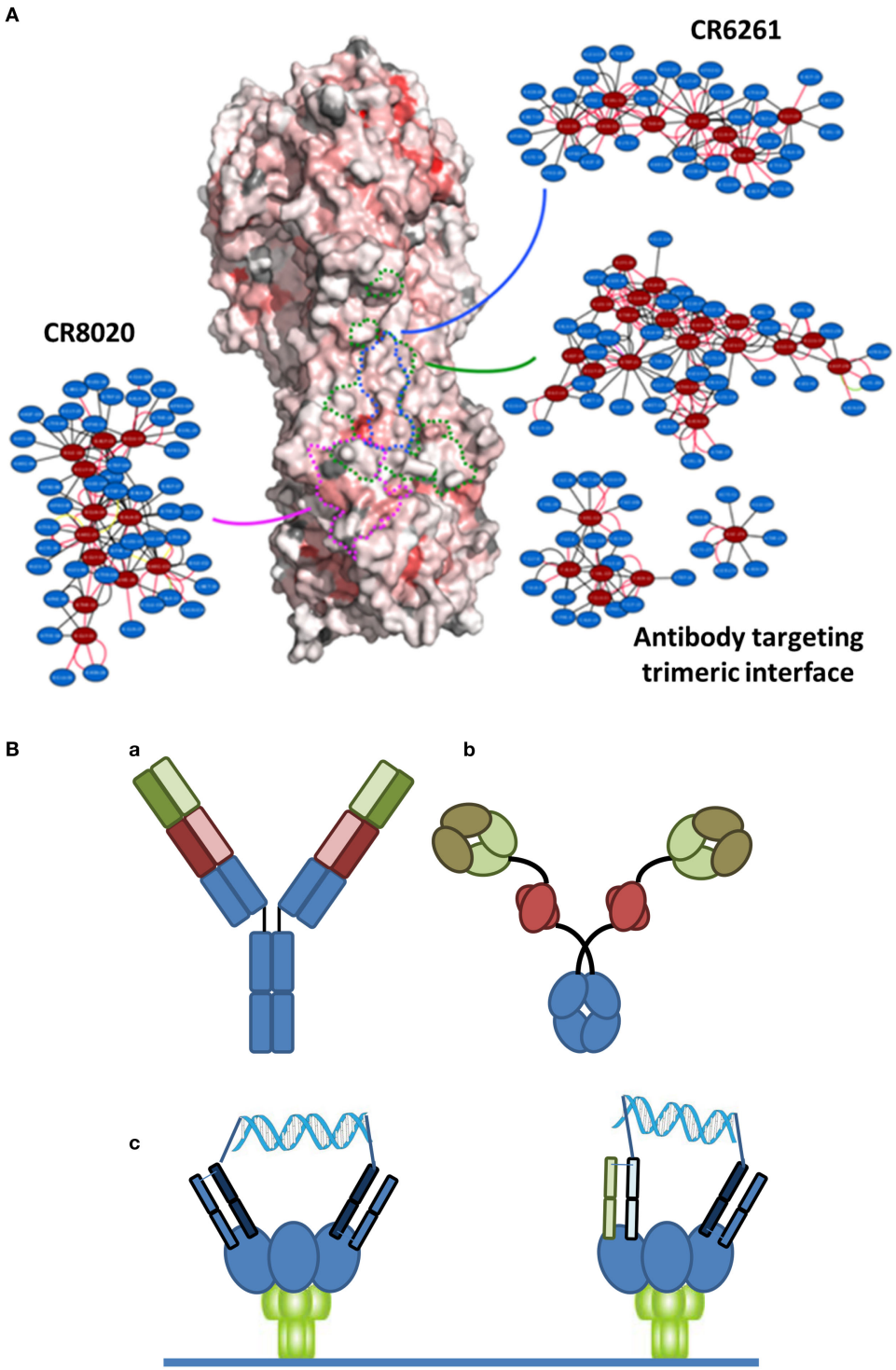


**FIGURE 1 | (A)** Network-view of bNAb epitopes. HA trimer is represented in a solvent accessible surface format and colored based on normalized residue network scores. Coloring varies from white to red where white indicates poorly networked residues and red indicates highly networked residues. The three bNAb epitopes are highlighted by dotted borderlines (green: antibody targeting trimeric interface; blue: CR6261; pink: CR8020). The 2D network map of the epitope is also shown. A network is made up of nodes and edges. Nodes colored in red indicate functional epitope residues whereas nodes colored in blue indicate residues that are in the network environment of the epitope residues. **(B)** Different bispecific formats that have demonstrated activity against infectious disease targets. (a) A dual-variable domain immunoglobulin format containing two distinct Vh-Vl pairings (one in red and one in green) has demonstrated activity against hepatitis B. (b) A bispecific format where a single chain variable region against Psl (red) targets the antibody to the cell surface of *Pseudomonas* enables engagement of a traditional Vh-Vl paratope with the rarer PcrV target. (c) Crosslinking of binding domains of variable and constant regions ($V_H$-$CH_1$/$V_L$-CL; Fabs), either homotypic (left) or heterotypic (right) with a defined DNA-based spacer enables more potent neutralization of HIV virus.

activities due to distinct mechanisms of action (36). Furthermore, as has become apparent in other viral diseases, such as HIV or hepatitis C, combination approaches are less likely to elicit resistance. Finally, recent studies have indicated that the activity of bNAbs is enhanced in the presence of the natural immune response (37).

The second important point to an ideal strategy is that there are a number of engineering-based strategies outside of the variable or binding region that can be used to enhance the efficacy of the antibody-based solutions. Certainly, one of the relevant approaches is Fc engineering to enhance the recruitment of complement and/or innate immune cells. In the context of bNAbs against influenza, it is known that the various stem-binding antibodies are able to recruit complement and that Fc effector functions are critical to their protective effect (38). However, the efficiency of complement recruitment is based on the geometry of engagement, with some antibodies being able to better engage complement as compared to others (39). The effector functions can be further enhanced through engineering of Fc mutations and/or alteration of the glycosylation site to enhance ADCC. Finally, in other therapeutic areas, particularly oncology, there has been an emphasis on discovery of bispecific antibody formats (40) (**Figure 1B**). In the context of antibodies to infectious agents, initial examples have provided intriguing results. Alternative formats have been investigated including use of multiple antibody binding domains ($V_H$-$V_L$), or inclusion of antibody-like binding domains, such as scFv or Fab fragments. Recently, data have been reported for a bispecific antibody-like construct to *Pseudomonas* where one binding site binds to a high density ligand (Psl) and thus targets the antibody, and the other binding site targets a highly neutralizing epitope (PcrV) (41). Furthermore, with reference to viral diseases, a recent report of a bispecific antibody to hepatitis B reported synergistic activity compared to the activity of the parent antibodies alone (42). Finally, in HIV, where the density of the gp140 spike protein at the viral surface is highly limiting, bridging through the use of a bispecific antibody resulted in much higher activity (43). Such a dual-targeting strategy may also be useful for other viruses such as influenza.

## Conclusion

If technologies can identify high affinity, bNAbs, passive immunization can likely provide an important adjunctive prophylactic and therapeutic option to supplement vaccination technologies. Antibody-based therapies are generally safe and well-tolerated, particularly when the antigen is an exogenous target. Even one of the more common effects of therapy, which is the development of anti-drug antibodies, at most serves to limit drug exposure rather than resulting in significant adverse effects. Recent maturation of several tools in antibody characterization, discovery, and engineering may enable a resurgence of passive immunotherapy strategies. With several antibody candidates that are currently in clinical development for influenza (**Table 1**) and potentially others, it is likely that we will determine in the near future whether an old idea becomes a new powerful tool to counteract the rapidly evolving threat of influenza and other virus infections.

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## Supplementary Material

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00315

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REVIEW
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# Modulating the innate immune response to influenza A virus: potential therapeutic use of anti-inflammatory drugs

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Infection by influenza A viruses (IAV) is frequently characterized by robust inflammation that is usually more pronounced in the case of avian influenza. It is becoming clearer that the morbidity and pathogenesis caused by IAV are consequences of this inflammatory response, with several components of the innate immune system acting as the main players. It has been postulated that using a therapeutic approach to limit the innate immune response in combination with antiviral drugs has the potential to diminish symptoms and tissue damage caused by IAV infection. Indeed, some anti-inflammatory agents have been shown to be effective in animal models in reducing IAV pathology as a proof of principle. The main challenge in developing such therapies is to selectively modulate signaling pathways that contribute to lung injury while maintaining the ability of the host cells to mount an antiviral response to control virus replication. However, the dissection of those pathways is very complex given the numerous components regulated by the same factors (i.e., NF kappa B transcription factors) and the large number of players involved in this regulation, some of which may be undescribed or unknown. This article provides a comprehensive review of the current knowledge regarding the innate immune responses associated with tissue damage by IAV infection, the understanding of which is essential for the development of effective immunomodulatory drugs. Furthermore, we summarize the recent advances on the development and evaluation of such drugs as well as the lessons learned from those studies.

**Keywords: influenza virus, inflammation, innate immunity, ARDS, cytokines, anti-inflammatory therapy**

## Introduction

Influenza A virus (IAV) infection usually results in a mild and self-limiting disease that in some individuals, commonly those with underlying medical conditions, can result in complications leading to severe disease and death. Pneumonia, bronchitis, sinus infections, and ear infections are examples of influenza-related complications (1). Thus, influenza has a significant economic impact and is a very important public health concern, with a rate for the 2014–2015 season of 57.1 laboratory-confirmed influenza-associated hospitalizations per 100,000 people reported as of March 14th, 2015 (2). The highest rate of hospitalization is among adults over 65 years old, followed by children under 4 years old, and the average annual influenza-associated deaths in the United States from 1976 to 2007 are 23,607 (3).

Characteristics of the IAV genome, such as its negative-sense, single-stranded segmented RNA, and its airborne transmission in humans provides this virus with a great pandemic potential. The co-circulation of different subtypes in animal reservoirs leads to reassortment (antigenic shift), which may result in a novel subtype that is able to transmit to the human population (4). The circulating IAV subtypes in humans as of 2015 are H1N1 viruses, which caused a pandemic in 2009, and H3N2 viruses; however, several different subtypes have circulated in humans during the last century. The natural hosts of IAV are aquatic birds, which may sporadically transmit viruses to poultry. Humans are, on occasion, infected by these viruses, causing what is known as avian influenza, which is associated with severe disease and high fatality rates (5, 6). The human-to-human transmission in these cases is very limited, and the most important of these IAV are the H5N1 and H7N9 subtypes.

Uncomplicated cases of influenza are limited to attachment and viral replication in the upper respiratory tract, and the symptoms in these cases are nasal obstruction, cough, sore throat, headache, fever, chills, anorexia, and myalgia. These symptoms are the consequences of the inflammation induced upon viral infection (7). Complications of IAV infection are more frequent in people with underlying comorbidities, such as chronic pulmonary or cardiac disease, asthma, immunosuppression, or diabetes mellitus. These complications begin when the viral infection reaches the alveolar epithelium in the lower respiratory tract, where severe tissue damage may occur and affect gas exchange. In alveolar tissue, type I pneumocytes prevent fluid leakage across the alveolar–capillary barrier, and type II pneumocytes resorb fluid from the alveolar lumen and produce lung surfactant (8). Thus, damage of the alveolar epithelium leads to respiratory dysfunction or acute respiratory distress syndrome (ARDS), which often occurs in cases of severe influenza. More extensive discussion on the contribution of the different cell types to tissue damage during influenza infection has been recently published in a very interesting review (9). Most of the lung pathology during influenza virus-induced ARDS is associated with the release of cytokines and other pro-inflammatory mediators, and the contribution of the direct viral cytopathic effect to the alveolar damage is still unclear (10, 11). H5N1 viruses have also been reported to spread to extra-respiratory tissue, although with limited or no viral replication (12).

Influenza complications are also frequently associated with secondary bacterial infections, which may be explained by a series of changes that the virus induces in the lung epithelial cells of the host that predisposes to adherence and invasion as well as changes in the immune response (13–15). For example, it is believed that IAV infection upregulates CD200 receptor in lung myeloid cells, which is involved in negative immune regulation upon interaction with the ligand CD200, resulting in predisposition to secondary bacterial infection (16).

Disease severity caused by IAV infection is greatly associated with high levels of inflammation, with increasing evidence that tissue damage is produced by an exaggerated innate immune response. Thus, many researchers have proposed that treatment with anti-inflammatory therapy could be beneficial. The primary challenge to successfully establish this type of therapy is to down-regulate specific mediators of the immune system that have a detrimental effect while avoiding increased levels of viral replication. Here, a review of the innate immunity processes associated with severe cases of IAV infection is provided. Specifically, we discuss clinical studies that have been published regarding the cytokines and chemokines shown to be upregulated in serum or lung tissue of patients with severe disease. We also provide a brief review of the most frequent of those immune mediators, including signaling pathways activated by them and the cellular processes that might lead to tissue damage and disease progression. Finally, anti-inflammatory therapies that have been proposed and tested, either in clinical, preclinical, or *in vitro* studies, are also discussed.

## Innate Immunity to IAV

The first barrier that IAV encounters when invading the host is the mucus layer covering the respiratory and oral epithelia. If the virus successfully overcomes this barrier, it can bind the respiratory epithelial cells, be internalized, and start replicating (17). The cellular defense mechanisms that are initiated upon pathogen invasion involve the sensing of components of pathogens, or pathogen-associated molecular patterns (PAMPs), by pattern-recognition receptors (PRRs) in host cells. This recognition leads to activation of subsequent signaling events that result in the secretion of inflammatory cytokines, type I interferon (IFN), chemokines, and antimicrobial peptides. There are several types of PRRs with a cell-type specific distribution and sub-cellular localizations that may be cytoplasmic, endosomal, or in the plasma membrane.

Cytoplasmic receptors include the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), the nucleotide oligomerization domain (NOD)-like receptors (NLRs), and the less-characterized cytosolic DNA sensors (18). These receptors are particularly important in the context of viral infection. Within the RLR family, the most important proteins are RIG-I, melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), all of which are expressed in the cytosol of most cell types and participate in the recognition of single-stranded and double-stranded RNA (19). The most studied of the NLRs in the context of virus infection is the NLR family pyrin domain containing 3 (NLRP3), which upon stimulation leads to the activation of the inflammasome system, with important implications in inflammation (20). NLRP3 is expressed in myeloid cell types, such as monocytes, macrophages, dendritic cells (DCs), and neutrophils and in lung epithelial cells (21). Several PAMPs and damage-associated molecular patterns (DAMPs) have been proposed to activate this receptor, including dsRNA (22), the M2 protein of influenza virus (23), and reactive oxygen species (ROS) (24).

Another very important family of PRRs is the toll-like receptors (TLRs). Some of these receptors, such as TLR1, TLR2, TLR4, TLR5, and TLR6, are located in the plasma membrane and are activated mainly by lipids, lipoprotein, and proteins. Other TLRs, namely, TLR3, TLR7, TLR8, and TLR9, are expressed in endosomal compartments and recognize nucleic acids (25). TLRs are highly expressed in antigen-presenting cells, such as DCs and macrophages, and they are also known to be expressed in several T cell subsets (26). For IAV and other RNA viruses, the most

important of these TLRs are TLR3 and TLR7/8, which recognize dsRNA and ssRNA, respectively (27).

Other PRRs that are expressed on the cell surface of antigen-presenting cells are the C-type lectin receptors (CLRs), such as the DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) or dectin-1 and dectin-2. CLRs recognize carbohydrate ligands and are also mainly expressed in antigen-presenting cells (28). Several reports have shown an interaction between IAV and DC-SIGN (29–31), which would facilitate infection of DCs.

Recognition of PAMPs by these PRRs leads to the activation of multiple signaling cascades initiating the innate immune response. This response leads to the production of type I and type III IFNs. Binding of these IFNs to their receptors in a paracrine or autocrine manner leads to the establishment of an antiviral response, characterized by the expression of hundreds of genes that will counteract viral replication (32). Also, PAMP sensing results in the release of pro-inflammatory cytokines and chemokines by the cells that will contribute to the development and modulation of specific T cell responses and recruitment of different immune cells, such as monocytes, neutrophils, and natural killer (NK) cells, to the infected tissue. In the case of antigen-presenting cells, such as DCs and some subtypes of macrophages, they also undergo maturation and migrate to the secondary lymphoid organs where antigen is presented to T (33) and B cells (34). These adaptive immune responses initiated upon innate immune activation are known to be necessary for protection and viral clearance, as recently reviewed by Chiu and Openshaw (35).

Hence, in the current model of IAV-induced ARDS, IAV particles invade a new host and if, able to cross the mucosal barrier, will infect tissue cells in the upper respiratory tract. In some cases, the virus reaches the lung, where it can infect type I and II pneumocytes, endothelial cells, and immune cells (9, 36–38). The presence of the virus is detected by infected cells, which release cytokines, chemokines, and other mediators in order to control the infection and remove dead cells and stimulate the initiation of adaptive immune responses. However, other effects of those mediators, which are described in detail below, are detrimental for the integrity of the tissue (11, 39).

While these alert systems are aimed to mount an effective immune response to clear viral infection, there are also important negative consequences of those responses that might compromise tissue integrity. One of the most described of those consequences is the production of ROS. Pro-inflammatory mediators induce intracellular ROS by activating the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Also, work by Ye et al. has shown that inhibiting ROS production *in vitro* results in attenuation of release of pro-inflammatory cytokines (40, 41), thereby amplifying the immune response. In addition to reacting with DNA, proteins, and lipids resulting in structural cell and tissue damage, ROS are known to be the second messengers that participate in several signaling pathways and function as transcriptional regulators (42). It is also known that pro-inflammatory responses activate signaling pathways that result in the activation of apoptosis and necrosis (43, 44). Accordingly, apoptotic alveolar epithelial cells have been observed by histochemistry of lung tissue from two patients who died by H5N1 infection (12).

## Hypercytokinemia and Pathogenesis in Severe Cases of Human Influenza

Several studies have characterized the profile of cytokines in human cases of influenza in order to understand the connection between innate immunity and pathogenesis. In cases of seasonal influenza, complications are mostly associated with secondary bacterial infection. Most cases of severe primary viral pneumonia have been associated with pandemic influenza, such as 2009 H1N1 or 1918 H1N1 influenza virus, and cases of avian influenza, such as infections by H5N1 or H7N9 influenza viruses (45, 46).

Acute respiratory distress syndrome is the main cause of death in IAV-infected patients (47, 48). Histopathology caused by complicated IAV infection in the absence of bacterial pneumonia consists of inflammation, congestion, epithelial necrosis of the larger airways, and diffuse alveolar damage characterized by hyaline membranes, interstitial and intra-alveolar edema, necrotizing bronchitis and bronchiolitis, and in some cases, hemorrhage (49, 50). Autopsies from fatal cases of 1918 H1N1, H5N1, and the 2009 H1N1 pandemic virus show comparable pathological characteristics (47, 50). Fatal infection with H7N9 influenza viruses in humans also showed diffuse alveolar damage as one of the main histopathology findings (51).

The majority of the patients infected by pandemic 2009 H1N1 virus experienced a mild disease with influenza-like symptoms that typically resolved in a few weeks (47, 48). However, due to the lack of pre-existing immunity against this virus, complications of the disease occurred in some patients, mostly those with underlying conditions (47). Gao et al. found the levels of seven proteins markedly upregulated in lung tissue in fatal cases of influenza virus 2009 H1N1 infection. Those proteins are interleukin (IL)1 receptor antagonist protein (IL1RA), IL6, tumor necrosis factor (TNF)-α, IL8, monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein (MIP) 1β, and IFNγ-inducible protein-10 (IP10)(52). In this work, they also found high levels of apoptosis in the lungs and airway by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, as well as marked levels of cleaved caspase 3 (52). A similar study by To et al. showed significantly higher levels of granulocyte colony-stimulating factor (G-CSF), IFNα2, IL1α, IL6, IL8, IL10, IL15, IP10, and MCP1 in plasma samples of patients that developed ARDS and died than in those patients that developed mild disease at early times after onset of symptoms (48). High levels of IP10, MCP1, and MIP1β were also found in a separate group of patients infected by 2009 H1N1 influenza virus (53). In this study, elevated levels of IL8, IL9, IL17, IL6, TNFα, IL15, and IL12p70 were found specifically in patients that required hospitalization, and IL6, IL15, and IL12 were markers of severe disease. In agreement, other studies reported high levels of IL6, IL8, IL10, and the chemokine MCP1 in 2009 H1N1 virus-infected patients (54) and correlated serum levels of IL6 and IL1β with disease severity in children infected by the same virus (55). An additional report showed elevated levels of IL2, IL12, IL6, IL10, IL17, and IL23 in patients with severe disease and correlation between clinical manifestations and IL6 and IL10 serum levels in patients infected by 2009 H1N1 IAV (56). Other studies reporting similar results are summarized in **Table 1** (54, 57, 58).

**TABLE 1 | Cytokines and chemokines detected in serum or lung tissue samples of human subjects with severe disease infected by IAV.**

| IAV subtype | Cytokines | Chemokines | Reference |
|---|---|---|---|
| 2009 H1N1 | IL6, TNFα, IL9, IL17, IL15, and IL12 | IL8 | (53) |
| 2009 H1N1 | IFNα2, IL1α, IL6, IL10, and IL15 | IL8, IP10, and MCP1 | (48) |
| 2009 H1N1 | IL6, TNFα, and IL15 | IL8 | (57) |
| 2009 H1N1 | IL2, IL12, IL6, IL10, IL17, and IL23 | | (56) |
| 2009 H1N1 | IL6 and TNFα | IL8, IP10, MCP1, and MIP1β | (52) |
| 2009 H1N1 | IL6 and IL1b | | (55) |
| 2009 H1N1 | IL6 and IL10 | IL8 and MCP1 | (54) |
| H3N2 | IL6, TNFα, and IL33 | | (58) |
| H5N1 | | IP10 and MIG | (59) |
| H5N1 | IL6, IL10, IFNγ | IL8, IP10, MCP1, and MIG | (60) |
| H5N1 | IFNβ, IL6, IFNγ, and TNFα | IL8, IP10, MCP1, RANTES, MIP1α, and MIG | (61) |
| H7N9 | IL6 | IL8 and MIP1β | (62) |
| H7N9 | IL6 | IL8 and MIP1β | (63) |

Cytokine responses in H5N1-infected patients have also been studied. Peiris et al. found elevated levels of IP10 and monokine induced by IFNγ (MIG) in serum of H5N1-infected patients (59). Similarly, de Jong et al. found the levels of IP-10, MIG, and MCP1 elevated in patients with H5N1 infections (60). Interestingly, in both studies, they found large numbers of macrophages infiltrated in the lung, in accordance with the functions of those chemokines. de Jong et al. also found elevated levels of IL6, IL8, IL10, and IFNγ in those patients (60). The level of cytokines was associated with elevated levels of viral replication. Another study that evaluated the levels of cytokines in two fatal cases of H5N1 infection found high levels of regulated on activation, normal T cell expressed and secreted (RANTES), MCP1, MIP1α, IP10, IL8, MIG, IFNβ, IL6, IFNγ, and TNFα in the lungs and serum in one patient, while no cytokine expression was detected in the other case (61). However, the patient who did not show cytokine expression was pregnant and treatment with glucocorticoids was provided in both cases, which may have affected the immune response although it is unclear how these or other factors could have affected the results (61).

Information regarding H7N9 IAV infections in humans is more limited given the recentness of the outbreak. However, a study evaluating the cytokine responses in infected patients identified early high levels of IL6, IL8, and MIP1β in serum as predictive parameters of severe or fatal outcome (62). Another study found a positive correlation of the same molecules (IL6, IL8, and MIP1β) with pharyngeal virus load in H7N9-infected patients (63).

Most of these studies with human samples point to elevated levels of cytokines and chemokines in IAV-infected patients. Interestingly, there is a clear overlap in the cytokines that are observed in most of those studies. A summary of the cytokines and chemokines found to be upregulated in humans infected by IAV is provided in **Table 1**. Experiments performed *in vitro* also have identified the production of similar cytokines in different systems, including IL6, TNFα, IFNs, IL1β, RANTES, IL8, MIP1β, and MCP1 (64, 65). Since the reported data indicate that the induction of these molecules might be associated with pathogenesis, understanding the effects of those proteins in receptor-expressing cells and the signaling pathways that they induce is important for eventually translating that information to the identification of efficient and safe treatment alternatives. Therefore, in the next section, we focus on the functions of each one of those cytokines and chemokines in more detail, as well as their participation in tissue damage in other diseases or other models as an additional indicator of their pathogenic potential.

## Cytokines and Chemokines with Increased Expression During Severe Influenza: How They Work and Their Involvement in Tissue Damage

Upon influenza infection, viral PAMPs are sensed by the cells and multiple signaling pathways are activated as a part of the innate immune response. The purpose of the innate immune response is to lead to the clearance of viruses and infected cells, as well as the activation of the adaptive immune response. However, these events can also result in tissue destruction as a consequence of excessive activation. Data discussed in the previous section indicate an association between the activation of the innate immune response, typically measured as the production of cytokines and chemokines in serum, and a more severe pathogenesis or fatality in many cases, supporting the hypothesis of causative relationship between innate immunity and severe disease. To provide deeper insights into these events and their connection, in this section we will review the effects and signaling pathways associated with the production of the main cytokines upregulated during influenza infection. Because of the broad and numerous functions of these cytokines, it is a challenging task to parse their functions as many of them are redundant and regulated by complex networks involving multiple transcription factors, adaptors, or secondary mediators. In terms of their potential as therapeutic targets, some therapies using monoclonal antibodies to neutralize the damaging effects of those proteins have been developed and are already in the clinic for treatment of anti-inflammatory diseases, while other approaches, such as administration of pro-inflammatory cytokines, small molecules, siRNA or shRNA, or gene therapy, are under study (66).

### Cytokines

#### TNFα

TNFα is the most studied of the cytokines, since it is involved in a large number of functions with multiple effects, such as activation of inflammatory responses, stimulation of adaptive immunity, cell survival, apoptosis, proliferation, and cell differentiation (67, 68).

The receptors for TNFα are TNF-R1, which is constitutively expressed in most cell types, and TNF-R2, which is expressed in immune cells (67). Binding of TNFα to its receptor results in the activation of multiple intracellular signaling pathways, which have been extensively reviewed elsewhere (68, 69). Therefore, in this review, we provide a general overview of these processes and the related outcomes in terms of tissue damage and pathogenesis.

TNFα leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, which promotes the expression of a large number of inflammatory genes. In the classical NF-κB pathway, which is the one activated upon TNFα engagement, NF-κβ is a dimer made up of two subunits, p50 and p65. In a resting state, NF-κB is inactive in the cytoplasm, forming a complex with the inhibitor of nuclear factor κB (IκB). Stimulation that activates the pathway results in the degradation of IκB, allowing the p50/p65 dimer to translocate to the nucleus where it interacts with DNA, leading to the regulation of gene expression (70). Other stimuli that activate NF-κB transcription factors are viral genomic RNA or DNA, bacterial products, acidic pH, and stress-related molecules, such as ROS among others. In addition, it is known that endogenous or host-derived ligands that are generated during tissue damage are also sensed by cell surface receptors, leading to NF-κB activation (71).

Engagement of TNFα to its receptor also leads to the induction of apoptosis. This occurs by several mechanisms, but the major one involves recruitment of pro-caspase 8 to TNF-R1 through the adaptors fas-associated death domain (FADD) and the TNF-R superfamily member 1A (TNFRSF1A)-associated death domain (TRADD), which leads to the auto-cleavage of caspase 8 to its active form. These events then result in caspase 3 activation and induction of apoptosis. Caspase 8 also leads to the release of cytochrome *c* from the mitochondria, contributing to apoptosis induction through the "intrinsic pathway" (68, 72). Interestingly, TNFα also induces caspase-independent cell death by a mechanism involving receptor-interacting serine/threonine-protein kinase 1 (RIPK1) by a kinase-regulated process, and it is known as necroptosis (73–75).

Another described function for TNFα is to stimulate the production of ROS (73, 74, 76), which are also inducers of apoptosis and necrosis (76). In addition, TNF signaling stimulates the activity of the NADPH oxidases (Nox) in neutrophils and macrophages, such as NOX2, resulting in the generation of superoxide ($O_2^-$) (77, 78), which is important for clearing intracellular microorganisms (74, 79).

On the other hand, TNFα signaling leads to activation of the c-jun NH2-terminal kinase (JNK) that also regulates several cellular functions including apoptosis, survival, and cell growth by phosphorylating downstream transcription factors, such as c-jun, activating transcription factor 2 (ATF2), or nuclear factor of activated T cells (NFAT). Interestingly, ROS has also been shown to be a co-activator of TNF-induced JNK activation and cell death (76).

Given the multiple functions of TNFα in inflammation and tissue damage, it is a very important target for immunomodulatory therapy in general. Indeed, antibodies that block its function are used as a primary treatment in some autoimmune disorders, such as rheumatoid arthritis (RA) and Crohn's disease, and several blocking agents are already approved and used in the clinic for such disorders (80). In the case of influenza disease, TNFα-blocking agents have also been tested for treatment of IAV-induced inflammation. Mice treated with one of these agents, etanercept, showed reduced lung inflammation and morbidity after challenge with influenza virus (81).

## IL6

IL6 has been attributed to both pro-inflammatory and anti-inflammatory effects (82, 83). In addition, IL6 is involved in the regulation of metabolism, bone homeostasis, and neural processes. The production of IL6 is tightly regulated, and its continuous production has been associated with numerous chronic and autoimmune diseases. The synthesis of this cytokine is upregulated during infection or stress, and its major roles involve the production of acute phase proteins by hepatocytes and stimulation of the adaptive response by inducing the differentiation of activated B cells and $CD4^+$ T cells (84).

Activation of IL6 signaling may take place through classic or trans-signaling pathways. In the classical activation, IL6 interacts with membrane-bound IL6 α-receptor (IL6R), while in the trans-activation pathway, IL6R is soluble. In both scenarios, the signal-transducing β-subunit glycoprotein gp130 forms part of the receptor complex and plays a fundamental role in initiating the signal. IL6R is expressed in a limited number of cells types, namely macrophages, neutrophils, some types of T-cells, and hepatocytes (85). However, gp130 is ubiquitously expressed, allowing IL6 signaling to take place in a broad range of tissues. It is believed that trans-signaling accounts for the pro-inflammatory effects of IL6, while the classic signaling is more associated with anti-inflammatory effects. Therefore, this dual activity has interesting implications when considering IL6/IL6R as a therapeutic target. A very interesting review by Scheller et al. discusses the dual functionality of classic versus trans-signaling (83).

Dimerization of gp130 leads to Janus kinases (JAK) activation, which results in phosphorylation of tyrosine residues in the cytoplasmic region of gp130. Next, the signal transducer and activator of transcription (STAT) 3 is phosphorylated, dimerizes, and translocates to the nucleus to regulate the expression of multiple genes associated with the induction of cell growth, differentiation, and survival (86). On the other hand, phosphatase Src homology domains containing tyrosine phosphatase (SHP)2 are recruited, leading to activation of the mitogen-activated protein kinase (MAPK) pathway, including ERK1/2 (associated with survival), p38, and JNK (associated with stress). IL6 can also lead to the activation of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) (87), which is classically associated with survival and cell growth. An important group of genes that are also regulated by JAK/STAT3 IL6-mediated activation is the suppressor of cytokine signaling (SOCS) family. Specifically, SOCS1 and 3, which are the most related with IL6 activation, inhibit the phosphorylation of gp130, resulting in a blockade of the JAK/STAT pathway (86).

Primarily, IL6 (but also IL1α/β and TNFα) is a potent inducer of the synthesis and release of approximately 30 proteins known as the acute phase proteins (88, 89). Acute phase proteins are secreted mainly by hepatocytes, and have multiple immunomodulatory effects. These proteins include the C-reactive protein (CRP), serum amyloid P component (SAP), mannose-binding protein, α1 antitrypsin, α1 antichymotrypsin, α2 macroglobulin, fibrinogen, prothrombin, and complement factors, among others. They are structurally and chemically unrelated, and there is a broad amplitude in their physiological functions, which ranges from inhibition of pathogen growth, facilitation of their removal by phagocytic cells, and elimination of infected cells to other

unrelated functions, such as providing anti-inflammatory feedback to the immune system or modulation of coagulation (90). CRP is perhaps the most studied of these proteins, and it is frequently used as a diagnostic marker for inflammation. Interestingly, CRP is known to be released locally by cells of the respiratory epithelia and the liver in response to cytokine stimulation and that patients with ARDS have high levels of CRP (91). CRP was identified as a biomarker of disease severity in patients hospitalized with IAV infection at the time of admission (92). However, another study indicates that, although the levels of CRP are elevated in patients with acute lung injury, a higher level of plasma CRP predicts a more favorable outcome in adult patients (93). This protein has both pro-inflammatory and anti-inflammatory functions, and its function remains to be well characterized. Chronic overexpression of these acute phase proteins is also characteristic of some chronic, autoimmune pro-inflammatory diseases, such as RA.

Excessive production of IL6 has been associated with several pathological manifestations, such as Castleman disease or RA. For this reason, IL6 has been extensively investigated as a therapeutic target, leading to the development of monoclonal antibodies, such as tocilizumab, which has already been approved for the treatment of these diseases (94). However, in the case of IAV infection, IL6 seems to have a protective role in the mouse model, promoting viral clearance and limiting inflammation (95), indicating that IL6 blocking agents might not be adequate for inflammation treatment during IAV infection.

### IL1β

IL1β belongs to the broad IL1 family. The precursor of IL1β (pro-ILβ) is processed by caspase 1, which activation is mediated by the action of the inflammasome. IL1β is produced by immune cells, such as monocytes, tissue macrophages, and skin DCs in response to TLR activation, complement components, and other cytokines, such as TNFα (96).

The receptor for IL1β, as well as for IL1α, is IL1 receptor type I (IL1RI). The IL1RI presents a toll-IL1-receptor domain (TIR), which is also present in TLRs, and it is necessary for signal transduction. Engagement of IL1β/α to IL1RI leads to the recruitment of the co-receptor chain IL1R accessory protein IL1RAcP. Next, the adaptor protein myeloid differentiation primary response gene 88 (MyD88) interacts with the TIR domain, leading to phosphorylation of the IL1RI-associated kinases, IRAKs. Further phosphorylation steps involving the inhibitor of NF-κB kinase α and β (IKKα/β) and the NF-kappa-B essential modulator (NEMO) lead to the subsequent activation of the NF-κB transcription factors (97). JNK and p38 MAPK pathways are also activated upon IL1RI engagement (98). These events result in the induction of the expression of inflammatory genes including IL1α and β, as well as IL6 and RANTES among others, leading to an amplification loop.

While the main function of this cytokine is to mediate inflammation through activation of the NF-κB transcription factors, IL1β signaling has other additional consequences. For instance, the activation of IL1RI include increased expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthases (iNOS), prostaglandin 2 (PEG2) (71), and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) on mesenchymal cells and vascular-cell adhesion molecule-1 (VCAM-1) on endothelial cells. This latter property promotes the infiltration of inflammatory cells into the extravascular space (99).

IL1 cytokines are highly associated with acute and chronic inflammatory afflictions. As such, therapies to counteract the effect of this cytokine have been developed and are under study. In particular, treatment with an IL1R antagonist (IL1Ra), known under the generic name anakinra, has been approved to relieve symptoms and pain in patients with RA, and it is a standard therapy for autoimmune syndromes in general (96, 100).

Several studies suggest that IL1β has important roles in tissue damage in several mouse models of inflammation, including induction of systemic inflammation with turpentine or zymosan-induced peritonitis (101). There is also data indicating an excessive activation of the inflammasome in lung pathology – which is activated by the PB1-F2 protein of influenza virus (102), probably as a consequence of subsequent NF-κB activation. Interestingly, another study showed a positive effect on survival after administrating the IL1Ra to influenza virus-infected mice (103). However, in another model of influenza virus infection, while IL1β-infected mice showed reduced body temperature, mortality was higher in IL1β knock out mice (104). In agreement with this, more recent studies have suggested that the inflammasome, in which IL1β has an important role, is important for mediating healing and reducing lung damage, while it is not necessary for virus clearance or humoral adaptive immune responses (105). Indeed, there is increasing evidence that IL1β has an important role in epithelial repair in patients with ARDS (106–108) and this effect seems to be mediated by epidermal growth factor (EGF)/transforming growth factor-α (TGF-α) pathway (109). More recently, it has been shown that IL1β activates the expression of the early growth response (Egr)1 transcription factor through activation of the EGF receptor (EGFR) (110).

Therefore, excessive IL1β responses might contribute to lung injury during severe cases of influenza, but its role in tissue repair seems to be necessary to ensure recovery. Therapeutic strategies targeting this aspect of pathogenesis are complicated given the dual role of IL1β signaling in inflammation and in tissue repair, and a better understanding of the mechanisms of action of IL1β and consequences of altering its functions is needed.

### Type I and Type III IFN

The most important function of type I and type III IFN is to induce the activation of an antiviral state in infected and neighboring cells. For this reason, these cytokines are very important for protecting against acute viral infections. In addition, type I IFNs have also an important role in the stimulation of adaptive immunity (111, 112).

The most studied type I IFNs are IFNβ, expressed by virtually all cells, and IFNα, produced primarily by hematopoietic cells. Both IFNα and β interact with the IFNα/β receptor (IFNAR), which results in the activation of the receptor-associated protein tyrosine kinases (JAK1) and tyrosine kinase 2 (TYK2). Then, the transcription factors, STAT1 and STAT2, are phosphorylated, dimerize, and translocate to the nucleus (113), where they assemble with IFN-regulatory factor 9 (IRF9) to form the complex IFN-stimulated gene factor 3 (ISGF3). This complex binds specific sequences in the DNA and promotes transcription of hundreds of ISGs, which leads to numerous changes in the transcriptome of the

cell thus activating the antiviral response (114, 115). Also, under certain conditions, type I IFNs are able to induce the formation and phosphorylation of STAT1 homodimers, which may bind gamma-activated sequences (GAS) and induce the expression of a different set of genes (116). This GAS-stimulated gene response is mainly activated by type II IFN as described below, and is composed principally of pro-inflammatory genes. Interestingly, type I IFN signaling also leads to STAT3 phosphorylation, which downregulates type I IFN-mediated induction of inflammatory mediators (such as MIG and IP10) while supporting ISGF3-dependent induction of antiviral genes (117).

Type III IFN or IFNλ is a more recently discovered antiviral IFN that triggers STAT1 activation through engagement of an independent heterodimeric receptor, IL-28 receptor α/IL-10 receptor β (IL28Rα/IL10Rβ) complex (118, 119), found primarily on epithelial cells of both the respiratory and gastrointestinal tract. There are three IFNλ proteins, IFNλ-1, -2, and -3 (also known as IL29, IL28A, and IL28B, respectively), all of which signal through the same receptor. Signaling through type III IFN receptor complex results in a cascade of signals similar to that produced by ligation of the type I IFN receptor, which are mediated by JAK1 and TYK2, leading to the formation of a transcription factor complex, ISGF3. Therefore, the biological responses induced by type I and type III IFNs are very similar and mainly characterized by the induction of antiviral responses with the main difference between them being the expression of the receptor in different cell types (120).

While type I IFN is known to be a key mediator of virus clearance during influenza virus infection (121), excessive IFN signaling has detrimental effects on disease severity, mostly as a result of overall increased inflammation (pro-inflammatory cytokines and lung-infiltrating cells), cell death, and oxidative stress that might have damaging effects on the host (122, 123). The production of type I IFN and its pathological effects are supported by its role in other immune diseases. In particular, genetic and transcriptomic analysis of blood from systemic lupus erythematosus (SLE) patients, has attributed type I IFN a central role in the pathogenesis of this disease (124). Type I IFN has also been implicated in the pathogenesis of RA (125) and a type IFN I signature has been documented in patients with Aicardi–Goutieres syndrome (126). It has been shown that the type I IFN receptor sensitizes macrophages to death caused by *L. monocytogenes* infection (127). Interestingly, type I IFN also has been associated with endothelial dysfunction through induction of endothelial nitric oxide synthase (128). However, the mechanisms of type I IFN-mediated regulation of oxidative stress have not been analyzed in detail.

While the damaging potential of type I IFN is evident, the main feature of this family of cytokines is that they are crucial inducers of the antiviral response and therefore they are absolutely required to fight IAV infection. Studies performed in mice clearly indicate that viral replication and disease severity are increased in the absence of IFN, indicating that both type I and type III IFN having protective roles (129). Given the importance of type I IFN induction in defeating viral infection at the cellular level, desirable anti-inflammatory therapies to treat IAV or other viral infections should not fully blunt this type of response.

### Type II IFN

Interferon-γ is the only member of the type II IFN family and is mainly produced by T cells and NK cells. The production of IFNγ is controlled by IL12 and IL18 released by antigen-presenting cells, such as DCs and macrophages. Type II IFN plays important roles in the stimulation of antigen presentation by macrophages, in activating the cellular Th1 responses upon infection by intracellular pathogens, and in regulating B cell functions (130).

The IFNγ receptor (IFNGR) comprises two different subunits, IFNGR1 and IFNGR2. Activation results in signal transduction through JAK1 and JAK2 and subsequent phosphorylation and homo-dimerization of STAT1 transcription factors. STAT1 dimers subsequently translocate to the nucleus and activate the GAS elements (131), which lead to the expression of IFNγ-related genes. Interestingly, some of these genes are transcription factors (such as IRF1) that can lead to the activation of a second wave of genes (such as IFNβ) (130) and thus there is significant overlap between the IFNα/β- and IFNγ-regulated genes.

One of the most important functions of IFNγ is that it stimulates antigen presentation by several mechanisms. Thus, IFNγ upregulates the expression of the major histocompatibility complex (MHC) class I (132) and MHC class II (133). Interestingly, IFNγ also facilitates antigen processing by stimulating the expression of several molecules associated with this function, such as proteasome subunits, including LMP2 and LMP7 (134, 135) or of the regulator of the immunoproteosome proteasome activator (PA) 28 (136). At the cellular level, activation of IFNγ signaling promotes cell growth and proliferation, but also it has been shown to be important in the upregulation of pro-apoptotic molecules [such as protein kinase R (PKR), the death associated proteins (DAPs), cathepsin D, and surface expression of the TNFα receptor]. A very important consequence of the activation of macrophages and neutrophils by IFN is the enhancement of microbial killing processes, mainly mediated by induction of the NADPH-dependent phagocyte oxidase system or respiratory burst (release of ROS), stimulation of NO production, and upregulation of lysosomal enzymes (137, 138). This defense mechanism, however, is also damaging for infected tissues and has been shown to enhance the pathogenesis during IAV infections (41, 139). IFNγ has also implicated in the pathology of diseases, such as systemic lupus erythematous (140) or multiple sclerosis (141).

## Chemokines

Chemokines are small chemotactic cytokines that play important roles in driving many components of inflammation, the most important of which is leukocyte migration. Chemokine receptors in the cell surface are transmembrane G protein-coupled receptors (GPCRs), and their activation leads to the transduction of intracellular signaling pathways that promote migration toward the chemokine source. Other functions mediated by chemokines include regulation of cell viability, proliferation, differentiation, and migration (142). The chemokine system is very promiscuous in providing flexibility and specificity in the trafficking of immune cells, and a specific chemokine may act on several leukocyte populations to coordinate the recruitment of cells with related functions.

### RANTES

RANTES also known as chemokine (C–C motif) ligand 5 (CCL5), plays an active role in recruiting leukocytes to inflammatory sites. In particular, it has been shown to induce the migration and recruitment of T cells, DCs, macrophages, monocytes, eosinophils, NK cells, mast cells, and basophils (143–146). Also, it induces the proliferation and activation of certain NK cells. RANTES is produced by macrophages, DCs, T lymphocytes, platelets, eosinophils, fibroblasts, endothelial, and epithelial cells. In general, production of RANTES is associated with viral infections. Interestingly, RANTES is a co-receptor for HIV (147) and for this reason, there is a field of intensive research to develop pharmacological inhibitors of this receptor with the ultimate goal of producing a therapeutic agent (148, 149). High levels of RANTES have also been associated with extensive inflammation of the lung in cases of avian influenza (150) and other viral infections. Deficiency of the receptor for this chemokine, CCR5, which is also the receptor for MIP1α and MIP1β, resulted in increased mortality in IAV-infected mice, suggesting that the function of those chemokines is important for virus clearance, and therefore, they are not promising targets to reduce inflammation (151).

### IP10

IP10 or (C–X–C motif) ligand (CXCL) 10 is a protein highly associated with the presence of viral infection. Several cell types release IP10, including T lymphocytes, neutrophils, monocytes, DCs, endothelial and epithelial cells, and fibroblasts. IP10 expression is induced by IFN-γ and the gene features ISRE and NF-κB binding sites in the promoter (152), allowing for direct upregulation upon virus infection (153). IP10 interacts with the C–X–C receptor (CXCR) 3 to activate the main target cells, which include T and B lymphocytes, NK cells, DCs, and macrophages. As a consequence of this interaction, signal transduction leads to chemotaxis toward inflamed or infected areas, apoptosis, and proliferation or cell growth inhibition (154). IP10 is known to contribute to the pathogenesis of several infectious diseases (154) and of many autoimmune diseases, such as type 1 diabetes, RA, psoriatic arthritis, or SLE (155). Experiments in mice have shown that the lack of IP10 or its receptor reduces the severity of ARDS during influenza virus infection, suggesting the potential of this signaling pathway as a therapeutic target for ARDS treatment (156).

### IL8

IL8 or CXCL8 is a potent neutrophil attractant and activator, but also acts on monocytes and mast cells, which express the IL8 receptors, CXCR1 and CXCR2. This chemokine is mainly produced by macrophages, epithelial cells, and endothelial cells (157). Interestingly, monocytes and macrophages produce low amounts of IL8 during influenza virus infection (158), while epithelial cells produce high levels of IL8 *in vitro* (159). Several transcription factors activated upon viral recognition have been shown to bind IL8 promoter and stimulate IL8 production. These include NF-κB, the activator protein 1 (AP-1), the CCAAT-enhancer-binding protein (C/EBP)-β, IRF1, and IRF3 (160, 161). IL8 has a significant role in ARDS, which is characterized by a large influx of neutrophils to the lung during severe influenza (162). Neutrophils protect against microbial infection through the release numerous factors such as ROS, proteinases, and neutrophil extracellular traps, molecules that, when produced in excess, might also have damaging effects (163). In addition to the contribution of IL8 to pathogenesis through increased inflammation via neutrophil recruitment, patients with ARDS also have been shown to present auto-IL8 antibodies that complex with IL8. These complexes are also able to induce chemoattraction of neutrophils, but interestingly, they trigger superoxide and myeloperoxidase release (neutrophil respiratory burst and degranulation) from human neutrophils in a FcγRIIa-dependent way (164).

### MCP1

MCP1 or CCL2 regulates the migration and infiltration of cells expressing the receptor CCR2, which includes monocytes, memory T lymphocytes, and NK cells, and is produced either constitutively or after induction by oxidative stress or pro-inflammatory mediators. It also participates in the phenotypic polarization of memory T cells toward a Th2 phenotype (165, 166). MCP1 is produced by several different cell types, including endothelial, fibroblast, epithelial, smooth muscle, and monocyte cells among others, monocyte and macrophages being the main sources (167). This chemokine has been implicated in the pathogenesis of several diseases, such as asthma (168), RA, cardiovascular diseases, cancer (169), and some neuropathologies (170). CCR2 signaling results in the activation of PI3K, MAPK, and protein kinase C, and therefore, elicits a broad range of cellular responses (171, 172). In the context of IAV infection, conflicting results have been reported regarding MCP1 function. On the one hand, one study showed that neutralization of MCP1 *in vivo* reduced the immunopathology in a mouse model (173). However, a different report showed increased alveolar epithelial damage and apoptosis upon a similar treatment (174). A separate report showed that $CCR2^{-/-}$ mice infected with IAV presented decreased pathological signs, but higher pulmonary titers early after infection (151). Thus, further characterization on the role of this chemokine is necessary to determine its potential as a target for anti-inflammatory therapy.

### MIP1β

Macrophage inflammatory protein-1β or CCL4 is also involved in the recruitment of multiple immune cells, such as monocytes, T-lymphocytes, monocytes, eosinophils, basophils, DCs, and NK cells (175). It also induces activation of these cells and increased adhesion (176). Low levels are constitutively expressed but its production is activated by multiple inducers (such as PAMPs and cytokines) in different cell types, including monocytes, macrophages, neutrophils, DCs, epithelial cells, fibroblast, and multiple cells from the nervous system (175). The receptor for MIP1β is CCR5, although it is known that a natural truncated form of MIP1β, which lacks two N-terminal amino acids, also binds and signals through CCR1 and CCR2B (177). Associations with autoimmune diseases, such as SLE, have been also reported for MIP1β (178).

## Modulating the Innate Immune Response During Severe IAV Infection

As described above, the current literature indicates a clear role for hypercytokinemia during severe IAV infection. Initially, cytokine

production is induced following detection of the virus by cellular PRRs (**Figure 1**). Therefore, the primary treatment of IAV infection should be antiviral compounds, such as neuraminidase inhibitors, which will limit viral replication and spread, and therefore minimize the production of pro-inflammatory cytokines. Inflammation results in the induction of multiple cellular processes that lead to increased oxidative stress, apoptosis, necrosis, altered adhesion, and migration of immune cells to the lung. In addition, these processes lead to the release of additional secondary mediators and induction of cytokines, which results in amplified inflammation leading to increased damage (**Figure 1**). Therefore, it is worth considering therapies that modulate these detrimental processes in combination with antiviral agents. Targeting some of the most prevalent cytokines or related signaling pathways in severe influenza in mouse models, using either knock out animals or blocking agents, have been shown to reduce lung damage and mortality in multiple studies as indicated in the previous section, supporting the idea that anti-inflammatory agents inhibiting the same pathways could be beneficial in humans. One of the most important parameters that should be evaluated among these anti-inflammatory agents is that the treatment should reduce the negative effects of inflammation but not the innate and adaptive immune arms that are responsible for restricting viral replication and spread. However, the pathways initiated by the most prevalent cytokines are very redundant and dissecting these complex responses is very challenging. Specifically, blockade of TNFα and IL1β have shown a potential benefit in the mouse model, while blocking other cytokines, such as type I or III IFN or IL6-worsened disease outcome. Inhibition of specific chemokines or their receptors are also possible strategies. A few reports have been reported evaluating the consequence of blocking their function, which indicated that IP10 and MCP1 might have beneficial effects on reducing morbidity due to inflammation, while deficiency in RANTES expression seems to be detrimental. Further studies in animal models should be performed to better understand which of these pathways could be targeted as an anti-inflammatory therapy during severe influenza disease. In addition to cytokines and chemokines, other elements of the inflammatory response are under consideration for this purpose. In this section, we review those therapies that have been evaluated in the clinic or that have shown promising results in preclinical studies, such as broad-spectrum therapies, other signaling mediators or their receptors, or molecules involved in the generation of oxidative stress.

## Corticosteroids

Corticosteroid treatment has been proven to be safe in patients with ARDS and is associated with reduced inflammation and improved clinical status (179). For this reason, the use of these drugs has been considered for the treatment of severe influenza

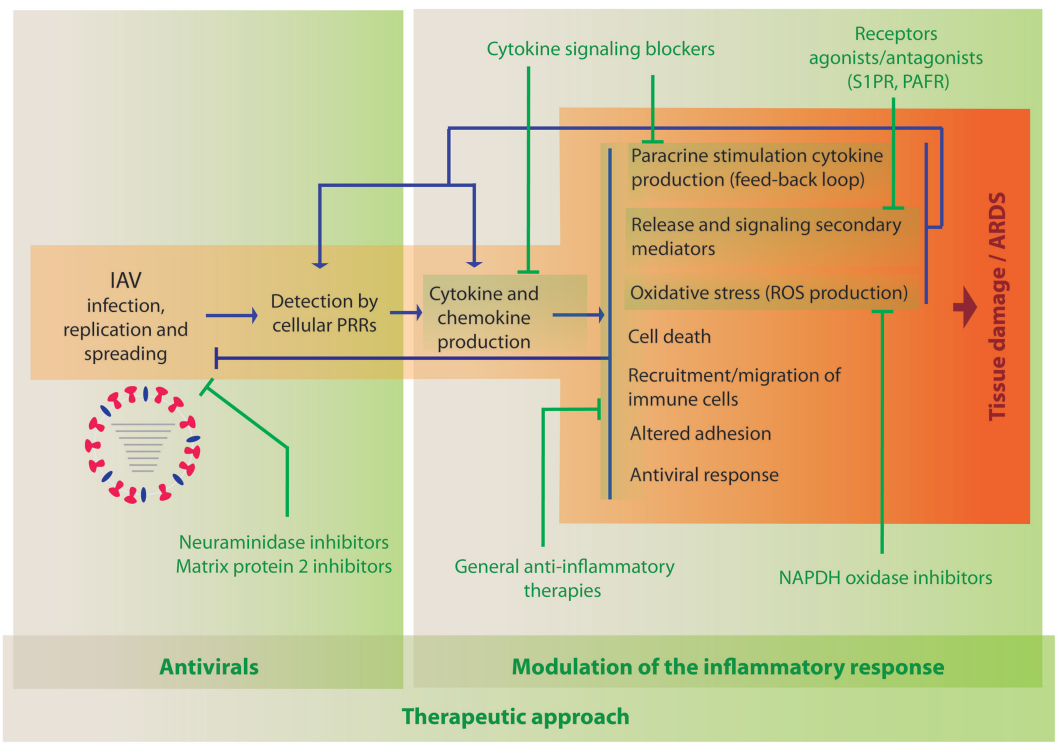


**FIGURE 1 | Activation of innate immune processes by IAV and therapeutic opportunities to modulate the immune response**. When IAV invades a new host, it infects and replicates in cells of the respiratory tract. Cellular sensors, such as TLRs, RLRs, NLRs, and CLRs, recognize the virus PAMPs and initiate immune responses leading to the activation of defense mechanisms to counteract viral infection. The development of the inflammatory response is accompanied by multiple changes in gene expression that also result in damage of the infected tissue. Antiviral treatment is the first opportunity to reduce viral load and inflammation (indicated in green, left panel). The use of anti-inflammatory drugs to reduce cytokine- and chemokine-induced damage that could be used in combination with antiviral therapies is under investigation (indicated in green, right panel).

and has, in fact, been used in several cases of avian influenza (H5N1) virus infection (180). In addition, corticosteroids are regularly used in long-term treatment for asthma and chronic obstructive pulmonary disease (COPD). Thus, understanding the effect of these drugs during influenza virus infection is very relevant not only for their anti-inflammatory use in cases of IAV infection but also to determine the best methods to manage these high risk patients in the clinical setting.

Several studies have evaluated the consequences of using corticosteroids in humans with influenza infection, with varying results. A study by Quispe–Laime reported a reduction in lung injury and multiple organ dysfunctions in H1N1 influenza virus-infected patients treated with corticosteroids (181). However, as recently reviewed by Hui and Lee, several clinical trials indicate that the administration of these steroids during influenza virus infection, either in the presence or absence of neuraminidase inhibitors, has either no effect or even a detrimental effect (182). A retrospective study by Kudo and colleagues evaluated the effect of corticosteroid administration in patients with 2009 H1N1 IAV infection with pneumonia and did not find a negative effect of the steroid treatment (183). On the other hand, Lee et al., in a prospective study with adult patients infected with H3N2 IAV, showed that the administration of systemic corticosteroid to reduce exacerbation of asthma or in patients with COPD correlated with delayed viral clearance (184). Accordingly, another retrospective study in adults infected with 2009 H1N1 influenza virus showed an association of corticosteroid treatment with increased mortality (185). An interesting study by Thomas et al. showed that glucocorticosteroid treatment prior to IAV infection inhibits antiviral responses and the release of cytokines when tested in cultured primary human airway cells, which resulted in increased viral replication (186). Similarly, an *in vivo* experiment in the same study showed higher replication in a mouse model after treatment, which resulted in enhanced inflammation. This is in agreement with the recent meta-analysis of the literature performed by Zhang et al., where they concluded that corticosteroids are likely to increase mortality and morbidity by influenza 2009 H1N1 virus (187).

Consequently, the current literature suggests that the corticosteroid treatment is not a good choice for the treatment of acute inflammation during influenza virus infection, probably due to increased viral replication as a consequence of reduced antiviral responses (188). Accordingly, WHO discourage the use of corticosteroid drugs as routine treatment for severe influenza due to the paucity of evidence for beneficial effects (189). Further research in this field should be done, given the routine use of chronic corticosteroids treatment in some other medical conditions, such as asthma and COPD, both of which are considered high risk populations for influenza disease.

## Statins

Statins are also a class of drugs with extensive use in the clinic given their ability to decrease cholesterol levels, thus reducing the risk of cardiovascular disease. These drugs are inhibitors of the hydroxyl methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme, acting in the cholesterol synthesis pathway. Interestingly, these drugs also have anti-inflammatory properties (190), which have been analyzed in the context of influenza infection (191). By altering the cholesterol synthesis route, statins also reduce the synthesis of lipid intermediates necessary for isoprenylation of multiple proteins. Consequently, multiple intracellular signaling pathways activated during the development of the inflammatory response are also affected (192).

An observational study that included more than 3000 patients hospitalized with influenza in the United States identified an association of statin use with reduced mortality (193). Other clinical studies have also shown that statin use could be beneficial in the treatment of influenza (194, 195), while yet other studies did not find supporting evidence for the use of this type of drug (196, 197). These retrospective studies, however, have the limitation that patients who are prescribed statins are some of those who are already at a higher risk for developing severe disease (due to pre-existing cardiovascular disease) and timing, duration and dose of the statin treatments are difficult to control. An interesting review by Mehrbod et al. (198) provides more detailed information on clinical trials evaluating the use of statins in IAV infections.

While the literature on this topic shows varied results, there is increasing evidence for a possible beneficial effect of the use of statins during influenza treatment, and further experimentation to confirm a positive effect should be developed. This is supported by several *in vitro* and *in vivo* studies that have indicated that, in addition to diminishing the production of cytokines upon influenza virus infection, statins also seem to result in decreased levels of viral replication (198–200).

## *N*-acetylcysteine

*N*-acetylcysteine is also a commonly used compound, which is mainly known for its mucolytic as well as anti-oxidant properties. Interestingly, anti-inflammatory properties have been also attributed to this molecule, which are probably associated with its anti-oxidant function by diminishing oxidative stress during inflammation. Related to this, animal models of systemic endotoxin-induced shock or acute lung injury showed reduced production of cytokines and tissue damage upon treatment with N-acetylcysteine (201–203).

Although the effect of this molecule in the context of influenza treatment has not been broadly studied, there are some reports indicating a possible beneficial effect. One study by Geiler et al. showed reduction of viral replication and pro-inflammatory cytokines in human lung epithelial cell lines upon infection with H5N1 influenza virus (204). The mechanism of inhibition seemed to be related to reduced NF-κB and MAPK p38 activation. These data were confirmed by a similar study where an H3N2 IAV and an influenza B virus strain were evaluated (205). Data from *in vivo* studies also seem to indicate that *N*-acetylcysteine might help to protect against IAV-induced pathology (206). It is important to note that in this case, contradictory reports have also been published, such as the study by Garigliany and Desmecht, which did not find an effect of the treatment in the mouse model (207). In humans, de Flora et al. (208) showed a long-term positive effect of *N*-acetylcysteine administration on the development of influenza or influenza-like symptoms. Therefore, although the amount of data reported is scarce, there seems to be evidence for a possible safe and beneficial effect for the use of *N*-acetylcysteine to treat

inflammation by influenza without enhancing viral replication. However, studies evaluating this molecule in humans are very limited, and more extensive work is needed to obtain conclusive information.

## Macrolides

Macrolides, which are generally used for their antibacterial activity, also have immunomodulatory properties. They have been shown to reduce the expression of several cytokines and chemokines, such as IL6, IL8, and TNFα during different inflammatory processes. The ability of macrolides to interfere with multiple signaling pathways accounts for these immunomodulatory properties. For instance, some macrolides suppress NF-κB and AP-1 signaling (209, 210), affect intracellular $Ca^{2+}$ dynamics (211), and inhibit the ERK1/2 pathway (212).

*In vitro* studies have shown that clarithromycin clearly reduces viral replication in epithelial cell lines approximately 4–7 h after viral adsorption (213). This effect is therefore also independent of the anti-inflammatory activity and might be mediated by alteration in cell signaling pathways. *In vivo* studies also support a potential role for the macrolides in improving recovery upon infection with IAV (214).

In the clinic, macrolides are sometimes administered in cases of influenza to treat secondary bacterial infections and because of their anti-inflammatory effects, clarithromycin being the most frequently prescribed first-line drug (215). Higashi et al. (216) analyzed the benefits of clarithromycin treatment in combination with neuraminidase inhibitors in patients with influenza infection. Their data indicated a possible effect in reducing fever, but they did not observe any differences in IL6 serum levels. However, another study could not find any association between significant improvement of symptoms and the use of macrolides.

In general, the number of studies evaluating macrolides in IAV infection is very limited. While *in vitro* and *in vivo* data showed promising results as indicated by a reduction of pro-inflammatory molecules alongside reduced viral replication, the small number of clinical studies does not suggest a significant benefit. Also, the use of antibiotics should be limited to cases with secondary bacterial infections, given the risk for emerging resistances. In addition, mice studies have shown that treatment with a combination of several antibiotics leads to impaired innate and adaptive immune responses and delayed virus clearance as a consequence of changes in the respiratory microbiota (217, 218) and therefore its use during influenza virus infection in humans should be further analyzed and cautiously used during severe infections.

## COX-2 Inhibitors

Cyclooxygenase enzymes catalyze the conversion of arachidonic acid to prostaglandins, which play important roles in modulating immune responses and inflammation. While the isoform COX-1 is constitutively expressed, COX-2 is induced by several stimuli, such as LPS, pro-inflammatory cytokines, and growth factors (219). Importantly, COX enzymes are main targets for non-steroidal anti-inflammatory drugs including aspirin, ibuprofen, diclofenac, naproxen, and for selective COX-2 inhibitors, such as celecoxib and nimesulide, and are therefore very available and frequently used as treatment for other conditions.

Considering the well-described pro-inflammatory role of COX-2, studies to understand its function in influenza pathogenesis have been performed. COX-2 knock out mice infected with IAV showed reduced levels of pro-inflammatory cytokines and mortality, but also increased levels of replication (220). Interestingly, COX-1 ablation showed opposite results, with augmented and earlier inflammatory responses. COX-2 expression was observed to be elevated in autopsy tissue samples from patients infected by H5N1 IAV (182, 221). *In vitro* experiments have shown that COX-2 inhibitors play a regulatory role in mediating pro-inflammatory responses after H5N1 infection (221, 222) and have been shown to have a direct antiviral effect in human macrophages infected with H5N1 influenza virus (223). However, another *in vivo* study did not find a beneficial effect from celecoxib treatment in mice infected with an H3N2 virus. Therefore, data regarding COX-2 inhibitors are also controversial. Another *in vivo* study did observe a positive effect of celecoxib administration when used in combination with mesalazine or 5-aminosalicylic acid (another anti-inflammatory drug) in addition to a neuraminidase inhibitor in mice challenged with H5N1 IAV (224), supporting the idea that a combination treatment might be more efficient.

To date, there are no systematic human studies evaluating COX-2 inhibitors for influenza treatment. The event that these studies move forward is important to consider the selectivity for COX-2 inhibitors, since COX-1 inhibitors would have an opposite effect, increasing inflammation, and pathogenesis. Indeed, an increased risk of mortality during influenza virus infection was associated with aspirin, paracetamol, and diclofenac in animal models in a meta-analysis of the literature (225).

## Peroxisome Proliferator-Activated Receptor Agonists

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors and ligand-activated transcription factors that control a number of target genes upon assembly of a transcriptional complex. There are several PPAR, but in general, they are regulators of energy balance, including glucose homeostasis, fatty acid oxidation, and lipid metabolism, and are frequently used in the treatment of diabetes (226).

Several *in vivo* studies point to a possible benefit of the use of these drugs in treating influenza infection. Moseley at al. showed a reduction in morbidity and mortality in mice infected with two different H1N1 strains and treated with PPAR agonists (227). Similarly, PPAR agonist treatment of mice challenged with an H5N1 or an H2N2 IAV led to decreased inflammation and morbidity, and increased survival (228–230), using a cyclopentenone prostaglandin (prostanoid 15-deoxy-Δ12,14-prostaglandin-j2), observed a reduction in the levels of cytokines and chemokines in a mouse model of influenza in addition to a reduction in viral titers, and this effect was shown to be mediated by PPARγ (230).

While these drugs have not been thoroughly studied for influenza treatment and no human studies have been performed so far, exploring their potential would be of great interest given their current use in the clinic and availability, which would facilitate their study in clinical trials (231).

### Sphingosine-1-Phosphate-1 Receptor Agonists

Sphingosine-1-phosphate (S1P) is a lipid signaling mediator synthesized from ceramides. The laboratory of Dr. Oldstone at The Scripps Research Institute (La Jolla, CA, USA) has focused on the use of S1PR agonists as a possible therapeutic to alleviate the inflammatory response arising during IAV infection, providing very interesting insights about the mechanisms of immunopathogenesis. They were first able to demonstrate that the administration of a promiscuous S1P receptor agonist led to a significant reduction of cytokines and chemokines upon influenza infection in the mouse model (232–234). This reduction of the inflammatory response correlated with a decrease in lung injury and improved survival upon infection (235). Importantly, the reduction of inflammation was not accompanied by a delayed clearance of the virus, indicating a potential for the use of these drugs as a therapeutic agent (234). Further work using S1PR agonists led them to describe a central role for endothelial cells in the generation of the cytokine storm (236). They further searched for the signaling pathways that the S1PR agonists might use to exert these anti-inflammatory-protective functions during IAV infection and found that the effect observed is independent of TLR3, TLR7, or cytosolic signaling pathways (237). In addition, they found an essential role for IL1R and MyD88/TRIF signaling in cytokine amplification (237). Therefore, although S1PR agonists are under investigation in mice and ferrets for influenza treatment (238), results from these studies are promising as a possible future treatment for hypercytokinemia in severe cases of influenza. One S1PR agonist has been approved in the clinic by the FDA for the treatment of relapsing–remitting multiple sclerosis. However, adverse effects have been noted in the use of this drug, and the safety profile of this and other S1PR agonists should be further investigated (239).

### Platelet-Activating Factor Receptor Antagonists

Platelet-Activating Factor (PAF) is a phospholipid mediator involved in many cellular processes including cell motility and synthesis of cytokines and other signaling mediators (240). PAF signaling occurs through the PAF receptor (PAFR), which is a single GPCR, expressed in the plasma and nuclear membranes of leukocytes, endothelial cells, epithelial cells, smooth muscle cells, and platelets (240). It is known that expression of PAFR in the airway is upregulated by IAV infection, and it is believed that this facilitates bacterial adherence and therefore susceptibility to *Streptococcus pneumonia* (241).

The use of PAFR antagonists has been proposed in different pathological settings, including influenza, mainly due to their anti-inflammatory properties (242). Using *PAFR* knock out mice and antagonists, Garcia et al. demonstrated that eliminating or counteracting these receptors reduced lung injury, infiltration of mononuclear cells and neutrophils, and the expression of IL12, RANTES, and IFNγ while not affecting the levels of IL6 and increasing IL1β production (243). This overall reduced immune response did not result in an elevated level of viral replication. A mechanistic analysis showed activation of TLR7/8 during infection was dependent on PAFR. While according to these data, PAFR antagonists could be candidates to treat inflammation during influenza, further characterization of the effect of these drugs should be performed.

### Other Candidates

Other anti-inflammatory therapies have been tested in animal models resulting in reduced inflammation, morbidity, and mortality. While these studies support the potential positive effect of immunomodulatory therapy in severe influenza, the scientific data in this field are very preliminary, and extensive investigation is needed to develop these treatments for human use. Here we discuss some of these treatments.

NADPH oxidases, enzymes that are involved in ROS production, have also been proposed as targets for reducing IAV-induced inflammation. There is evidence that activation of NOX2 promotes lung oxidative stress, inflammation, injury, and dysfunction resulting from infection with IAV ranging from low to high pathogenicities (244). Apocynin, a NOX2 inhibitor, inhibited influenza-induced hypercytokinemia and ROS production in airway epithelial and immune cells *in vitro*, while not affecting viral replication (41).

A study by Sharma et al. analyzed the effect of other two orally available and approved anti-inflammatory drugs, a phosphodisesterase-4 inhibitor and a selective serotonin reuptake inhibitor. This study showed a clear reduction in the levels of cytokines and chemokines, lung infiltration, alveolitis, and overall lower mortality in H1N1-infected mice, all while not affecting the levels of viral replication (245).

Another research group further explored the combination of antiviral and anti-inflammatory therapy and generated a novel compound with these two properties by conjugating two drugs, zanamivir (a neuraminidase inhibitor) and caffeic acid (cytokine suppressor) (246). This innovative method provided improved protection in mice against H1N1 and H5N1 IAV.

## Concluding Remarks

There is substantial information in the literature supporting the association of influenza pathogenesis with high levels of inflammation and production of cytokines and chemokines, highlighting the opportunity to identify immunomodulatory drugs that could reduce the inflammation-associated damage in the lung seen in severe cases of influenza. These therapies should be evaluated in combination with antivirals, which control virus replication and spread. Reduction of viral load with antiviral drugs also acts to decrease inflammation by lowering the presence of PAMPs to be sensed by cellular PRRs. In addition, one crucial aspect to assess when testing these drugs is to assure that the treatment does not provide an environment for enhanced replication due to a general shutdown of the innate and adaptive immunity.

To date, the therapies studied in humans have commonly used broad-spectrum anti-inflammatory drugs, which are frequently used for other affections. Corticosteroids are a good example of those therapies, which are frequently used in patients with asthma and COPD, and have been evaluated in multiple studies with conflicting results. Some of those studies point to a possible detrimental role of treatment with corticosteroids, and their use

should be avoided if possible until their effect is better understood. Other broad-spectrum anti-inflammatory drugs that could be beneficial are statins, *N*-acetylcysteine, and COX-2 inhibitors. However, there is no sufficient data in the current literature to justify their use. More specific treatments that have been explored in animal models include blocking cytokines, such as TNFα or IL1β, reducing the oxidative stress through NADPH inhibitors, or the use of inhibitors for receptors for secondary inflammatory mediators, such as PAFR or S1PR. As for the last examples, targeting cell surface receptors in immune cells is an attractive approach since this would facilitate cellular accessibility of the drug. Further research to bring these therapies closer to the clinic in the context of IAV infection is needed, as well as for the identification of novel immunomodulatory agents.

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# Mast cells and influenza A virus: association with allergic responses and beyond

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Influenza A virus (IAV) is a widespread infectious agent commonly found in mammalian and avian species. In humans, IAV is a respiratory pathogen that causes seasonal infections associated with significant morbidity in young and elderly populations, and has a large economic impact. Moreover, IAV has the potential to cause both zoonotic spillover infection and global pandemics, which have significantly greater morbidity and mortality across all ages. The pathology associated with these pandemic and spillover infections appear to be the result of an excessive inflammatory response leading to severe lung damage, which likely predisposes the lungs for secondary bacterial infections. The lung is protected from pathogens by alveolar epithelial cells, endothelial cells, tissue resident alveolar macrophages, dendritic cells, and mast cells. The importance of mast cells during bacterial and parasitic infections has been extensively studied; yet, the role of these hematopoietic cells during viral infections is only beginning to emerge. Recently, it has been shown that mast cells can be directly activated in response to IAV, releasing mediators such histamine, proteases, leukotrienes, inflammatory cytokines, and antiviral chemokines, which participate in the excessive inflammatory and pathological response observed during IAV infections. In this review, we will examine the relationship between mast cells and IAV, and discuss the role of mast cells as a potential drug target during highly pathological IAV infections. Finally, we proposed an emerging role for mast cells in other viral infections associated with significant host pathology.

**Keywords: mast cell, mast cell activation, influenza A virus, dengue virus, inflammation, degranulation, viral infection, viral immunology**

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## Introduction

Influenza A virus (IAV) is a common human respiratory pathogen, which causes annual seasonal infections with a low frequency of morbidity and mortality, usually limited to the young (<5 years) and the elderly (>65 years) populations. Importantly, IAV has the potential to cause global pandemics, which can significantly increase morbidity and mortality throughout the entire population (1). In the past century, there have been four major IAV pandemics: the 1918 H1N1 "Spanish" influenza, the H2N2 "Asian" influenza in 1957, the H3N2 "Hong Kong" influenza in 1968, and more recently, the reemergence of a pandemic H1N1 (H1N1pdm) influenza in 2009 (2). Moreover, significant spillover infections from the zoonotic avian reservoir of IAV continue to have an impact on the human population, including the current avian H5N1 and H7N9 IAV outbreaks in Southeast Asia (3). To date, these

H5N1 and H7N9 outbreaks have remained a spillover event, but the potential of these novel avian IAV strains to develop the ability to efficiently transmit human-to-human through aerosol droplets exists (3–5); thus, increasing the threat of new global pandemics.

As an RNA virus that lacks proofreading capabilities, IAV has a high mutation rate, resulting in significant antigenic drift in the immunodominant hemagglutinin (HA) and neuraminidase (NA) proteins. Furthermore, owing to its segmented genome, IAV can undergo genetic reassortment (antigenic shifts), resulting in novel IAV strains with the potential to rapidly transmit between humans to cause a new pandemic. Given these factors, the next pandemic IAV strain is nearly impossible to predict, leading to many challenges in vaccine development. Current vaccine strategies take approximately 6 months for production. During the 2009 H1N1 pandemic, this delay resulted in no effective vaccine being available for the first wave of the pandemic (2). Thus, it is necessary to find alternative ways to alleviate and treat IAV-induced disease during the early wave(s) of a novel pandemic IAV outbreak.

Antiviral drugs are an obvious front line of defense against the emergence of novel IAV strains. Currently, two main classes of antiviral drugs are approved to treat IAV-infected patients. The first class of antiviral drugs targets the M2 ion channel (amantadanes), which is important for virus uncoating. However, amantadanes are no longer recommended for prophylaxis or treatment of IAV due to widespread resistance among current human seasonal H1N1 and H3N2 isolates (6–8). The second class of antiviral drugs targets the enzymatic active site of the viral NA. The viral NA is a sialidase capable of hydrolyzing terminal sialic acid residues from glycoproteins and glycolipids. The NA is crucial in allowing the IAV to traverse the glycan rich soluble mucins in the respiratory tract, as well as allowing newly formed virions to be released from host cells, to be shed into the extracellular space for dissemination within a host and transmission between hosts. NA inhibitors are becoming of limited efficacy as well, due to emerging resistance among IAV isolates found in humans and the requirement for early administration (within 48 h of the presentation of symptoms) for maximal effectiveness (2, 7, 9–12). Therefore, additional antiviral drugs are required to limit IAV-induced disease and fight the spread of IAV. Numerous drugs are currently in development, which target viral entry, viral transcription, or host factors necessary for IAV replication (9). However, the effectiveness of these drugs against IAV in the clinical setting is unknown.

An alternative front line defense against the emergence of novel IAV strains is to target the inflammatory pathways that lead to lung damage and loss of function (13, 14). Alveolar epithelial cells, endothelial cells, tissue-resident alveolar macrophages, dendritic cells, and mast cells protect the lungs, as these cells are readily able to respond to invading pathogens. Pandemic strains of IAV, including the 1918 "Spanish" influenza and the 2009 H1N1pdm influenza, and spillover infections with avian IAV isolates can produce excessive tissue damage and pathological changes to the lung architecture (1, 15, 16). Current evidence suggests the lung injury induced during IAV infection is the result of excessive leukocyte infiltration and an exaggerated inflammatory cytokine response that is disproportionately high relative to the level of viral replication, which has been termed a "cytokine storm" (16–21). Selectively dampening the inflammatory response in mice has been shown to increase survival following IAV infection without impairing viral clearance (16, 17, 19–22). Thus, understanding the inflammatory cascade responsible for the immunopathology observed following IAV infection is imperative for the development of novel immunotherapeutics aimed at limiting IAV-induced disease and pathology.

Macrophages and neutrophils are recruited at excessive levels following infection with the 1918 or H5N1 influenza strains (16). More recently, it has been demonstrated that mast cells play a pivotal role in initiating and/or amplifying the immunopathological "cytokine storm" and inflammatory leukocyte recruitment in the respiratory tract during IAV infection (23–25). Mice infected with either H1N1 or H5N1 IAV demonstrated elevated levels of inflammatory cytokines and chemokines during infection. Conversely, mice lacking mast cells or treated with mast cell stabilizing agents show a reduction in the levels of these inflammatory mediators that correlates with a decrease in the recruitment of inflammatory cells to the lungs during infection (23, 24). Therefore, it is crucial that the individual and collective roles of these inflammatory cells, with each other and with the epithelial and endothelial compartments, during pathological IAV and other pathological viral infection, be more thoroughly examined.

## Mast Cell Biology

Mast cells are tissue resident, granule-containing cells capable of regulating both the innate and adaptive immune response (26). Enrichment of mast cells at environmental interfaces allows these cells to be among the first to respond during pathogen invasion, along with dendritic cells and epithelial cells (27). Moreover, mast cells are typically situated near blood vessels, lymphatics, and nerve endings, enabling them to have long range effects on the host response to pathogens (27, 28). As such, mast cells are critical to immune surveillance, eliciting an immediate reaction to invading pathogens and initiating an appropriate innate and adaptive immune response.

### Phases of the Mast Cell Response

Mast cells have two distinct phases of activation: immediate degranulation, resulting in the release of pre-synthesized mediators, and delayed secretion of secondary *de novo* synthesized mediators (27, 29, 30). The delayed secretion of secondary *de novo* effector molecules produced by mast cells can be further segregated into two classes: (1) prostaglandins and eicosanoids released within minutes of activation, and (2) cytokines, chemokines, and growth factors that are released within hours of stimulation (**Figure 1**). Together, these mast cell outputs can increase epithelial and endothelial cell permeability and activation state, which together with chemotactic molecules, result in increased inflammatory cell recruitment to infected tissues (**Figure 2**).

Mast cell granules contain histamine, TNF-α, amines, β-hexosaminidase, serotonin, antimicrobial peptides, and proteases (tryptases and chymases) bound to either heparin or chondroitin sulfate through electrostatic interactions (29, 31–33). Upon stimulation, the granules are released from the cell via a calcium-dependent exocytosis process. Once expelled, the granules can either discharge the stored mediators into the immediate environment or intact granules can travel through the

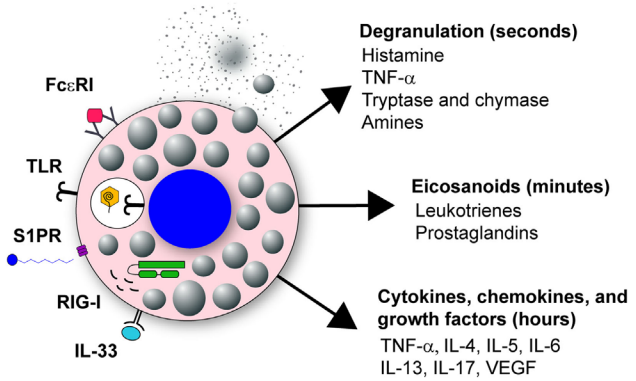


**FIGURE 1 | Mast cell activation in response to viral infection**. Mast cells are classically known for their response to polyvalent cross-linking of IgE in the FcεR1 receptor, which is important in protective immunity to helminth worm infection and pathologically associated with allergic disease. However, mast cells also are important tissue sentinel cells for initiating inflammatory response to pathogens. Mast cells can recognize and respond to viruses through several different receptors. These receptors include TLR signaling, such as TLR3 detection of dsRNA, sphingosin-1-phosphate (S1P) binding to its receptor S1PR, and RIG-I recognition of uncapped vRNA. Engagement of these receptors results in mast cell activation leading to immediate degranulation, the *de novo* synthesis of eicosanoids within minutes of activation, and the *de novo* synthesis of numerous cytokines, chemokines, and growth factors within hours of activation.

bloodstream and lymphatics, acting as a signaling mechanism to activate and recruit other cells to the infected tissue (34, 35). Histamine is a potent inflammatory molecule, which increases vascular permeability, induces vasodilation, and stimulates bronchial smooth muscle contraction. The inflammatory cytokine TNF-α promotes local and systemic inflammation while enhancing neutrophil recruitment to the site of infection. Granule proteases are capable of increasing vascular permeability and enhancing the recruitment of neutrophils to the site of inflammation (36–39), or can act directly to detoxify toxic proteins (40–43). Interestingly, the local homeostatic cytokine milieu of a tissue modulates the precise granule components, allowing mast cells to adapt to their local environment to mount a tissue appropriate inflammatory response (44, 45). Following activation, mast cells are unique in that they replenish their granules, usually within weeks of activation (46, 47). This ability to regranulate allows mast cells to tailor the composition of their granules, and thus be more prepared for reinfection (**Figure 2**) (27).

After the immediate mast cell degranulation response, the arachidonic acid-dependent inflammatory mediators, such as leukotrienes and prostaglandins, are rapidly produced and released from mast cells due to enzymatic, rather than transcriptional, changes within the mast cell (48). These lipid mediators contribute to local vascular permeability, tissue edema, and the recruitment of neutrophils and other inflammatory cells (49–51).

Finally, *de novo* synthesized cytokines, chemokines, and growth factors are released, hours following activation through transcriptional and translational up-regulation. The multitude of cytokines, chemokines, and growth factors released by mast cells include *de novo* synthesized TNF-α, IL-4, IL-5, IL-6, IL-13, IL-17, and VEGF (32, 52). These mediators activate tissue-resident cells, while recruiting additional effector leukocytes and lymphocytes to maintain the inflammatory state for a prolonged time. In conclusion, through the release of numerous chemotactic factors and vasodilators, mast cells are optimized for the rapid initiation and propagation of an acute inflammatory response through degranulation, production of bioactive lipids, and secretion of cytokines and chemokines. The resulting leukocyte and lymphocyte infiltrate can then help to maintain the inflammatory state if the infection persists (**Figure 2**).

### *De Novo* Mast Cell Recruitment During Inflammation

In addition to tissue-resident mast cells, mast cell progenitors can be recruited to sites of acute or chronic inflammation. How the recruitment of these mast cell progenitors is regulated is just now beginning to be understood. Mucosal mast cells (MMC), the dominant type of mast cell in the lung, develop from the bone marrow as mast cell progenitors (53). In an asthma model, following aerosolized challenge with ovalbumin, mast cell progenitors are rapidly recruited into the lungs, peaking day 1 after challenge (54). In this ovalbumin-induced allergic airway inflammatory disease, multiple pathways are critical for mast cell progenitor accumulation in the lungs. Integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ regulate the migration of mast cell progenitors to the lungs through VCAM-1 interactions (55). Moreover, CXCR2 expression in a radio-resistant cell population is important in regulating mast cell progenitor recruitment to the lungs, likely through its regulation of VCAM-1 on the pulmonary endothelium (56). NKT cells are also able to induce mast cell progenitor accumulation in the lungs through an IL-9 dependent pathway (57). Finally, both prostaglandin $E_2$ and leukotriene $B_4$ ($LTB_4$), which can be highly produced by mast cells, have been shown to enhance chemotaxis of mast cell progenitors (58, 59). In addition to their well-elucidated role in allergic airway disease, there is strong evidence for an accumulation of mast cells in the intestinal tract during helminth infections (60). Furthermore, mast cell precursors appear to accumulate at sites of viral infection including IAV, Sendai virus, infectious bursal disease virus (IBDV), and Newcastle disease virus (NDV) (61–65). Accumulation of mast cell progenitors occurs either in a mast cell degranulation-dependent (24, 62–65) or -independent manner (61, 66). Therefore, mast cell activation can result in the local accumulation of mast cells in infected tissue, further augmenting the role these cells can play during infection (**Figure 2**).

### Expression of Pattern-Recognition Receptors by Mast Cells for Sensing Invading Microbes

Mast cells express a large array of innate cell surface and cytosolic receptors that mediate their activation, and as such are integral cells in initiating appropriate immune responses to infectious agents. Notably, mast cells express a large array of Fc receptors including FcεRI, FcγRI, and FcγRIII (67). Mast cells are also able to respond through a wide variety of pattern-recognition receptors (PRR), including toll-like receptors (TLR), nod-like receptors (NLR), retinoic-acid inducible gene 1-like receptors (RLR), and C-type lectin receptors (CLR), each of which play an essential role in innate immunity by detecting conserved molecular patterns expressed by pathogens (68–82). Mast cells can also be activated through

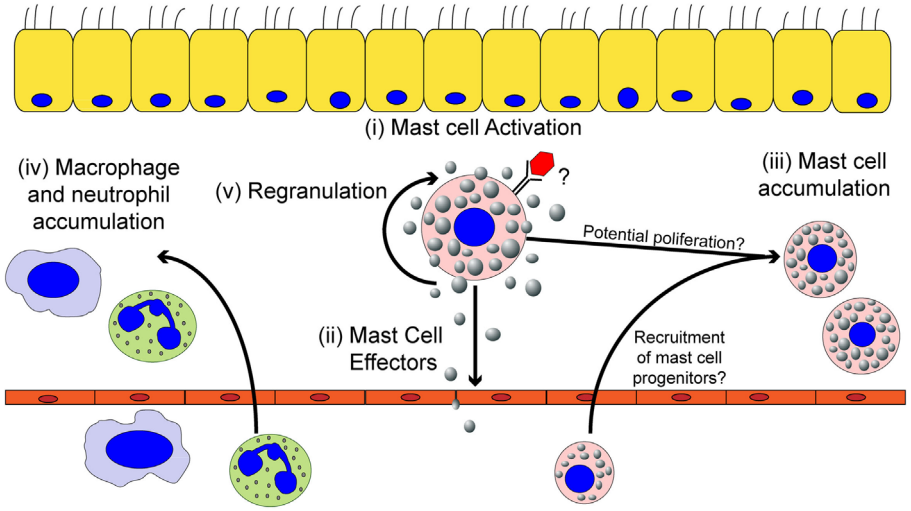


**FIGURE 2 | The effects of mast cell activation on the inflammatory environment induced by viruses**. Within the tissues, mast cells can be activated by viruses (i) resulting in the secretion of effector molecules (ii). Mast cell-derived effector molecules act within the local tissue environment or at distal site to mediate the accumulation of mast cell progenitors (iii) and leukocytes (iv) to the site of infection. Mast cell accumulation in the infected tissues could be due to either the recruitment and differentiation of mast cell progenitors to the infected tissue and/or proliferation of the tissue-resident mast cell population. Mast cell activation can participate in limiting viral replication in the local tissue and viral dissemination, but if left unchecked can cause significant tissue damage, vascular leakage, and tissue edema. Finally, activated mast cells can survive the pathogenic insult and replenish mast cell granules to return the mast cell to a basal state to survey the tissue for future pathogenic insults (v).

engagement of complement receptors (28), CD48 (83, 84), and integrins (85). Lastly, mast cells can respond to pathogens indirectly through the IL-33 signaling pathway (48). Thus, mast cells are capable of responding to a broad range of pathogen-derived or pathogen-induced stimuli (**Figure 1**). Interestingly, mast cells do not respond uniformly to all input stimuli (86). For example, signaling through TLR4 leads to a strong pro-inflammatory cytokine response, but limited mast cell degranulation. Conversely, signaling through TLR2 induces both an inflammatory cytokine response and mast cell degranulation (87). Mast cell activation therefore is an important rheostat for the immune system, which will likely modulate to the appropriate response. However, aberrant activation or prolonged activation may elicit tissue immunopathology.

### Role of Mast Cells in Allergies and Asthma

Mast cells are most frequently recognized for their detrimental role during an allergic response. Following an initial exposure to antigen (Ag), activated B cells can undergo class switching, resulting in the secretion of IgE. The high-affinity IgE receptor, FcεRI, expressed on the surface of mast cells binds to the Fc portion of IgE, sensitizing the mast cells. Upon subsequent exposures, polyvalent Ag cross-links the surface bound IgE resulting in mast cell degranulation and the production of bioactive lipids and cytokines and chemokines (67, 88, 89).

Mast cells have also been recognized for their role in asthma. Asthma is a pleomorphic disease characterized by recurrent airway restriction, shortness of breath, wheezing, and coughing. Within asthma patients, including both atopic (allergic) and non-atopic (intrinsic), the number, localization, and phenotype of mast cells are altered. Repeated activation of the pulmonary mast cells by the allergen results in mast cells, which are more likely to degranulate compared to non-asthmatic patients (90, 91). Overall, the mast cell response contributes to the bronchial constriction, chronic inflammation, and tissue remodeling typical of asthma patients.

It is now well-documented that infection with respiratory viruses, including IAV, rhinovirus, and respiratory syncytial virus (RSV), often exacerbates asthma (92–96). These upper respiratory tract infections frequently lead to hospital admission for asthma patients (97). Interestingly, asthma was the most common comorbidity among hospitalized patients during the 2009 H1N1pdm IAV pandemic (98–101). A state of hyperresponsiveness in the asthmatic patients, as well as increased levels of inflammatory molecules (e.g., histamine, IL-6, and leukotriene), are believed to contribute to asthmatic exacerbation from viral infection (102). Thus, it is critical we understand the interactions of mast cells with viruses in both naïve hosts and those with chronic inflammatory conditions, which alter mast cell numbers and function.

## Is There a Role for Mast Cell Activation and Mediators During Pathological Viral Infections?

Numerous highly pathological viral infections cause significant disease through immune-mediated pathology to tissue and/or induction of vascular permeability. For example, during dengue virus infections there is significant vascular permeability, which is associated with severe disease and mast cell activity (51, 103). Additionally, severe respiratory virus infection can induce acute respiratory disease syndrome (ARDS), which is associated with significant epithelial–endothelial dysfunction and excessive

activation of macrophages and neutrophils (104). ARDS has been observed during experimental IAV infection of animal models, as well as in people naturally infected with highly pathological IAV isolates, such as the 1918 H1N1 "Spanish" influenza strain and the recent zoonotic outbreaks of avian H5N1 and H7N9 IAV strains (105–107). An eloquent transcriptome analysis by Josset et al., which compared highly pathological versus seasonal IAV infections, detected a strong transcriptional signature of macrophages and neutrophils in the lungs of mice with severe IAV infection (108), which fits with prior histological observations (16). Intriguingly, Josset et al. also saw a strong transcriptional contribution of mast cells during these severe IAV infections (108); however, these authors did not explore the role this cell population might play in the observed disease. We propose that, in addition to macrophages and neutrophils, mast cells may contribute to the excessive inflammatory response and vascular problems observed not only during highly pathogenic IAV but also in a range of highly pathogenic viral infections as further discussed below.

## Influenza Virus

Pandemic isolates and the emerging highly pathogenic avian strains of IAV are capable of inducing a robust inflammatory response, which causes significant damage within the lungs and the ultimate restructuring of the lung architecture (1). In humans experimentally infected with IAV, detection of histamine metabolites correlates with clinical symptoms (109, 110). Moreover, emerging data in the murine model of IAV suggests a link between mast cell recruitment and activation with lung immunopathology. Following inoculation with a mouse adapted strain of the 2009 H1N1pdm IAV (A/California/04/2009), mice develop significant pathology and inflammation, recapitulating clinical observations from the 2009 pandemic in humans, while mice infected with a non-adapted strain do not (108, 111). In those mice inoculated with the mouse-adapted 2009 H1N1pdm IAV, an enrichment of genes for activated macrophages, neutrophils, and mast cells was observed when compared to mice inoculated with the non-pathogenic strain (108). Moreover, this same observation was made during infection with recombinant 1918 H1N1 (108). Thus, it appears that early accumulation of activated macrophages, neutrophils, and mast cells correlates with the immunopathology associated with pandemic IAV infections.

As this prior transcriptomic study suggested (108), increased mast cell density was observed in the nasal mucosa, trachea, lung parenchyma, and mediastinal lymph node following infection with a highly pathological H5N1 isolate (A/chicken/Henan/1/2004) (24). While these data demonstrated that mast cells are increased in the lungs of mice during highly pathological IAV infection, their role in the inflammatory response induced by IAV remained elusive. In this regard, recent data demonstrates that mast cells can play a detrimental role during IAV infection in a strain specific manner. Specifically, following infection with A/WSN/1933, B6.Cg-*Kit*$^{W\text{-}sh}$ mice, which lack mast cells (112), exhibit a reduction in weight loss, lung pathology, and pulmonary inflammation compared to wild-type mice (23). Importantly, when mast cells are reconstituted into B6.Cg-*Kit*$^{W\text{-}sh}$ mice, the weight loss and inflammatory response are restored to wild-type levels (23). In studies using a highly pathogenic H5N1 virus (A/chicken/Henan/1/2004), mice administered ketotifen, a mast cell stabilizing agent, demonstrate reduced lung inflammation and epithelial cell apoptosis than untreated mice (24). Furthermore, combination therapy with ketotifen and oseltamivir (an NA inhibitor) improves survival better than either drug alone (24). Taken together, these data show mast cells can contribute to the pathology observed during IAV infection in mice. The newly emerging zoonotic strains of highly pathogenic IAV, such as H7N2, are also presenting with high cellular infiltrate and damage within the lungs of mice, suggestive of mast cell activation (25, 107). If mast cells participate in the immunopathology elicited by these emerging zoonotic IAV isolates remains to be seen.

## Dengue Virus

Human infection with dengue virus can result in a wide range of pathologies. In its most severe forms, dengue virus induces dengue hemorrhagic fever and dengue shock syndrome, both of which are characterized by increased vascular permeability. The production of cross-reactive antibodies during a primary infection can lead to more severe disease upon secondary infection with a heterologous serotype (113, 114). The urine and blood of infected patients display elevated levels of histamine (115, 116), the presence of vasoactive factors (117, 118) and increased serum levels of chymase, a mast cell specific enzyme (103). As each of these mediators is released by mast cells, numerous studies have examined the role mast cells play during dengue virus infection. Upon exposure, dengue virus induces both degranulation and cytokine production by mast cells (82, 103, 119, 120). Mast cell derived $LTB_4$ and granule proteases increase vascular permeability (82, 103), while the synthesis and release of TNF-α, IL-6, IFN-α, CCL2, CCL3, CCL5, and CX3CL1 recruit NK cells and T cells to the site of infection (82, 121–123). Mast cell deficient mice show a reduction in symptoms, demonstrating that mast cells play an important role in dengue virus-induced immunopathology (103). Moreover, administration of the mast cell stabilizing drugs, cromolyn and ketotifen, or the $LTB_4$ antagonist montelukast results in reduced vascular leakage compared to untreated mice (103). Current data suggests that early after infection, mast cell activation by dengue virus is beneficial, as it recruits NK and T cells to promote viral clearance (82, 122, 123). However, widespread mast cell activation is detrimental, as it increases vascular leakage, leading to the more severe forms of dengue-induced disease (103). In a murine model, the presence of non-neutralizing IgG enhances mast cell degranulation during dengue infections through interactions with FcγRIII (124). Therefore, dengue virus can activate mast cells both directly, through an as yet unidentified mechanism, or indirectly through FcγRIII.

## Hantavirus

The zoonotic transmission of hantavirus to humans can result in hemorrhagic fever with renal syndrome or hantavirus cardiopulmonary syndrome, both of which are characterized by increased vascular permeability and thrombocytopenia (125). Patients with hemorrhagic fever with renal syndrome exhibit significantly elevated histamine levels, indicating a possible role for mast cells in potentiating this syndrome (125). Endothelial cells, epithelial cells, and dendritic cells are all permissive to

hantavirus infection *in vitro* (125–127), and recent evidence suggests mast cells are also susceptible to this virus (125). Inoculation of *in vivo* differentiated mast cells results in productive infection and mast cell activation, though the ability of hantavirus to directly induce degranulation is not known (125). Furthermore, the ability of various strains of hantavirus to infect and replicate within mast cells directly correlates with the pathogenicity of the strains (125). Thus, mast cells may be an important factor during hantavirus-induced disease.

### Sendai Virus

Sendai virus is a respiratory parainfluenza virus that is highly transmissible in both rodents and swine. In neonatal rats, Sendai virus causes viral bronchiolitis and airway hyperresponsiveness, which are associated with elevated levels of bronchiolar mast cells and eosinophils (66, 128–130). The elevated numbers of bronchiolar mast cells observed after Sendai virus infection result from both the proliferation of tissue-resident mast cells and recruitment of mast cell progenitors to the airways (61). Sendai virus can also infect human mast cells, resulting in their activation (131). While the release of β-hexosaminidase (a major granule component) has not been detected from human mast cells, both histamine release in rats and tryptase release in pigs have been detected following Sendai virus challenge (131–133). Following Sendai virus infection, human mast cells produce type I and III interferon (131), which have been implicated in asthma exacerbations (134). Interestingly, in the rat model, animals previously infected with Sendai virus subsequently sensitized to ovalbumin 1-month later display heightened allergic airway inflammatory cell reactions (66). Thus, mast cells are important contributors to the inflammatory response to parainfluenza viruses, and participate in their pathological role during allergic airway disease.

### Infectious Bursal Disease Virus (IBDV)

IBDV is a contagious disease with a high mortality rate, which impacts the poultry industry worldwide. IBDV infected chickens have increased inflammatory lesions, which lead to susceptibility to secondary infections (135–137). Mast cell numbers are increased at the site of infection during IBDV. Moreover, these mast cells are activated, as mast cell tryptase accumulates in the infected tissue (64). Treatment with ketotifen not only decreases mast cell numbers in infected birds but also correlates with reduced injury during infection without altering expression of IBDV Ags (65). Thus, by reducing the release of mast cell mediators, one can decrease mast cell accumulation in the infected tissue, and ultimately decrease tissue damage, and increase survival during IBDV infection.

### Newcastle Disease Virus (NDV)

NDV is another highly contagious poultry disease, which infects the gastrointestinal tract, resulting in high mortality and economic losses (138). Similar to IBDV, mast cells are found in and around NDV lesions during infection, correlating with an increase of mast cell tryptase levels in the tissues (63). Chickens pretreated with ketotifen show a reduction in tissue damage during NDV infection (62). Thus, similar to IBDV, inhibition of mast cell mediators reduces mast cell accumulation in the infected tissue and decreases tissue damage, increasing survival following NDV infection.

### Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV) is associated with high mortality in pigs. Infection with low pathogenic PRRSV (LP-PRRSV) results in minimal histopathological changes with no mortality. In contrast, infection with a high pathogenic strain of PRRSV (HP-PRRSV) results in significant mortality associated with extensive tissue damage within the lungs (139, 140). Pigs infected with HP-PRRSV display significant respiratory distress, which is associated with pulmonary lesions characterized by inflammatory cell infiltrates, interstitial and alveolar edema, and hemorrhaging, which is not observed following LP-PPRSV infection. Infection with the HP-PRRSV results in higher virus titers and higher levels of pro-inflammatory cytokines and immune cell infiltrate, including neutrophils, mononuclear phagocytes, and mast cells. Both histamine and $LTB_4$ are significantly increased in the serum of HP-PRRSV infected pigs (141). Because, these mediators play an important role increasing blood vessel permeability and disease severity during dengue virus infection (51), it is likely they contribute to the increased lung edema and hemorrhage observed during HP-PRRSV (141).

## How are Mast Cells Activated by Viruses?

### Are Virus Entry and Replication in Mast Cell Required for Activation?

Both pathogenic and non-pathogenic hantavirus nucleoprotein can be detected in mast cells (125). In addition, the human mast cell lines KU812 and HMC-1 are permissive to dengue virus in the presence of human dengue virus immune sera (119). This data demonstrate that these highly pathogenic viruses can infect mast cells. RSV activates mast cells resulting in the production of cytokines and chemokines including CXCL10, CCL4, CCL5, and type I interferons (142). RSV Ag can be detected in both primary cord blood mast cells and the human mast cell lines following infection (142). However, similar to many other pathogenic viruses, mast cell infection does not result in the release of infectious progeny virions (142).

While respiratory epithelial cells are the primary target for IAV replication, IAV can infect a wide range of cells, including endothelial cells (21), macrophages (143), dendritic cells (144), and mast cells (23, 121, 145). In mast cells, IAV is able to mediate viral entry, but largely appears to undergo an abortive infection. Inoculation of murine bone marrow derived mast cells (BMDMC) with A/WSN/1933 results in *de novo* expression of the viral NS-1 protein, but does not produce any new infectious particles (23). Interestingly, treatment of murine BMDMC with another H1N1 isolate, A/PR/8/1934, does not result in detectable NS-1 expression (23). On the other hand, infection of the human mast cell line LAD and human cord blood derived mast cells with the A/PR/8/1934 strain results in viral mRNA and protein synthesis, but does not produce *de novo* infectious particles (145). In contrast, recent data demonstrate the murine mastocytoma cell line P815 can be productively infected with A/WSN/1933 (H1N1),

A/Chicken/Henan/1/2004 (H5N1), and A/Chicken/Hebai/2/2002 (H7N2), producing infectious virus over the first 24 h of infection, as measured by qRT-PCR, hemagglutination assay, and plaque forming assay (25). These differences likely reflect the different types of mast cells used for these studies and the infectious dose of the virus. Overall, these data demonstrate that IAV, dengue virus, RSV, and hantavirus can at least bind to and enter mast cells, which is likely important for mast cell activation. More studies are needed to understand the fine specificity of these viruses, and specifically the different IAV isolates for distinct mast cell populations, and the cellular factors that may be present in some of these populations that limit IAV propagation.

### How are Viral Particles Recognized by Mast Cells?

Mast cells express a wide range of PRR which allows these cells to respond to a variety of stimuli, including bacteria, parasites, fungi, and viruses (**Figure 1**) (86). RIG-I is a cytosolic receptor that can detect IAV RNA and many other single stranded RNA viruses (73, 80). Once RIG-I detects vRNA, it signals through the mitochondrial adaptor MAVS resulting in an antiviral response. In mast cells, signaling through the RIG-I/MAVS pathway is important for the secondary response to IAV, but not for the immediate degranulation of mast cells (23). Virus recognition through RIG-I by mast cells is also important during dengue virus and vesicular stomatitis virus (VSV) infections (82, 121, 146). However, our studies suggest the RIG-I dependent responses in mast cells do not significantly contribute to the pulmonary immunopathology associated with IAV infection (Graham and Obar, unpublished observation); rather, mast cell degranulation appears to be the dominant mediator of immunopathology (24). In addition to RIG-I detection, TLR3 is also important for the recognition of IAV, type I reovirus, RSV, VSV, and NDV by murine BMDMC for the production of secondary mast cell mediators (79, 147, 148). Moreover, viral recognition by both Mda5 and 2′-5 oligoadenylate synthase (OAS) can participate in the initiation of the secondary response of mast cells induced by VSV (148). Thus, detection of viral nucleic acids appears to be central for production of *de novo* synthesized mast cell mediators following viral infection. Alternatively, infection can be detected indirectly by mast cells, as occurs with herpes simplex virus (HSV). Infected epithelial cells secrete IL-33, which is in turn detected by mast cells, resulting in the secretion of IL-6 and TNF-α without degranulation (149).

Mast cell degranulation not only appears to play a critical role in regulating mast cell dependent inflammation following IAV infection (23, 24) but also in a number of other viral systems (62, 65, 103). The mast cell degranulation inhibitor, ketotifen, reduces inflammation in response to H5N1 IAV infection of mice (24), and the inflammation associated with IBDV and NDV in poultry (62, 65). Additionally, mast cell stabilization using cromolyn limits dengue virus induced immunopathology (103). Together, these data strongly support a role for mast cell degranulation in the mast cell-dependent inflammatory response to highly virulent viral infections. Thus, it appears critical we understand how viruses drive mast cell degranulation to appropriately target these cells pharmacology.

How mast cells degranulate in response to viral infections remains largely unknown. Degranulation still occurs in response to A/WSN/1933 infection in RIG-I-deficient BMDMC, demonstrating that degranulation is a RIG-I-independent response (23). As degranulation occurs within 30 min following treatment with IAV, other PRR and/or early signaling events necessary for the virus attachment and/or entry processes are likely important in regulating mast cell degranulation. With dengue virus, degranulation of mast cells occurs prior to RIG-I signaling (82). Moreover, UV-inactivated dengue virus (82) and IAV (147) retain the ability to activate mast cells, suggesting this occurs early in the viral replication cycle. While FcγIII-deficient mast cells are able to degranulate in response to dengue virus, mast cells pre-treated with anti-dengue IgG demonstrate enhanced degranulation in response to all four serotypes of dengue virus compared to dengue virus alone, suggesting that antibody binding enhances degranulation in response to dengue virus (124). Although mast cell degranulation appears to be pivotal for the immunopathology associated with highly pathological IAV (24) and dengue virus infections (103), we do not understand how degranulation is initiated. To date, the only virus for which the mechanism of mast cell degranulation has been well elucidated is vaccinia virus. The activating event is fusion of the viral envelope with the mast cell plasma membrane (31). Specifically, the vaccinia virus envelope contains sphingomyelin (150), which is converted to sphingosin-1-phosphate (S1P) and signals through the S1PR2 G-coupled receptor to cause degranulation (31). Signaling through the S1PR2 has also been shown to regulate mast cell responses in general (31, 151–154). However, the role of S1P receptor signaling in other viral infections remains unknown. Further understanding the molecular signals necessary for mast cell degranulation could lead to novel therapeutic avenues for these highly virulent viral infections.

## Mast Cells as Drug Targets for Limiting Virus-Induced Immunopathology

Predicting the next pandemic IAV strain is nearly impossible, as IAV has a high mutation rate resulting in significant yearly antigenic drift and can randomly reassort resulting in antigenic shift. Even deciding which IAV strains to produce for the yearly vaccine is difficult, as the strains must be chosen months ahead of the yearly influenza season. If these predictions are inaccurate or the seasonal IAV strains drift significantly, then the vaccine will not be highly effective resulting in a high incidence of IAV-induced disease (2). The current antiviral treatments against IAV are becoming increasingly ineffective due to the emergence of resistant strains. Therefore, alternative therapeutics avenues are needed. Targeting host-derived factors necessary for viral replication or host factors participating in the excessive pathological inflammatory response during highly pathogenic IAV are promising alternatives (2).

The literature review presented here shows the strong correlation between mast cell accumulation and degranulation at local sites of infection with the observed tissue damage and pathology, not only during highly pathological IAV infections but many other pathogenic viral infections of humans and animals. Additional

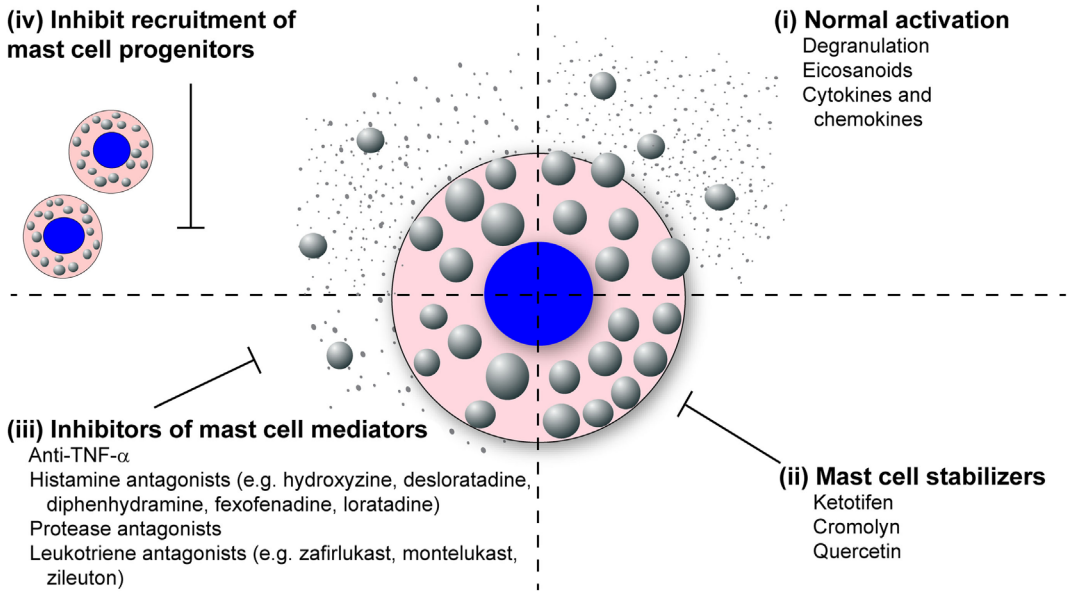


**FIGURE 3 | Mast cell inhibitors**. Various classes of mast cell inhibitors already exist for the treatment of various conditions. (i) Uninhibited, activated mast cells will degranulate and synthesize eicosanoids, cytokines, and chemokines which are released into the surrounding tissue. (ii) The mast cell stabilizing drugs (e.g., ketotifen, cromolyn, and quercetin) block the release of mast cell granules following activation. (iii) Second broad class of mast cell inhibitors target the activity of specific mast cell mediators. These includes anti-TNF-α compounds, anti-histamines (e.g., hydroxyzine, desloratadine, diphenhydramine, fexofenadine, loratadine), protease antagonists, and leukotriene antagonists (e.g., montelukast, zafirlukast, zileuton). (iv) A potential third class of mast cell inhibitors could target the recruitment of mast cells to inflamed tissue following infection.

studies examining other highly pathological viruses that are known to cause ARDS and/or vascular leakage are thus warranted, which would include the emerging coronaviruses, SARS-CoV, and MERS-CoV, and hemorrhagic viruses such as Marburg and Ebola. Overall, we hypothesize that excessive mast cell activation may be a common feature of highly pathological viral infections that cause ARDS and/or vascular leakage. This novel pathway could be pharmacologically targeted to limit the morbidity and mortality associated with these infections. Additionally, understanding how mast cells accumulate in the infected tissues, through mast cell proliferation and/or mast cell progenitor recruitment, could provide additional therapeutic targets (**Figure 3**).

Because mast cells and their products are known to play a dominant role in both allergic and asthmatic reactions, many drugs that stabilize and neutralize mast cells are already approved for human use (**Figure 3**). The mast cell stabilizing drugs, which inhibit the release of granules following mast cell activation, have proven effective at reducing vascular leakage and limit inflammatory cellular recruitment, thus increasing survival in the murine dengue virus and IAV models (24, 103, 155). Furthermore, these compounds have proven very effective at limiting lung pathology following IBDV and NDV in poultry (62, 65). Compounds are also available which block the activity of specific mast cell products including TNF-α, histamine, mast cell proteases, and leukotrienes (**Figure 3**). Many anti-TNF-α compounds are already approved for the treatment of inflammatory arthritis. Numerous anti-histamines, including hydroxyzine, desloratadine, diphenhydramine, fexofenadine, and loratadine, are approved to treat allergy symptoms. Drugs are currently in development, which target the mast cell proteases, especially the mast cell derived chymase which has been implicated in cardiovascular disease. Finally, there are two classes of leukotriene antagonists, the leukotriene-receptor antagonists (zafirlukast and montelukast) and the leukotriene synthesis inhibitors (zileuton).

In addition to stand alone treatments targeting mast cell activation and mediators, adjunct therapies utilizing both antiviral and mast cell targeting compounds might be fruitful. Earlier studies using human peripheral blood leukocytes exposed to NAs or IAV at the time of IgE stimulation resulted in significantly greater histamine release (156–158). These data suggest the presence of multiple stimuli may result in an additive or synergistic effect. Therefore, mast cell targeting drugs could be used in parallel with antiviral drugs for greatest efficacy. Following infection with a highly pathogenic H5N1 IAV strain, the only cohort of mice which survived infection were those treated with both antiviral and mast cell stabilizing compounds (24). This approach may prove especially beneficial during asthmatic exacerbations following viral infection.

## Concluding Remarks

Mast cells are important players in pathogen defense. Their location at environmental barriers allows them to quickly respond to invading pathogens. In parasitic and bacterial infections, mast cells are essential in preventing the spread of infection (26–28). While in certain viral infections mast cells can be protective (31, 122, 123, 149), in highly pathogenic viral infections, such as IAV

or systemic dengue infections the data demonstrate that mast cells are more detrimental than beneficial (23, 24, 103). If the role of mast cells during IAV infections, and other highly pathogenic viral infections, can be elucidated, these cells may serve as a lucrative target for new therapeutics. Activation and release of mediators from mast cells in response to these viruses correlates with severity of disease in mice. Application of existing allergy medications that target mast cells may decrease the severity of IAV infections, limiting the morbidity and mortality associated with future pandemics.

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# Pseudotype-based neutralization assays for influenza: a systematic analysis

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The use of vaccination against the influenza virus remains the most effective method of mitigating the significant morbidity and mortality caused by this virus. Antibodies elicited by currently licensed influenza vaccines are predominantly hemagglutination-inhibition (HI)-competent antibodies that target the globular head of hemagglutinin (HA) thus inhibiting influenza virus entry into target cells. These antibodies predominantly confer homosubtypic/strain specific protection and only rarely confer heterosubtypic protection. However, recent academia or pharma-led R&D toward the production of a "universal vaccine" has centered on the elicitation of antibodies directed against the stalk of the influenza HA that has been shown to confer broad protection across a range of different subtypes (H1–H16). The accurate and sensitive measurement of antibody responses elicited by these "next-generation" influenza vaccines is, however, hampered by the lack of sensitivity of the traditional influenza serological assays HI, single radial hemolysis, and microneutralization. Assays utilizing pseudotypes, chimeric viruses bearing influenza glycoproteins, have been shown to be highly efficient for the measurement of homosubtypic and heterosubtypic broadly neutralizing antibodies, making them ideal serological tools for the study of cross-protective responses against multiple influenza subtypes with pandemic potential. In this review, we will analyze and compare literature involving the production of influenza pseudotypes with particular emphasis on their use in serum antibody neutralization assays. This will enable us to establish the parameters required for optimization and propose a consensus protocol to be employed for the further deployment of these assays in influenza vaccine immunogenicity studies.

**Keywords: influenza, hemagglutinin, pseudotype, neutralization assay, universal vaccine, lentiviral vector, retroviral vector**

## INFLUENZA PSEUDOTYPES

Influenza is a respiratory syndrome caused by three of six genera in the orthomyxoviridae family, influenza A, B, and C. A putative fourth genus (influenza D) has recently been characterized and proposed (1). Influenza A is the most widespread, its various subtypes are classified according to their antigenically variable surface glycoproteins: hemagglutinin (HA, H1–H18) and neuraminidase (NA, N1–N11). The virion consists of a segmented negative sense genome encapsidated in ribonucleoprotein complexes, which are surrounded by a matrix shell and lipid envelope containing the two surface glycoproteins and the M2 ion channel. Influenza A is the primary source of the human seasonal form of the disease, responsible for up to 500,000 deaths per annum as well as deaths caused by pandemics such as those occurring in 1918, 1957, 1968, and 2009 (2). Consequently, vaccines against influenza need to be regularly updated to match predicted circulating strains that are constantly escaping from vaccine protection through a mechanism known as antigenic drift. Influenza A is primarily associated with wild fowl/birds in the case of the majority of subtypes and can reassort with human strains through antigenic shift to yield human compatible viruses with previously un-encountered surface epitopes. Pigs are usually considered to be the mixing vessel for reassortment as they express a mixture of α-2,3 and α-2,6 sialic acid linkages. Influenza virus research is often hindered by the requirement for expensive biosafety precautions, especially in the case of the highly pathogenic avian influenza (HPAI, e.g. H5N1, H7N1) or pandemic strains.

Pseudotypes or pseudotype particles are chimeric "viruses" consisting of a surrogate virus core surrounded by a lipid envelope with the surface glycoproteins of another virus, such as HA. By removing the genetic element of the virus being studied and replacing it with a suitable reporter, viruses, especially HPAI, can be studied in this safer, single cycle system. The comparative safety of pseudotype viruses circumvents the need for restrictive, expensive, and widely unavailable high-category biosafety facilities, increasing access to research groups interested in highly pathogenic viruses.

This review is a systematic analysis encompassing a wide range of peer-reviewed literature in English concerning the production and use of pseudotypes bearing influenza glycoproteins to date. For the purpose of this review, pseudotypes will be defined as replication-deficient viruses containing a viral core from one species and bearing glycoproteins from another that are not represented in the genome. Literature was gathered by searching for "influenza pseudotypes" using Google Scholar and NCBI PubMed. The resulting list of publications was expanded by following up cited references and finally, those falling outside of our pseudotype definition or not specifically using influenza pseudotypes were excluded from the sections on production, transduction, and neutralization.

This review will be useful to those interested in the production of pseudotypes for use in immunogenicity testing of preclinical influenza vaccines, whether in human or animal settings, and including "universal vaccine" candidates. Influenza serological studies such as the measurement of seroprevalence will benefit from this manuscript, which will also help to inform the process of validation of pseudotype-based assays to clinical end-point. Furthermore, studies utilizing chimeric HA proteins in order to differentiate between stalk and head directed antibodies will be discussed.

## PSEUDOTYPE COMPONENTS

### CORES AND REPORTERS

The core and its associated genome containing a reporter are the backbone of the pseudotype system, which can be used to study the properties of selected entry proteins. The use of cores from lentiviral human immunodeficiency virus (HIV) and gammaretroviruses such as murine leukemia virus (MLV) predominate in the influenza pseudotype literature. Recent development of systems involving rhabdoviruses, in particular the vesicular stomatitis virus (VSV), has also been used to produce pseudotype cores with promising results (3, 4).

### RETROVIRAL AND LENTIVIRAL CORES AND VECTORS

Retroviral and lentiviral vectors are complex systems, which will be explained in simple terms specific to the production and use of pseudotypes. Pseudotype core and vector systems have been reviewed in detail (5, 6).

The primary genes provided by retroviral and lentiviral systems are *gag* and *pol*. In the case of HIV, *gag* provides the structural proteins p18, p24, and p15, whereas *pol* provides the integrase and reverse transcriptase in conjunction with the p10 protease required for cleavage and maturation of each distinct protein from their respective polypeptide chain (7, 8). Reporter constructs are associated with their respective cores based on the Psi (Ψ) packaging element incorporated in the vector design process, making them specific to the surrogate species used.

Human immunodeficiency virus cores are derived from several different origins between laboratory groups. First generation pNL4-3 vectors are well represented and the pNL4-3-Luc.E-R- variant is the most commonly used (9–14). The pNL4-3.Luc.E-R- replication deficient proviral HIV-1 clone is derived from the pNL precursor but has inhibitory frame shifts in the *env* and *vpr* genes as well as a luciferase reporter gene cloned into *nef* and the entire construct is incorporated into progeny pseudotypes. The vector's life cycle mimics that of HIV, using the Ψ element to allow encapsidation into nascent pseudotypes and long terminal repeat (LTR) regions bearing the U3 promoter, which with the aid of *tat*, permit the expression of the viral proteins after integration into the host genome. The rev responsive element (RRE) allows nuclear export of viral messenger RNA (mRNA), including the reporter gene transcript, which is the measure of output for this system. Due to the incorporation of the HIV core genes into the same integrated construct as the reporter, transduced cells may possibly produce luciferase containing cores alongside its transcribed enzyme, which could potentially interfere with luciferase activity.

Another commonly used HIV core vector is pCMV ΔR8.2, a relation of pCMV ΔR8.9, which still contains intact *vif*, *vpr*, *vpu*, and *nef* genes (15–20).

A further approach uses the second generation HIV vector p8.91 that also originates from pCMV ΔR8.9 and ΔR9 (15, 21). The p8.91 vector is a modified HIV-1 clone, lacking the Ψ sequence as well as the *env*, *vif*, *nef*, *vpu*, and *vpr* genes and is widely used in the articles studied (22–25). The cytomegalovirus promoter is used in lieu of LTR-based promotion, meaning that p8.91 provides the necessary genes for the production of the core but the proviral and packaging elements (LTRs, RRE, and Ψ) are transferred to a separate plasmid bearing the reporter gene. Thus, the reporter construct will be incorporated into nascent virions and integrated into the transduced cell's genome, whereupon the LTRs and RRE will act to enhance expression. In the case of the commonly used firefly luciferase or green fluorescent protein (GFP) plasmids pCSFLW or pCSGW, a safety component is incorporated through a deletion in the 3′ LTR (U3 promoter region), creating so called self-inactivating (SIN) vectors (26, 27).

Third generation vectors have also been used. In this instance, HIV structural and accessory genes are separated from *rev*, which is provided in *cis* on an additional plasmid. The third generation Invitrogen ViraPower Lentiviral Expression System was used in several cases using the plasmids pLP1 and pLP2 (28–31).

Murine leukemia virus cores are less widely used but provide similar *gag* and *pol* elements to HIV vectors (32–38). One MLV core used consists of *gag* and *pol* under the effect of a CMV promoter, a vector which has been shared across various laboratories (39–41). In this instance, the vector originates from pCI G3 N, B, or NB, which are differentially restricted in certain murine cells based on the mouse resistant gene alleles $Fv1^N$ and $Fv1^B$ (42). The reporters used in this system are derived from CLONTECH vectors LNCX and pIRES2-EGFP (39, 41). Another described MLV plasmid, pkatgagpolATG originates from the ecotropic Moloney MLV and strain 4070A (17).

Minor differences have been observed when pseudotyping HIV or MLV cores with influenza glycoproteins (43). Therefore, the question of which core to use to produce pseudotypes is often down to choice, preference, and availability (44).

See **Figure 1** for schematic representations of packaging constructs and vectors.

### RHABDOVIRUSES

Recombinant VSV viruses are produced expressing GFP in place of the resident VSV envelope glycoprotein (VSV-G). In certain cases,

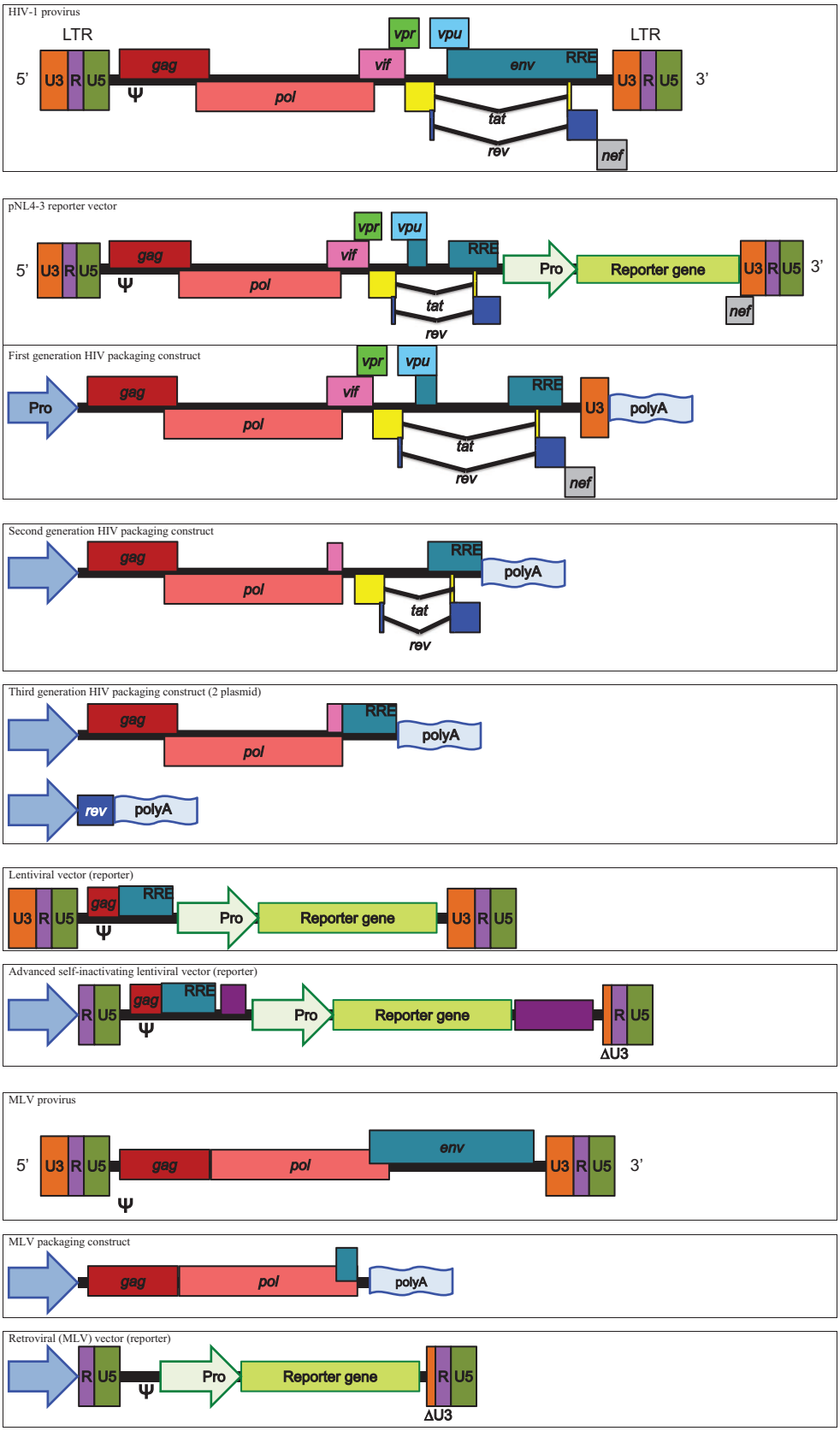


**FIGURE 1 | Schematic representation of HIV and MLV derived packaging constructs and vectors**.

HA and NA or simply HA are also added to the VSV genome. These additions produce a replication-competent virus, which will promote GFP production in infected cells (4). As these recombinant viruses are not limited to a single cycle of replication, they lack the safety element found within other systems.

A safer VSV-based alternative involves transfection of surface protein encoding plasmids (HA/NA) into cells and subsequent infection with a recombinant VSV. In this way, one can produce VSV pseudotyped with influenza surface proteins, which lack entry-glycoproteins in its resident genome, rendering the second generation of virus infection-incompetent (3).

## REPORTER SYSTEMS

The output of the pseudotype system is based on the incorporated reporter, which mimics the genome of the surrogate virus. In the case of HIV or MLV surrogates, the reporter will often be incorporated into the pseudotype in RNA form, which upon transduction will be reverse transcribed, translocated to the nucleus, and integrated into the host cell genome. The reporter will then be produced by the host cell and can be used to measure transduction efficiency.

The primary reporter used in influenza pseudotypes is firefly derived luciferase (45–53). Relative luminescence units (RLU) or relative luciferase activity (RLA) are used as output, measured by lysing transduced cells and adding substrate for the luciferase enzyme, the signal from which is then read using a luminometer.

Green fluorescent protein is also commonly used, in which case transduction efficiency is determined by counting the number of fluorescing cells via epifluorescence microscopy or fluorescence-activated cell sorter (FACS) (54, 55).

Other reporters such as *lacZ* (29, 54, 56, 57) as well as *Gaussia* (58) and *Renilla* (59, 60) luciferase are also used to a lesser extent.

## INFLUENZA ENVELOPE PROTEINS: HEMAGGLUTININ

The trimeric attachment and fusion protein HA is the principal constituent of the influenza virus envelope, alongside NA and M2. Attachment to sialic acid residues on target cell membranes triggers endocytosis and pH-dependent exposure and engagement of the fusion peptide, mediating entry of the virus (61). This process is the basis on which influenza neutralization assays are founded – the exploitation of attachment and entry for the study of HA-directed antibodies and their neutralizing ability. Analysis has permitted classification of influenza A subtypes into two distinct groups: group 1 containing subtypes 1, 2, 5, 6, 8, 9, 11, 12, 13, 16, 17, and 18 and group 2 containing 3, 4, 7, 10, 14, and 15 (62–64). Subtypes within each group are often subdivided into clades with further sequence dissimilarity.

See **Figure 2** for a phylogeny of influenza groups and **Figure 3** for influenza strains pseudotyped with HA compared to HA sequence entries currently in NCBI GenBank. A wide variety of influenza A strains exist and have been pseudotyped, influenza B is grouped into two distinct lineages (Yamagata and Victoria) and has yet to be pseudotyped. Influenza C pseudotypes have been produced using a VSV core (65).

### *Codon optimization, synthesized genes*

Codon optimization has been employed for several commercially synthesized genes, which are sometimes used concurrently with extracted wild type viral sequences depending on availability [Genscript, Gene Art, Integrated DNA technologies (54, 68–73)]. Recursive PCR has been used in some cases to produce the same end product (16, 68, 74, 75). In the context of pseudotype production, codon optimization is performed based on the assumption that conforming to codon-bias within producer cells will increase production of proteins and pseudotype yields.

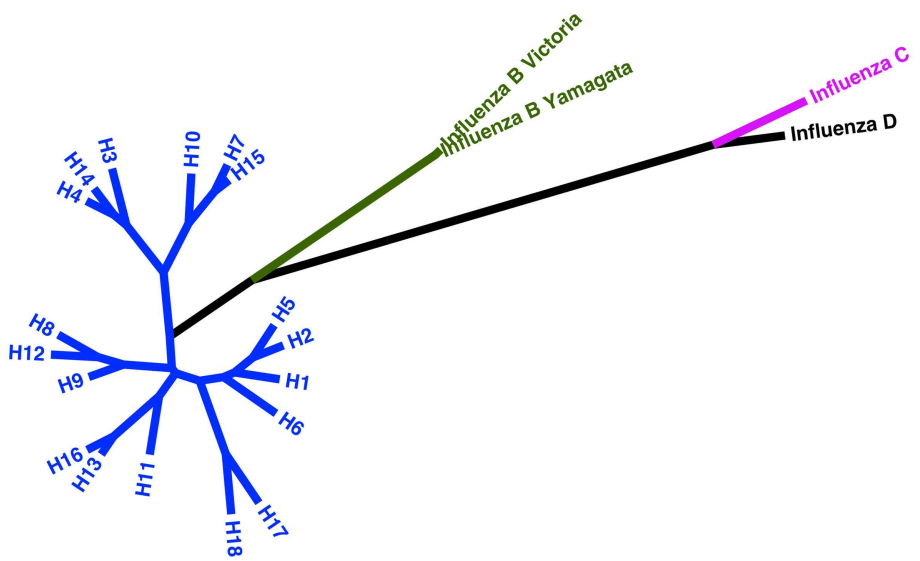


**FIGURE 2 | Phylogeny of current influenza subtypes using the HA glycoprotein**. Maximum likelihood tree representing amino acid sequences of the HA glycoprotein for influenza A, B, and C virus as well as putative influenza D. The tree inferred is based on MUSCLE alignment of downloaded sequences conducted using MEGA 5.2 under the WAG + G model (four categories). The phylogenetic tree with the highest log likelihood (−16773.4044) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 538 positions in the final dataset (66, 67).

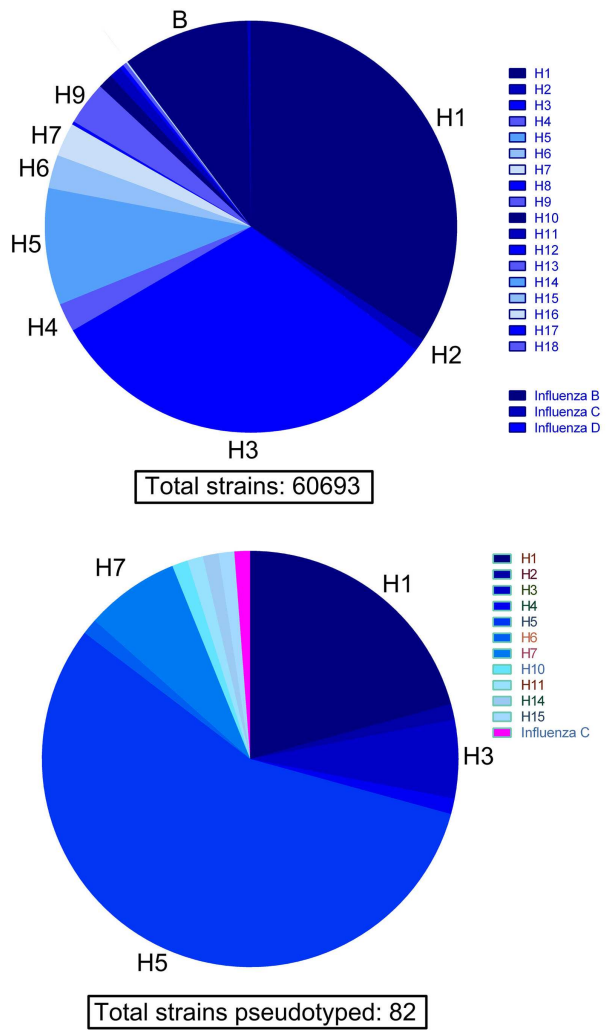


**FIGURE 3 | Comparison of influenza HA sequences described against strains pseudotyped**. Out of a total of 60,693 HA amino acid sequences extracted from NCBI GenBank, the vast majority come from subtypes H1, H3, H5, and influenza B. Conversely, the current number of different subtypes and strains of HA used to produce pseudotypes is 82. The majority of pseudotyped strains come from subtypes H1, H3, and especially H5.

## INFLUENZA ENVELOPE PROTEINS: NEURAMINIDASE

As with wild type influenza virus, NA is required for the exit of influenza pseudotypes via its cleavage of surface sialic acid molecules on producer cells. However, it is common to circumvent the requirement of NA expression for pseudotype production by the treatment of cultured cell lines with commercial exogenous bacterial NA 24 h after transfection (59, 76–78). This 24 h time period requires optimization to allow maximal budding of pseudotypes and minimal loss through transduction of producing cells. Exogenous NA treatment is often used in neutralization studies in order to prevent NA directed antibodies from providing a neutralization signal. However, several studies opt to incorporate an NA plasmid such as that from influenza B/Yamagata/16/88, A/Shanghai/37T/2009, A/Thailand/1(KAN)-1/04, or A/Puerto Rico/8/1934 (16, 58, 79–85).

Several recent articles have characterized sialic acid binding attributes of neuraminidases sharing particular genetic characteristics. New mutations have been characterized such as G147R in the A/WSN/33 strain that has been shown to rescue HA-binding deficient viruses. The G147R mutation is present in a range of strains including representatives of pandemic H1N1 and chicken H5N1 (86, 87).

## INFLUENZA ENVELOPE PROTEINS: M2

It is also possible to incorporate the M2 ion channel into influenza pseudotypes in order to study its effect on the production process. However, the M2 role in acidification of the wild type influenza virus core is not required for the dissociation of pseudotype cores as they are derived from non-influenza viruses, which achieve release of their genetic material (i.e., a luciferase reporter gene transcript) through different mechanisms. Therefore, M2 is not required for the production of influenza pseudotypes despite being shown to have an effect on yields and infectivity (88, 89). There are reports of M2 incorporation increasing pseudotype particle yields such as H7 A/FPV/Rostock, and for H1N1 pseudotypes (29, 88). M2 has been shown to influence the budding of wild type influenza and consequently, this may be the mechanism through which M2 expression increases the reported pseudotype yields (90).

## PROTEASES

As HA is produced and trafficked through the secretory pathway it requires proteolytic cleavage in order to become fusion competent. Proteolytic cleavage is mediated by certain host cell proteases and restricts certain subtypes to epithelial cells where these required proteases are expressed. While this is achieved naturally in wild type infection, a cleavage component must be incorporated into pseudotype production workflows in order to achieve optimal yields. This is because in producer cell lines the required proteases are either not expressed or are expressed, but not at sufficient levels to make the pseudotypes fusion competent.

In order to mimic the proteolytic properties of the natural host cells of influenza, protease encoding plasmids can be transfected alongside the other requisite plasmids in order to induce transient expression within the same timeframe as the production of pseudotypes. The serine transmembrane protease (TMPRSS2) and the human airway trypsin (HAT), which cleave wild type influenza (91) have been used successfully in several studies for pseudotype production (17, 28, 70, 78, 92–96). TMPRSS4, another serine protease has also been used to successfully cleave wild type and influenza lentiviral pseudotypes (97).

However, the addition of a protease encoding plasmid can be side-stepped through cleavage post-production using tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (17, 28, 92, 98). TPCK inhibits the less specific proteolytic elements of chymotrypsin, restricting the treatment process to the cleavage of peptide bonds required for HA maturation (99, 100).

TPCK-trypsin concentrations used for the production of pseudotypes generally ranged from 1 to 50 μg/ml. However, one

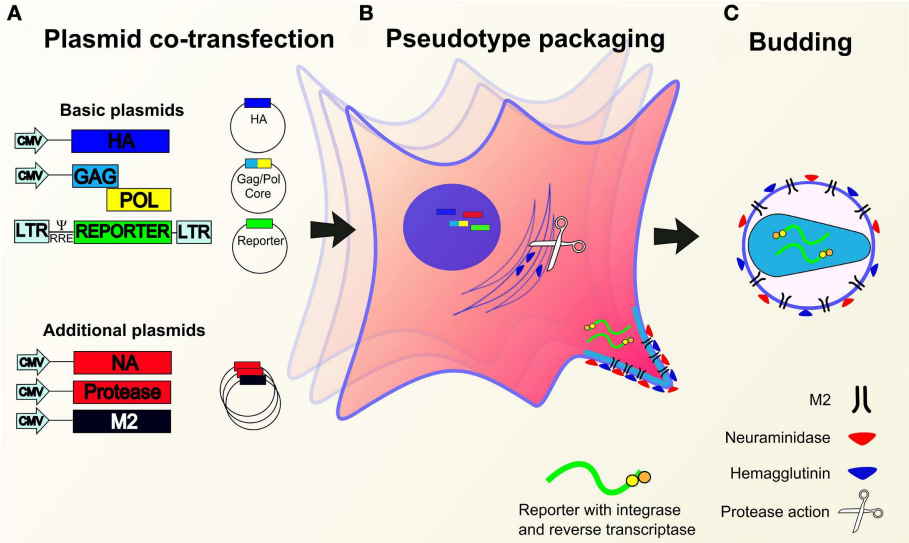


**FIGURE 4 | Production of lentiviral or retroviral pseudotypes**. **(A)** Essential (containing HA, packaging construct *gag pol*, reporter construct) and/or additional (NA, protease, M2) expression plasmids are co-transfected into HEK293T producer cells. **(B)** Plasmids migrate to the nucleus whereupon genes are expressed leading to the production of pseudotype proteins and the reporter RNA construct. Cleavage of HA is mediated by transfected or cellular proteases. **(C)** Pseudotype proteins are packaged by the cell and budding occurs at the cell membrane to yield pseudotypes bearing desired glycoproteins and incorporated reporter.

study reported increased transduction when used at concentrations above 40 μg/ml for H1N1 pseudotypes (101). Incubation ranged from 10 min at room temperature to the more usual 1 h at 37°C. TPCK-trypsin treatment is typically carried out an hour before transduction. The enzyme is then neutralized before transduction using commercial trypsin inhibitors, in some cases originating from soybean (28, 92).

HA derived from HPAI strains that contain a polybasic cleavage sequence in the HA0 protein are cleaved by a wider range of proteases that are ubiquitous in cells. This allows the omission of protease plasmids or TPCK-trypsin treatment in HPAI pseudotype production (102). In some cases, the polybasic cleavage site of HPAI strains have been integrated into other HAs in an attempt to produce pseudotypes without the protease plasmid requirement, or to give strains similar entry characteristics (60, 73).

See **Figures 4** and **5** for representative drawings of the pseudotype production process and different cores used.

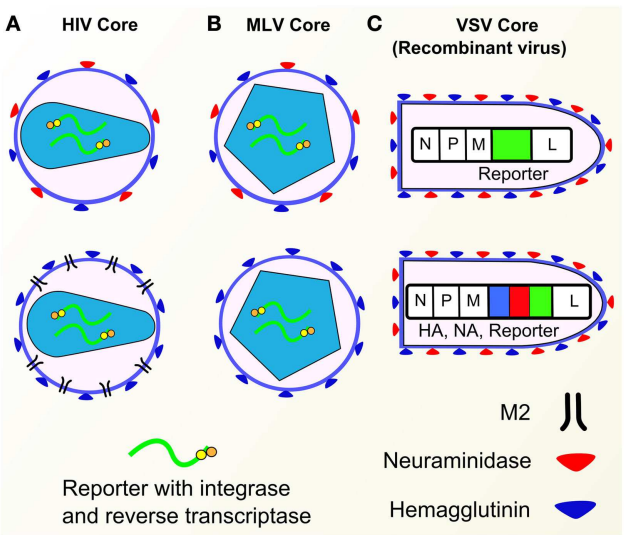


**FIGURE 5 | Pseudotype cores**. **(A)** HIV cores with various envelope glycoproteins (HA, NA, M2). **(B)** MLV cores with HA or HA and NA. **(C)** Recombinant VSV containing GFP gene (top) and HA/NA/GFP genes (bottom). Components of influenza pseudotypes can be varied according to need. Pseudotypes have been produced with HA, NA, and M2 influenza envelope proteins, with a range of core packaging constructs (HIV, MLV, VSV shown) as well as different reporters.

## PRODUCTION METHODS

### PLASMIDS RATIOS AND AMOUNTS

There is considerable variation between studies regarding choice of expression plasmids as particular systems are established within research groups and networks, inherited from previous studies and are often dependent on collaborations or gifts. The most popular system employed involves a multiple plasmid co-transfection approach using separate plasmids for the HA, reporter and retroviral *gag* and *pol* core genes. These genes are cloned into a range of expression plasmids such as pI.18, pcDNA3.1, phCMV, and pCAGGS (43, 74, 103, 104). Kozak consensus sequences are very rarely mentioned and only defined in one study, in which a kozak consensus sequence derived from the pHW2000-N1 (Kan) plasmid was used (3).

Additional plasmids encoding NA and M2 are sometimes used when studying the relevant aspects of influenza infection or

pseudotype production, but more rarely in the case of neutralization (93, 105). In one study, a 10- to 30-fold increase in pseudotype production was achieved through expression of M2 using lentiviral cores and a 5-fold increase was achieved when an MLV core was used (29).

Plasmid ratios are crucial to pseudotype production but specific to plasmids used as well as transfection methods. In order to attain the highest quality and yields, optimization is required. Typically, the "core:HA:reporter" plasmid ratio is 1:1:1.5. However, the "HA:NA" ratio ranges from 3:1 to 8:1 and protease gene bearing plasmids (HAT, TMPRSS2) are often present at 50% (or below) the concentration of HA (e.g., 1 μg HA plasmid to 0.5 or 0.25 μg protease plasmid). Calcium phosphate precipitation requires the highest plasmid input, with as much as 25 μg of each plasmid per 100 mm dish used, whereas other methods [Fugene, polyethylenimine (PEI), Lipofectamine] require quantities of between 1 and 5 μg for each plasmid per 100 mm dish (20, 29, 69). Plasmid ratios are differentially affected by the composition of plasmids and therefore the quantities used to produce pseudotypes in the literature are justified based on optimization carried out by particular laboratories (17, 72, 88).

## PRODUCER CELLS

The producer cell lines used for pseudotype production are predominantly Human Embryonic Kidney 293 cells transformed with the SV40 large T antigen (HEK293T, 293T). These cells are highly susceptible to transfection and make good retroviral packaging cells (106). The clone 17 (HEK293T/17) of this cell line is also extensively used to produce high-titer influenza pseudotypes. Other cell lines used include 293FT cells (Invitrogen) used in the production of VSV–HA–NA pseudotypes (3).

Where mentioned, cell confluency at transfection varies between 60 and 90% with cells subcultured 24 h before transfection (74). Cell monolayers are grown on dishes ranging from 60 to 150 mm with the occasional study using T75 Flasks or multi-well plates (74, 107). Transfections are usually carried out using medium with serum such as fetal bovine serum (FBS) at concentrations of up to 10% (83, 108, 109).

## TRANSFECTION REAGENT/METHOD

The methods studied use the following chemical transfection reagents: Lipofectamine, Lipofectamine 2000 (Thermo Fisher Scientific), Fugene-6, Fugene-HD (Promega), PEI, jetPEI (Polyplus Transfection), or calcium phosphate precipitation. The choice of reagent is based on optimized lab protocol, cost, as well as the cytotoxicity of each reagent depending on requirements of pseudotype production. Of the above reagents, calcium phosphate precipitation and Fugene-6 are the most popular.

Calcium phosphate precipitation is a well-established transfection method of mammalian cells, developed in 1973 by Graham and van der Eb. This method involves mixing a comparatively high amount (5–25 μg) of plasmid DNA with calcium chloride and then adding this mixture slowly to a buffered saline solution. The mixture is incubated at room temperature whereupon a positively charged DNA and calcium phosphate precipitate is formed. The charge allows the precipitate to associate with the negatively charged cell membrane, entering by endocytosis or phagocytosis. The calcium phosphate precipitation process is sensitive to small differences in pH (20, 110, 111).

Polyethylenimine is a polymeric cation, which was first evaluated for its transfection capabilities in 1995. PEI acts at a range of pH values and associates with DNA to produce a complex with an overall positive charge that can then allow interaction with the cell membrane. Entry is by endocytosis and PEI has been shown to aid the delivery of nucleic acids to the cell nucleus of transfected cells. The original report states that PEI is non-cytotoxic at optimal concentration for transfection (112). However, when using PEI for transfection, it is commonplace to change cell culture medium within 24 h of transfection. JetPEI is a manufactured linear form of PEI, which is suited to high-throughput assays (84, 113).

Lipofectamine (or Lipofectamine 2000) are cationic lipids sold by Invitrogen that allow delivery of nucleic acids such as vectors into host cells through the formation of positively charged liposomes. The liposomes containing the pseudotype vectors are then able to fuse with cell membranes due to their positive charge and lipid constitution (114). Lipofectamine is among the most expensive transfection reagents used in influenza pseudotype production. The benefits of using this method are not readily apparent when the cost of the reagent is considered (107, 115).

Fugene-6 and Fugene-HD are cationic lipid complexes, which have low cell cytotoxicity. This allows laboratories to avoid replacing the transfection medium that may allow an increase in final titers of pseudotype when harvested. Fugene-HD has been shown to be more efficient than other transfection reagents (17, 96, 116, 117).

**Table 1** shows a list of transfection reagents, their cytotoxicity, cost, and plasmid input required.

## CELL WASHES AND MEDIUM REPLENISHMENT

In transfections where cytotoxic reagents are used, medium is replenished 6–24 h post-transfection, with most studies stating that media is typically replenished after overnight incubation (73). Media replacement can also be accompanied by a PBS wash. Where rhabdoviruses are used for pseudotyping, cell lines are washed using PBS 12 h after transfection with influenza surface glycoprotein plasmids. Helper virus is then added and 4 h later the helper virus containing medium is replaced after a further PBS wash step (3).

## SODIUM BUTYRATE

Sodium butyrate, a compound that can increase cell proliferation and pseudotype production is used in several studies with the concentrations ranging from 10 μM to 10 mM (18, 28, 57, 68).

**Table 1 | Transfection reagents, price, cytotoxicity, and plasmid input.**

| Transfection reagent | Price | Cytotoxicity | Plasmid input |
|---|---|---|---|
| Lipofectamine 2000 | High | – | Low |
| Fugene-6 | High | Low | Low |
| Fugene-HD | High | Low | Low |
| Polyethylenimine (PEI) | Low | Low | Low |
| Jet PEI | Medium | High | Low |
| Calcium phosphate precipitation | Low | – | High |

### HARVEST

Pseudotypes are harvested at various time intervals, typically 48 h post-transfection but sometimes also at 24 or 72 h. The supernatant is taken from the transfected cell monolayer and passed through a 0.45 μM filter to remove cell debris before being stored at −80°C. In many cases, centrifugation at low or high speed is used to concentrate harvested virus (20, 29, 57, 68, 77, 89, 105, 115, 118, 119).

One study has demonstrated that influenza pseudotypes are stable after five freeze–thaw cycles, retaining over 80% infectivity. Keeping pseudotype supernatant at −20°C for 6 months had a similar effect. However, storage at −4 or 20°C led to a reduction in infectivity of 50% in both cases (120). In environments lacking reliable refrigeration facilities, pseudotypes can be lyophilized and stored at a range of increased temperatures and humidity, maintaining viability and concentrations adequate for use in neutralization assays (121).

See **Figure 6** for a detailed depiction of methods used for production based on all pseudotype employing articles cited in this review.

## TRANSDUCTION

### TITRATION

As previously mentioned, with luciferase reporter pseudotypes RLU readings derived from titrations can be used as a secondary measure of pseudotype concentration within a sample. However, RLU readings are dependent on many variables surrounding the cells and the particular luminometer used.

Pseudotypes are titrated by 2-fold serially diluting 100 μl of harvested supernatant in a 96-well plate. After an incubation of 48 or 72 h, RLU can be measured by lysing the transduced cells and adding luciferin (luciferase substrate). This can then be used to calculate the RLU per well and the RLU/ml of the original sample.

Reverse transcriptase quantitative PCR (qRT-PCR) has also been employed in order to estimate transfected gene copies as well as mRNA copies in cells. This method is often used in conjunction with others described in this section in order to have comparative measurements of pseudotype quantity (55, 109, 122).

In many studies, pseudotype input is normalized via enzyme-linked immunosorbent assay (ELISA) detection of the principal component of the HIV core, p24 (16, 17, 28, 54, 59, 88, 89, 92, 95, 103, 109, 113, 115, 123, 124). However, as core budding is independent of surface HA, this method will detect cores lacking envelope glycoproteins as well as cores belonging to transduction competent pseudotypes. Pseudotype HA has also been detected using ELISA and used to normalize pseudotype input (82, 98, 125).

Quantification through hemagglutination assay has also been used frequently (28, 55, 58, 82, 84, 95, 98, 101, 122, 126–128).

Western blotting is used in some cases to determine the amount of glycoprotein or HIV p24 in a pseudotype sample (59, 72, 109). It is also used in a wider range of studies to ascertain glycoprotein or HIV p24 expression (17, 28, 55, 109, 122).

### CELL INPUT

The vast majority of studies involving neutralization assays titrate and transduce in 96-well plates with $1 \times 10^4$ cells (HEK293, HEK293T/17, or MDCK) per well. However, the amount of cells can range from $5 \times 10^3$ to $1 \times 10^5$. In some instances, 293A and MDCK-London cells are also used, whereas BHK-21 cells are frequently used for VSV-based pseudotype infection due to their comparative susceptibility (65, 73, 93, 95, 129–132). Specialized cells overexpressing α2,6-linked sialic acid (MDCK-SIAT) have also been used and compared to parental cells in the presence of soluble HA (77).

In one case, transduction was carried out in 96-well transparent culture plates, before lysates were then transferred to 96-well luminometer plates for analysis (69). The importance of pseudotype input in batch to batch variation is highlighted in Garcia et al. (133), the study suggests that an RLU of at least $1 \times 10^5$ per well should be used to ensure that antibody titer is independent of pseudotype input.

### SUBSTRATES

Steady-Glo or Bright-Glo (Promega) are the most common sources of luciferin. While expensive, these two substrates also serve a secondary purpose of lysing cells and releasing any expressed luciferase enzyme.

### EQUIPMENT: 96-WELL PLATES AND LUMINOMETERS

There is some disparity in the recording of equipment used in the articles studied for this review. Without this required information, reproduction of each study is hampered by these further variables relating to plate reading. Information relating to the color and manufacturer of 96-well plates is very important in the quantification of viable pseudotypes in order to prevent introduction of further variables between laboratories. While logistically difficult, the standardization of neutralization assay equipment across laboratories studying influenza would bring benefits to the interpretation of research data. Standardization of plate reading equipment is also required in order to ensure comparable data are obtained from different machines when reading the same experiment.

### HIGH-THROUGHPUT APPROACHES

A high-throughput approach has been used to evaluate antiviral compound effects on pseudotype transduction, testing a wide range of unique compounds in a single assay performed with 96- or 384-well plates (115, 134).

### INCREASED TRANSDUCTION EFFICIENCY

Polybrene (hexamethrine bromide) and polyfect (Qiagen) are used in several studies in order to increase transduction efficiency (17, 29, 58, 68, 76, 79, 80, 98, 135). 1 μg/ml, 8 μg/ml, or 16 mg/ml of polybrene is added to virus or virus/antibody mixes before the addition of cells in titration and neutralization assays or during incubation.

In two studies, spinoculation was used to increase transduction rate. To achieve the increased transduction rates, the pseudotypes and cells were centrifuged at 1250 rpm for 2 h or 3000 rpm for 1 h (3, 92).

## PSEUDOTYPE NEUTRALIZATION ASSAYS

### PROTOCOL

Pseudotype neutralization assays (pMN) are usually carried out in 96-well white plates. A measured amount of antibody in medium is

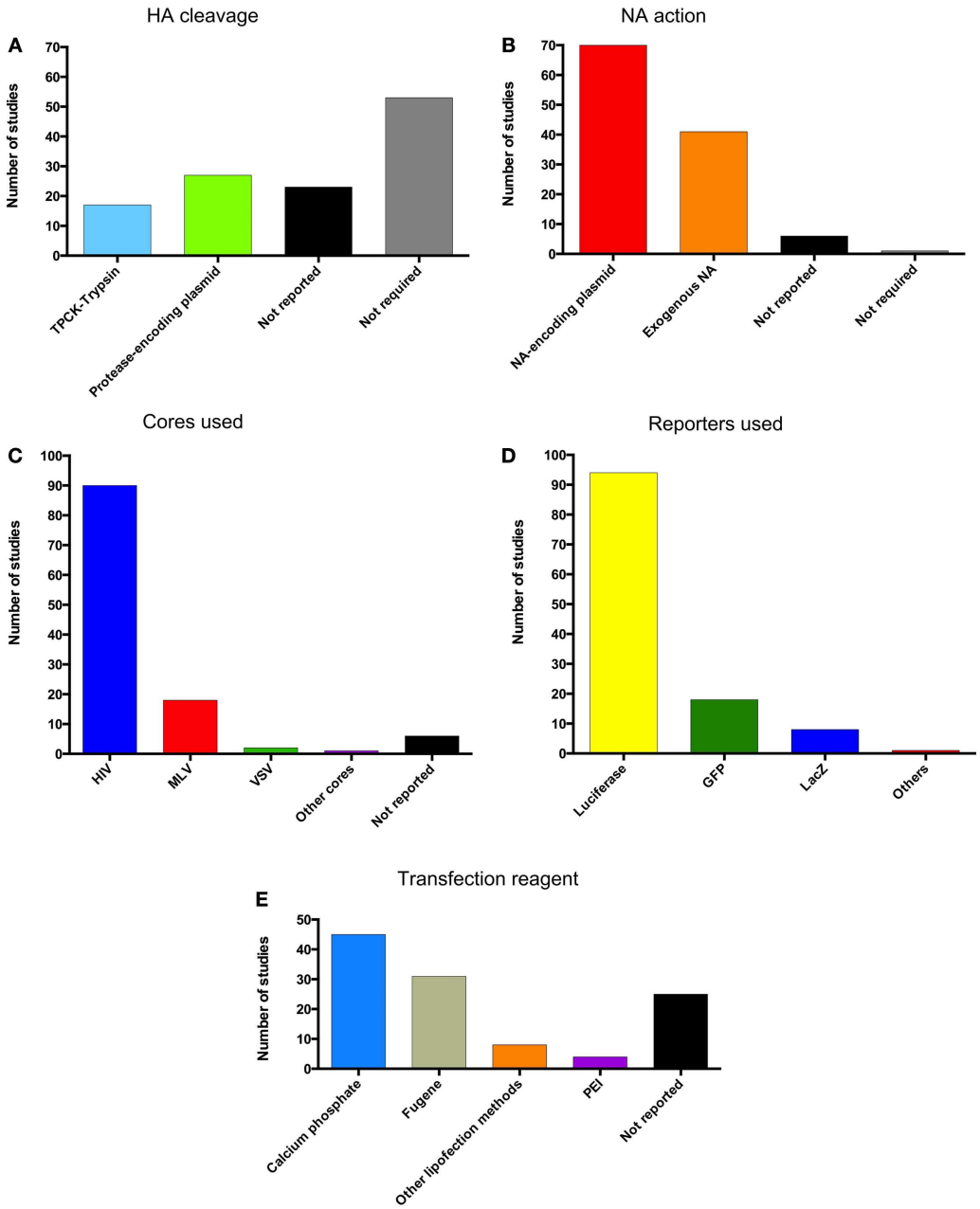


**FIGURE 6 | Pseudotype production methods**. Graphical representation of the methods used for pseudotype production in the literature cited in this review. **(A)** Method of HA cleavage used. **(B)** Method of NA action used. **(C)** Pseudotype cores used. **(D)** Reporters incorporated into pseudotypes. **(E)** Transfection reagents for the production of pseudotypes.

serially diluted across the plate and incubated with a set amount of quantified virus in medium, usually at a 1:1 virus:antibody ratio. Incubation is carried out at between 20 and 37°C for between 30 min to 2 h (43, 82, 96, 123, 133, 136). About $1 \times 10^4$ target cells are then added to each well, subsequently the plate is left to incubate at 37°C in 5% $CO_2$ for 48 or 72 h. A cell-only control as well as known positive and negative sera standards should be used as benchmarks for the neutralization assay (95, 113). See **Figure 7** for a depiction of the pMN assay.

## PSEUDOTYPE INPUT

The quantities of pseudotype used in neutralization assays, which were normalized based on p24 ELISA ranged from 6.25 to 50 ng/ml (17, 95). RLU or RLA values of between $1 \times 10^4$ and $1 \times 10^6$ per well were used (in a 96-well plate), sometimes in conjunction or normalized with p24 or qPCR methods (89, 121, 127). Estimates of copy number per set volume of original viral supernatant can also be used. It is important to note that RLU based values are affected by the make-up of the plasmid bearing the HA gene, as

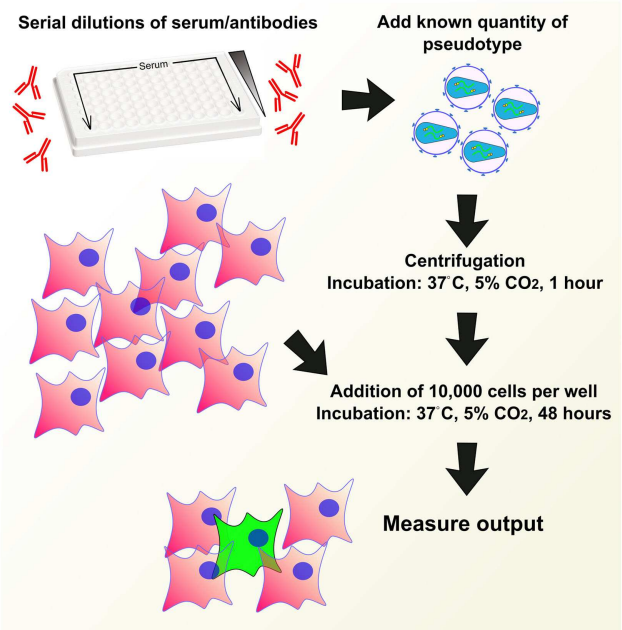


**FIGURE 7 | Example of a pseudotype neutralization assay (pMN).** Serum or antibodies are serially diluted across a 96-well plate, a known quantity of pseudotype is added and the plate is centrifuged and incubated to allow antibody binding. A set quantity of cells are added and plates are incubated for 48 h. Output is measured in a manner depending on reporter used.

well as a multitude of factors such as the luminometer, which is used to measure transduction.

### SERUM/ANTIBODY DILUTIONS AND START POINTS

Antibody input varies depending on availability, especially when taking into account the possibility of repeats and replicates. Antibodies are primarily diluted 2-fold in Dulbecco's modified eagle medium (DMEM), with or without FBS, across a 96-well plate, with the occasional three, four, or 5-fold dilution experiment (3, 18, 58, 137, 138). Where mentioned, starting antibody concentration ranged between 1:4 and 1:40.

### INCUBATION TIMES AND TIME PERIODS

When stated, serum complement inactivation varies from 30 min to 1 h at 56°C (68, 133). Pseudotype-antibody incubation times are generally consistent between studies, at 37°C for 1 h. Transduction times vary in 24 h increments at 24, 48, and 72 h, after which output is measured.

### CONTROLS

Positive sera or specific commercial antibodies are required as positive controls, which can be compared to tested sera and used to normalize between assays (see Approaches Toward Validation and Standardization). Reference sera from the National Institute for Biological Standards and Control (NIBSC), Office International des Epizooties (OIE), Animal and Plant Health Agency (APHA, previously AHVLA), and US Food and Drug Administration (FDA) are regularly used (88, 89, 127, 139, 140).

### NEUTRALIZING ANTIBODY TITER DETERMINATION

Antibody effect is displayed using one of many inhibitory concentrations ($IC_{50}$, $IC_{80}$, $IC_{90}$, and $IC_{95}$). The numerical value relates to the percentage point each particular study is calculating. For example, the $IC_{50}$ value can represent the concentration of an antibody that reduces RLU reading by 50%, when compared to 100 and 0% transduction controls (48, 68, 141, 142). These controls are essential to the calculation. About 100% inhibition can be benchmarked by a cell-only control and 0% by incubation of cells and virus in the absence of sera.

### HEMAGGLUTINATION-INHIBITION ASSAY

Hemagglutination-inhibition assay (HI) assays using pseudotypes utilize the same procedures as with wild type virus. A quantified amount of viral sample (as determined by hemagglutination assay) in phosphate buffered saline is added to serially diluted sera in a 96-well plate, to which 50 μl of a 0.5–1% chicken/turkey red blood cell suspension is added. After 30 min to 1 h, the HI plates are scored for agglutination. Pseudotype input is adjusted according to WHO guidelines at four hemagglutination units and sera is treated with receptor destroying enzyme to inactivate non-specific inhibition of agglutination (37, 98).

### POST-ATTACHMENT ASSAY

The post-attachment neutralization assay is used to identify antibodies that neutralize HA after it has bound to sialic acid. Oh et al. (143) modified the post-attachment assays, originally developed by Edwards and Dimmock (144), to allow wild type influenza virus to be replaced by influenza pseudotype particles.

In this assay, pseudotype particles are incubated at 4°C with cells to enable the synchronization of the attachment of virus to sialic acid on the cell surface and to block viral endocytosis. A diluted serum is then added, and following another 4°C incubation, plates are transferred to 37°C to permit transduction (143). Transduction is then measured using the same approach as that taken in a neutralization assay.

Antibodies detected by this assay have neutralizing activity via their ability to impede the endocytosis step and subsequent HA conformational changes necessary for virus–endosome fusion (143, 144). Antibodies that have neutralizing activity through impeding viral attachment will produce negative results in this assay. The post-attachment assay is useful for evaluating the neutralizing capacity of stalk-directed antibodies that do not inhibit viral attachment (143, 145).

### CROSS REACTIVITY

The issue of cross-reactive sera has been raised previously in traditional serological assays, serum samples produced by injection of wild-type virus into mice have been shown to lead to the presence of interfering antibodies directed toward NA or M2 epitopes (146, 147). It is expected that pMN will suffer from the same problems of cross-reactivity, an important issue, which must be addressed in the future in order to strengthen the usefulness of this assay as a competitor to the current gold standards.

## REPRODUCIBILITY

Reproducibility is a major issue in the field of serology. Serum samples are often finite, leading to an inability to reproduce

experiments or results in the same context as they were originally published. However, by standardizing methods for production, titration, and neutralization and the use of common reference standards it is possible to minimize variation between experiments and research groups.

## CORRELATION WITH OTHER SEROLOGICAL ASSAYS

Comparisons have been made between pMN assays and traditional serological assays with mixed results. Several articles report increases of between 31.9 and 200% in human antibody titers in comparison to microneutralization (MN) based results (148, 149). Buchy et al. (148) show a correlation between H5 pseudotypes and MN (spearman 0.79, $p < 0.001$), which is also seen in Du et al. (69) and Wang et al. (89), the latter presenting $r^2$ values of 0.9802 for A/Vietnam/1203/2004, 0.8193 for A/Anhui/1/2005, and 0.5244 for A/turkey/Turkey/1/2005 strains.

Alberini et al. (137) compared pMN assays to hemagglutination-inhibition (HI), single radial hemolysis (SRH), and MN assays using 226 different human serum samples. The Pearson correlation test produced significant correlation ($p < 0.001$) between the antibody titers calculated from each assay. The correlation coefficients between pMN and HI, SRH, and MN assays were 0.73, 0.70, and 0.78, respectively. Furthermore, the correlation between H5 MN and H5 pMN allowed the establishment of a threshold from which pMN titers could be based. pMN data were then analyzed based on the threshold, which showed protective titers in patients of 38–43, 54, and 79% after adjuvanted vaccination, second dose and booster, respectively (137).

Qiu et al. (81) show a range of correlations between HI and pMN using different HA subtypes. A/Moscow/10/1999 (H3N2) correlates well ($r = 0.8454$, $p < 0.0001$), A/Brisbane/59/2007 (H1N1), and A/Japan/305/57 (H2N2) poorly ($r = 0.1171$, $p = 0.7472$ and $r = 0.1171$, $p = 7472$) whereas A/Vietnam/1203/2004 (H5N1) correlates ($r = 0.7921$, $p = 0.0029$). In an additional study, HI and pMN ($IC_{50}$) correlate well in Qiu et al. (107) in the case of A/Shanghai/4664T/2013 (H7N9) (spearman $r = 0.88$, $p < 0.0001$) as well as in Whittle et al. (126) ($r^2 = 0.6491$, $p < 0.0001$).

A significant correlation of 65% ($p = 0.002$, $r = 0.65$) has also been reported between SRH and pMN using equine influenza pseudotypes and sera and another study showed the relationship between RLU and HA content (78, 119).

## APPROACHES TOWARD VALIDATION AND STANDARDIZATION

Approaches toward the standardization of pMN should follow the procedure that was required for MN standardization. Standardization of MN in general has focused on the use of pooled serum samples as reference standards. A/California/7/2009 (pandemic H1N1, pdm) standard was established by the WHO in 2010 with an assignment of potency of 13,000 IU/ml. A second pooled sera reference standard for H5N1 exists and has successfully been used in a number of studies (89, 137, 150). A cut off value for positive and negative H5N1 neutralizing sera exists for this set of H5N1 reference standards (137).

## CHIMERIC HEMAGGLUTININ AND STALK-DIRECTED ANTIBODIES

There has been considerable research into the stalk region of HA in relation to vaccine design and immunity to influenza. Various stalk-directed monoclonal antibodies (mAB) such as CR6261 have been characterized, opening up the potential use of chimeric HA to test for the presence of similar antibodies in serum samples (151, 152).

Stalk-directed antibodies were first identified in 1994 when the cross-reactive C179 mouse monoclonal antibody was identified and found to inhibit fusion of several HA subtypes (153). Since then many studies have focused on stalk-directed antibodies and their neutralization of multiple diverse subtypes of influenza (145, 152, 154–156). However, this range of heterosubtypic immunity is dependent on the characteristics of the epitope of each antibody tested, which will influence which subtypes, clades and whether they neutralize group 1 or 2 influenza.

The stalk region of HA is more conserved than the variable globular head to which the vast majority of neutralizing antibodies are directed. While residues in the head mediate attachment of the virus to target cells by binding to sialic acid, the fusion peptide in the stalk of HA is just as crucial to the HA function (157, 158). In order to test for neutralizing stalk antibodies, studies have employed a variety of chimeric HA constructs bearing stalks and heads from different subtypes. The concept behind this revolves around the use of HA heads that are largely unreactive to the antibodies used in the assay. Utilizing this approach, a neutralizing response can be detected in the absence of head-directed neutralization.

Several hybrids have been constructed and pseudotyped using HIV cores, these are generally constructed through PCR amplification and incorporation of complementary restriction sites, allowing ligation of different segments of HA genes. A wider variety has been used in reverse genetics approaches toward development of wild type virus bearing chimeric HAs (159–161). These chimeric HA are promising candidates for the testing of "universal" vaccines.

**Table 2** displays the regions and subtypes used in the construction of chimeric hemagglutinins. **Figure 8** is a visualization of chimeric HA construction in the form of a computer model.

## FUTURE OF INFLUENZA PSEUDOTYPES

Pseudotype neutralization assay offers the safety of using pseudotypes and the sensitivity of the MN assay. Further validation

**Table 2 | Examples of chimeric hemagglutinins originating from divergent subtypes and used for pseudotype production**.

| Reference | Head | Stalk |
|---|---|---|
| Hai et al. (58) | H5 A/Vietnam/1203/2004 | H1 A/Puerto Rico/8/1934 |
| | H1 A/California/04/2009 | H1 A/Puerto Rico/8/1934 |
| | H7 A/mallard/Alberta/24/2001 | H3 A/Perth/16/2009 |
| | H5 A/Vietnam/1203/2004 | H3 A/Perth/16/2009 |
| Pica et al. (80) | H6 A/mallard/Sweden/86/2002 | H1 A/Puerto Rico/8/1934 |
| | H9 A/guinea fowl/Hong Kong/WF10/1999 | H1 A/Puerto Rico/8/1934 |
| | **HA1** | **HA2** |
| Wang et al. (72) | A/Brisbane/59/2007 | A/New Caledonia/20/1999 |
| | A/New Caledonia/20/1999 | A/Brisbane/59/2007 |

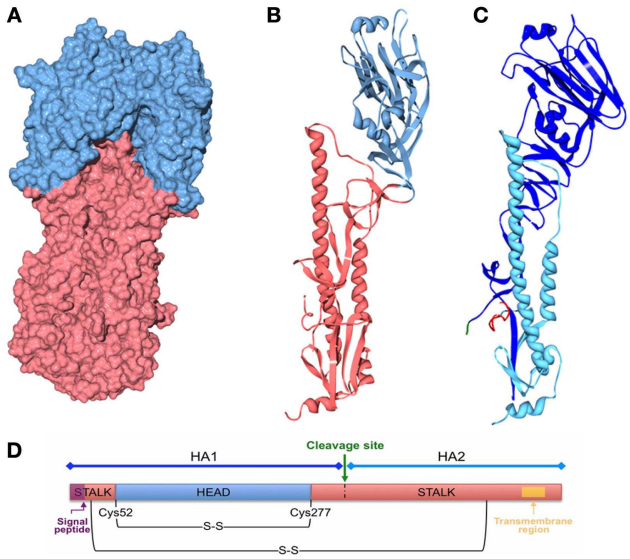


**FIGURE 8 | Computer models of chimeric HA**. Three-dimensional structures were generated with Swiss PDB viewer and POV-Ray 3.7 using the structure of the recombinant virus A/Hong Kong/1/1968 X-31 H3 [PDB ID: 2VIU (162)]. The signal peptide is not present in the HA. The transmembrane region is not resolved by X-ray crystallography. **(A)** Three-dimensional structure of the influenza HA trimer, showing the HA surface of the head (blue) and stalk (red) regions. **(B)** Three-dimensional ribbon structure of the influenza HA monomer showing the head (blue) and stalk (red) regions. **(C)** Three-dimensional ribbon structure of the influenza HA monomer showing HA1 (blue) and HA2 (light blue) subunits, the cleavage site and the fusion peptide are also shown in green and red, respectively. **(D)** Schematic of the HA polypeptide.

and standardization of the assay are required but once established, the assay should offer a robust and sensitive means of interrogating influenza vaccine trials for head and stalk-targeting antibodies. The production of vaccines that elicit stalk-targeting antibodies may in time lead to a universal vaccine, preventing 250,000–500,000 deaths from seasonal influenza and the emergence of pandemic strains, most recently the H1N1 2009 pdm, which caused an estimated 284,500 deaths (163). pMN currently offers the opportunity to batch test vaccines or commercialized antibodies in the absence of standardization.

Furthermore, the ability of the pMN assay to include chimeric HA, and also NA and M2 allows the pMN to be used to explain the pathogenicity of seasonal and pandemic influenza strains and perhaps elucidate the antigenic evolution of influenza further.

## OTHER USES OF PSEUDOTYPING INFLUENZA

### GENE THERAPY AND VACCINES

As the field of gene therapy progresses, influenza pseudotyping will benefit from the design of even safer and more effective vectors. As more sophisticated systems are developed they may become more easily standardized and comparable to wild type virus.

One aspect of gene therapy that may benefit the field of influenza is the use of viral entry proteins to target delivery of nucleic acids into specific cells, as vaccines or delivery systems. One delivery system study used influenza pseudotypes to transduce the respiratory epithelial cells of mice after nasal administration with promising results indicating that the method could be used in the treatment of cystic fibrosis (118). A similar study presented the rescue of ciliary function using influenza pseudotypes containing therapeutic cDNA (164).

Pseudotype-based influenza gene delivery vaccines are also becoming more widespread, with several candidates already cited in this review. Baculovirus pseudotyped with VSV-G has been used successfully to express HA in mammalian cells and provided an efficacious vaccine when tested in chickens and mice (165). Originally a popular vector for transgene expression in insect cells, baculovirus has been shown to be a useful tool for vaccine production in mammalian cells (166). In Wu et al. (165), delivery was achieved through VSV-G incorporation into baculovirus under the effect of the polyhedron promoter and HA under the effect of the CMV promoter in order to achieve expression and subsequent infection of mammalian cells. This is an interesting gene delivery system, which could be used as a method for the introduction of pseudotype genes into cells through a VSV-G bearing baculovirus in lieu of cytotoxic transfection reagents.

A further pseudotype vaccine has been developed which contains a modified HA gene, allowing expression in transduced cells but lacking the viral RNA sequences required for replication. This approach yields a particle bearing the desired glycoproteins, in this case A/Puerto Rico/8/1934 (H1) that consequently induces a robust T-cell response when given to mice via inhalation. Reduction in the severity of symptoms was also seen in mice infected with a different subtype: H3N2, A-X31 (71). While these approaches demonstrate the flexibility of the pseudotype platform, other more established methods including adenovirus or modified vaccinia viruses (e.g., modified vaccinia Ankara) may present a more attractive option for the delivery of influenza genes, and have been reviewed in great depth (167).

Pseudotypes used as immunogens, such as those bearing H5 have been tested in mice as a candidate vaccine, eliciting high levels of anti-HA antibodies as determined by HI. Mice that were vaccinated survived despite weight loss of approximately 12.8–21.1% whereas the non-vaccinated group lost approximately 25.5–26.2% of their bodyweight and perished 6 days after H5N1 virus challenge (20). A similar approach is taken by Szécsi et al. (168) in the production of H5 and H7 pseudotyped virus-like particles as immunogens tested in mice.

Influenza pseudotypes could also be used in vaccine design through the use of integrase defective lentiviral vector technology. Defective lentiviral vector technology allows transduction of target cells through maintenance of an episomal reporter construct without integration into the genome. This approach may bring benefits by reducing the chance of interrupting host genes and the eventual dilution of the delivered gene over time (169, 170).

See **Figure 9** for a depiction of the various pseudotype-based vaccines and immunogens.

## RECOMMENDED CONSENSUS PROTOCOL FROM SYNTHESIS OF PUBLISHED ARTICLES

### PRODUCTION PROTOCOL

A HEK293T cell monolayer of 60–90% confluence should be transfected using Fugene-6 or calcium phosphate precipitation

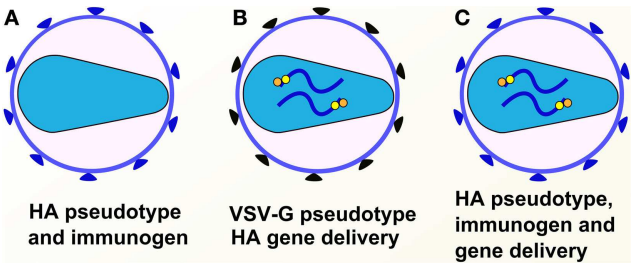


**FIGURE 9 | Pseudotypes used for gene delivery or as immunogens**. Pseudotypes can be employed as immunogens bearing the antigen of choice or as delivery systems for genes of choice. **(A)** HA-based pseudotype/virus-like particle immunogen. **(B)** VSV-G pseudotype delivery system for HA gene. **(C)** HA pseudotype delivery system for HA gene.

in medium containing 10% FBS. Plasmid ratios should be optimized based on the plasmids used. The use of second generation HIV packaging constructs is recommended. An NA encoding plasmid can be used or exogenous NA can be added at 24 h post-transfection to induce release of pseudotypes. The supernatant should be harvested at 48 h post-transfection and filtered through a 0.45 μm filter. Filtered supernatant should be kept at −80°C in single use aliquots if long-term storage is required.

### TITRATION PROTOCOL

Titration should be carried out using luciferase-based transduction in 96-well white plates, by p24 ELISA or other methods of quantification. Quantification of pseudotype particles using luciferase-based transduction involves the 2-fold serial dilution of 100 μl of pseudotype in 10% FBS medium. About $1 \times 10^4$ cells are then added in a 50 μl volume and the resulting solution is incubated for 48 h. After the 48-h incubation period luciferase substrate is added to each well and RLU values are read. Cell only, ΔEnv and VSV-G bearing pseudotypes can be used as negative and positive controls.

### PSEUDOTYPE-BASED NEUTRALIZATION PROTOCOL

Serum samples are serially diluted across a 96-well plate in 50 μl of media. Pseudotype virus should be added in a 50 μl volume at a concentration of $1 \times 10^6$ RLU. After 1 h incubation at 37°C, $1 \times 10^4$ HEK293T or MDCK cells should be added in a 50 μl volume. The plate is then incubated at 37°C for 48 h before luciferase substrate is added to each well, after which RLU values are read. Standards should ideally be used in the form of neutralizing antibodies or pooled serum samples.

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# Diagnostic potential of recombinant scFv antibodies generated against hemagglutinin protein of influenza A virus

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Human influenza A viruses have been the cause of enormous socio-economic losses worldwide. In order to combat such a notorious pathogen, hemagglutinin protein (HA) has been a preferred target for generation of neutralizing-antibodies as potent therapeutic/diagnostic agents. In the present study, recombinant anti-HA single chain variable fragment antibodies were constructed using the phage-display technology to aid in diagnosis and treatment of human influenza A virus infections. Spleen cells of mice hyper-immunized with A/New Caledonia/20/99 (H1N1) virus were used as the source for recombinant antibody (rAb) production. The antigen-binding phages were quantified after six rounds of bio-panning against A/New Caledonia/20/99 (H1N1), A/California/07/2009 (H1N1)-like, or A/Udorn/307/72(H3N2) viruses. The maximum phage yield was for the A/New Caledonia/20/99 (H1N1), however, considerable cross-reactivity was observed for the other virus strains as well. The HA-specific polyclonal rAb preparation was subjected to selection of single clones for identification of high reactive relatively conserved epitopes. The high-affinity rAbs were tested against certain known conserved HA epitopes by peptide ELISA. Three recombinant mAbs showed reactivity with both the H1N1 strains and one (C5) showed binding with all the three viral strains. The C5 antibody was thus used for development of an ELISA test for diagnosis of influenza virus infection. Based on the sample size in the current analysis, the ELISA test demonstrated 83.9% sensitivity and 100% specificity. Thus, the ELISA, developed in our study, may prove as a cheaper alternative to the presently used real time RT–PCR test for detection of human influenza A viruses in clinical specimens, which will be beneficial, especially in the developing countries.

**Keywords: influenza, recombinant, antibodies, scFv, HA, phage display, ELISA, diagnosis**

## Introduction

Flu is a respiratory illness, caused by influenza virus, with annual global attack rate of 5–10% in adults and 20–30% in children, causing significant levels of illness, hospitalization, and death (1). Influenza virus is an RNA virus with immunogenic surface receptors, hemagglutinin (HA), and neuraminidase (NA). Error-prone RNA dependent RNA polymerase (2) and segmented genome enable influenza

viruses to undergo antigenic shifts (major) and antigenic drifts (minor), which cause changes in the surface glycoproteins, HA and NA, and permit the virus to evade the adaptive immune response of the host cells. Such phenomena back the threat posed by influenza viruses for occurrence of frequent epidemics and pandemics (3), in spite of the available antivirals for treatment (4) or the vaccines for prevention and control of the disease (5). In view of such emerging and re-emerging outbreaks, timely diagnosis of the influenza virus infections in humans is essential. The developing countries, where limited resources are available, are in urgent requirement of alternate strategies to the expensive molecular real time RT–PCR test (6), which is currently used for diagnosis of flu. The cheaper alternative could highly minimize the loss of resources caused by frequent occurrence of the influenza virus infections. In the wake of such an issue, the present study was undertaken to develop recombinant antibodies (rAbs) against the HA antigen of human influenza A virus by phage-display technique for their subsequent use in diagnosis and/or therapy. Antibodies play important role in the course of natural protection against influenza virus infections; the HA antigen being the major target (7, 8).

Monoclonal antibodies (mAbs), since their advent in 1975 by Kohler and Milstein, using hybridoma technology, have proved to be potential diagnostic molecules (9–11). However, due to the well-known limitations of the conventional hybridoma technique, development of recombinant monoclonal antibodies (rAbs) by newer and more efficient molecular methods is preferred. Such rAbs have contributed immensely in medical and pharmaceutical research, cancer therapy, and diagnosis and treatment of infectious diseases (12–15). A major advancement in the production of rAbs has been the invention of phage-display technology, where high affinity and high specificity antibodies can be developed (16) against the antigen of interest.

In the present study, we developed recombinant single chain variable fragment (scFv) antibodies against the HA antigen of A/New Caledonea/20/99 (H1N1) virus. Anti-HA influenza mAbs have been developed for various applications, however, most of them are either hybridoma-based (17–20) or against HPAI H5N1 viruses (21, 22). The anti-HA rAbs produced in the current work showed cross-reactivity against the A/California/07/2009 (H1N1)-like or/Udorn/307/72(H3N2) viruses and helped in the development of a diagnostic ELISA test with 83.9% sensitivity and 100% specificity. The therapeutic efficacy of the one hetero-subtypic rAb (C5) is being evaluated in our laboratory, which has shown promising results so far (data not shown). Further assessment of the anti-HA influenza rAb developed in the present work would help to validate its efficiency as potential diagnostic and therapeutic agent.

## Materials and Methods

### Hyperimmunization of Mice

Five 6–11 weeks old Balb/c mice were immunized via intramuscular route with 1:128 HA titer of influenza A/New Caledonia/20/99(H1N1) virus, in a final volume of 100 µl, made up with 1× PBS (23). The virus administration was done on 20th, 35th, 42nd, and 51st days post first immunization. A day before each immunization, the mice sera were collected and tested for detection of anti-HA antibodies by indirect Enzyme Linked Immunosorbent Assay (ELISA) against the HA antigen of the influenza A/New Caledonia/20/99 (H1N1) virus. Permission for animal experiments was obtained by the Institutional Animal Ethics Committee (IAEC), which is registered under Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

### Indirect ELISA

The HA antigen of influenza A/New Caledonia/20/99(H1N1) virus diluted in $NaHCO_3$ coating buffer pH 9.6 was coated onto ELISA wells by incubation at 4°C for overnight. The wells were washed twice with PBST (1× PBS containing 0.5% Tween 20), and blocked with 1% BSA in PBS for 1 h at room temperature (RT). The wells were washed four times with PBST and then incubated with different dilutions of the immunized mice sera as primary antibody at RT for 2 h. The wells were washed vigorously with 1× PBST five times and incubated with HRP-conjugated rabbit anti-mouse antibody (1:1000 dilution in PBS) at RT for 1 h 30 min. The unbound secondary antibody was removed from the wells by 5–7 washings with PBST followed by addition of 50 µl of the TMB substrate solution and incubation for 25 min at RT. The reaction was stopped by the addition of 1M sulfuric acid and the absorbance was measured at 450 nm in the ELISA reader ($EL_X800_{MS}$, Biotek Instruments, Inc., Winooski, VT, USA).

### Collection of Spleen and Preparation of cDNA Template

After establishment of sufficient immune response against the virus strain, on 53th day, two of the five mice were given intranasal instillation with influenza A/New Caledonia/20/99 (H1N1) virus, followed by aseptic dissection on the 55th day for collection of spleen. Following RBC lysis, the spleen cells were re-suspended in 1 ml of RPMI medium and subjected to live cell count estimation using Neubauer's Hemocytometer. Aliquots of approximately $3.5 \times 10^6$ cells were made and re-suspended in five volumes of RNA*later* (Sigma-Aldrich). After overnight incubation at 4°C, one aliquot of the cell suspension was subjected to total cellular RNA isolation using RNeasy Mini Kit (Qiagen) and thereafter total mRNA isolation using the Oligotex mRNA Mini Kit (Qiagen) as per the manufacturer's instructions. Synthesis of cDNA was done using M-MuLV Reverse transcriptase enzyme. The reaction mix (final volume of 25 µl) consisted of 10 µl of the isolated mRNA, 800 µM dNTP mix, 200 U of M-MuLV enzyme, 0.2 µl RNase inhibitor, 2 µl oligo-dT primer, and 1× M-MuLV RT buffer. The reaction was set-up at 42°C for 2 h followed by heat inactivation of enzyme at 94°C for 5 min.

### Amplification and Cloning of scFv Gene Repertoire

The variable light ($V_L$) and variable heavy chain ($V_H$) genes were amplified from the cDNA using degenerate mouse IgG primer set (Cat. No. F2010, Progen Biotechnik, GmBH, Germany), consisting of 11 degenerate forward primers for either $V_H$ chain gene or $V_L$ chain gene amplification. Initially, a 10 µl reaction mix was

set-up using primer set I. The reaction consisted of 2.5 μM of each primer, 1× PCR buffer, 2.5 U of Hot Star *Taq*DNA polymerase (Qiagen), 0.5 mM of the dNTP mix, 2 μl of cDNA, and 2 mM of $MgCl_2$. Initial denaturation was carried out at 94°C for 15 min followed by 35 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 30 s), and a final extension at 72°C for 10 min. The reaction was finally held at 4°C and the PCR products were resolved on 1.5% agarose gel. Second set of PCR reactions were carried out, using the corresponding Set-II forward primers, which showed amplification in the Set-I reaction. The second set of PCR was performed for introduction of restriction sites *Nco*I (5′) and *Hin*dIII (3′) to the amplified $V_H$ gene fragments and *Mlu*I (5′) and *Not*I (3′) to the amplified $V_L$ gene products. The Set-II reactions were put up as described for the Set-I. After analysis on the agarose gel, DNA bands from positive PCR reactions were purified and pooled for cloning in the pSEX81 phagemid vector.

The purified $V_L$ gene pool was digested with *Mlu*I and *Not*I restriction enzymes (Fermentas) in appropriate buffer and ligated into 50 ng of the digested pSEX81 vector at 1:3 vector: insert molar ratio using 0.4 Weiss Units of T4 DNA ligase at 4°C overnight incubation. The ligated products were transformed into ultra-competent cells XL1-Blue strain of *E. coli* and plated onto ampicillin (100 μg/ml) supplemented nutrient agar plates followed by overnight incubation at 37°C. The colonies obtained over the agar plates were scraped and propagated in LB/amp (100 μg/ml) medium and subjected to phagemid isolation for restriction analysis with *Mlu*I and *Not*I REs, for confirmation of the $V_L$ gene cloning in the vector.

The pSEX81-$V_L$ vector was further digested with *Hin*dIII and *Nco*I R.E.s (Fermentas) in appropriate buffer and insertion of the RE digested pooled $V_H$ gene DNA at 1:3 vector: insert molar ligation ratio using 0.4 Weiss Units of T4 DNA ligase in an overnight reaction at 4°C. The ligation mix was transformed and the resulting colonies were scraped and propagated for restriction analysis by *Nco*I and *Not*I REs to confirm the cloning of the complete scFv cassette in the pSEX81 vector.

## Construction of Mouse Recombinant Phage-scFv Library

### Phage Rescue

The pSEX81-scFv transformed *E. coli* XL1-Blue cells were grown overnight in 10 ml SOB–GAT medium (SOB broth supplemented with 100 mM glucose, 100 μg/ml ampicillin and 10 μg/ml tetracycline) at 37°C with shaking at 200 rpm. The overnight culture was inoculated at 1:100 dilution in SOB–GAT medium, incubated at 37°C with shaking at 180 rpm and monitored every hour for bacterial growth till an $OD_{600}$ of 0.3 was obtained. The lyophilized hyperphage M13K07ΔPIII (Progen Biotechnik Cat. No. PRHYPE) was re-constituted in 2 ml of the autoclaved milliQ water just before use, as per the manufacturer's instructions and added to the log phase cells at an MOI of 20 (Multiplicity of Infection) representing the average number of phages per bacteria was calculated by using the following formula:

$$\text{MOI} = \frac{\text{No. of phage (ml added} \times \text{plaque forming units/ml)}}{\text{No. of bacteria added}}$$

The hyperphage-infected culture was incubated at 37°C without shaking for 30 min and then shaking at 260 rpm for 30–45 min. The cells were harvested by centrifugation at 3,000 rpm for 10 min at RT and re-suspended in equal volume of pre-warmed SOB–AKT medium (SOB broth supplemented with 100 μg/ml ampicillin, 50 μg/ml kanamycin and 10 μg/ml tetracycline). The cultures were incubated overnight at 34°C with shaking at 220 rpm (24).

### Phage Precipitation

The overnight culture was centrifuged at 3,000 rpm for 15 min at room temperature and the supernatant transferred in a fresh tube. The phage particles in the supernatant were precipitated by addition of one-fifth volume of 20% PEG, 2.5M NaCl and incubating on ice for 1 h, followed by centrifugation at 4,000 rpm/4°C for 40 min. The pellet was re-suspended in phage dilution buffer (10 mM Tris–HCl pH 7.5, 20 mM NaCl, 2 mM EDTA) and stored at 4°C until further use (24).

## Phage Quantification ELISA

For optimization, two-fold dilutions of the rescued phages were prepared in coating buffer (sodium bicarbonate buffer pH 9.6), coated onto ELISA wells in duplicate and incubated at 4°C overnight. After incubation at 37°C for 15–20 min, the wells were washed thrice with PBS and blocked for 1½ h with 2% skimmed milk in PBS (SMP) at room temperature. The wells were washed three times with PBS followed by addition of different dilutions of anti-M13 mouse monoclonal antibody B62-FE2 against PVIII coat protein of the M13 phage. After 1½ h incubation, the wells were washed three times with PBS and incubated with anti-mouse-HRP conjugated antibody (SIGMA, affinity purified antibody) 1:1000 diluted in SMP, for 1 h, followed by washing with PBS with 1–2 min hold per wash and incubation with 50 μl of substrate solution (5 mg OPD, 4 μl $H_2O_2$ in phosphate-citrate buffer) at 37°C for 15 min. The absorbance was measured at 492 nm; reference wavelength being 620 nm (Magellan V 7.1). All the incubation steps were done at RT unless individually mentioned (25).

## Bio-Panning

Bio-panning was optimized using protocols which differed in the choice of surface area, and type of elution buffer. The surface area tested included 96-well microtiter plate, immunotube and six-well tissue culture plate, while the elution buffers included 0.1M HCl (adjusted to pH 2.2 with glycine), trypsin (1, 2, and 5 μg/ml) and 100 mM triethylamine (pH 11.0).The optimized procedure, finally preferred for selection of HA-specific recombinant phages is as follows. A MaxiSorp Immuno Tube (Nunc, Thermo Fisher Scientific, USA) was coated with influenza A/New Caledonia/20/99 virus (1:128 HA titer) diluted in a total volume of 4 ml coating buffer [0.2M $Na_2CO_3$·$NaHCO_3$ (pH 9.6)], and kept at 4°C overnight followed by washing with PBS and then incubation with blocking solution (2% SMP) at 37°C/1 h. The tube was washed thrice with PBS and incubated with the rescued phage, vol 1 ml, at room temperature for 1 h with shaking at 35 rpm and then without shaking for 45 min. After washing 10 times each with PBST (PBS with 0.1% Tween 20) and PBS, the antigen-specific phage particles were eluted by incubation with 1 ml of

100 mM triethylamine (Sigma) for maximum 10 min. The eluted phage was aspirated into a tube containing 0.5 ml of Tris–HCl pH 7.5, for neutralization of the eluted phage (26). The panning procedure was repeated six times for selection of HA-specific scFv antibody-phages with stringency of washing increased for each round. Total phage yield (in triplicates) was assessed after each round of bio-panning by ELISA.

### Assessment of Cross-Reactivity

The phages precipitated after the last two rounds of bio-panning were also assessed (in duplicates) for reactivity against the A/California/07/2009 (H1N1)-like or A/Udorn/307/72(H3N2) viruses. After the sixth round of bio-panning, 56 phage clones were randomly selected and underwent small-scale phage rescue as previously described (24). The precipitated phage preparations from individual clones were tested for binding activity against HA of influenza A/New Caledonia/20/99 virus. The clones showing high reactivity were further analyzed for cross-reactivity against HA antigens of A/California/07/2009 (H1N1)-like or/Udorn/307/72(H3N2) viruses by phage ELISA as previously described (25). Briefly, of HA of each of the virus diluted in coating buffer were applied to ELISA plate wells and incubated overnight at 4°C. The wells were washed twice with 1× PBS and blocked with 2% SMP (2% skim milk powder in PBS) for 2 h at RT. HA-specific precipitated recombinant phage preparation (1:2 diluted in 2% SMP) was added to each well and incubated at RT for 1 h. After washing six times with 400 µl PBS per well, the bound phage were detected with the anti-M13 antibody B62-FE2 against PVIII coat protein of the M13 phage.

### Peptide ELISA

Five peptides (27) were commercially synthesized and used for testing the phage-scFv antibodies for analysis of the epitopes identified by them by peptide ELISA. The peptide sequences were chosen based on the previous report that these were linear HA epitopes and relatively conserved among different subtypes of influenza A viruses. The peptides (about 2 µg/well) in a final volume of 50 µl coating buffer ($Na_2CO_3$·$Na_2HCO_3$, pH 9.6) were coated onto ELISA wells and incubated overnight at 4°C. The wells were washed with PBST (1× PBS with 0.1% Tween 20) once, followed by three washes with PBS. The wells were incubated with blocking buffer (1% BSA in 1× PBS) at 37°C for 1 h. After washing, selected phage-bound scFv was applied onto each well. After incubation at 37°C for 2 h, the wells were washed with washing buffer (1M NaCl, 0.05% Tween 20) for four times, and incubated with anti-mouse HRP conjugated antibody (1:1000) for 1 h followed by one washing with PBST and three washings with PBS and detection with substrate, and absorbance measurement at 490 nm in ELISA reader. Sequences of the peptides tested against the scFv antibodies are mentioned in **Table 1**.

**TABLE 1 | Sequences of the peptides tested against the scFv antibodies.**

| Peptide | Position | Sequence |
|---|---|---|
| P1 | 38–52 | EKNVTVTHSVNLLED |
| P2 | 58–72 | LCKLRGVAPLHLGKC |
| P3 | 318–332 | GKCPKYVKSTKLRLA |
| P4 | 318–332 | GACPRYVKSNTLKLA |
| P5 | 318–332 | GECPKYVRSAKLRMV |

### ELISA for Diagnosis of Influenza A Virus Infection

A total of 53 human nasal/throat swab specimens were tested for development of the phage-scFv antibody-based diagnostic ELISA test. The samples were collected from various hospitals in Delhi, India and were already tested with the standard real time RT–PCR test. A total of 53 samples were tested. Forty-seven samples were from patients, who acquired natural infection of influenza A virus, of which 10 patients were diagnosed for pH1N1/09 virus infection and 37 for seasonal influenza A virus (14 H1N1 + 23 H3N2) infection by real time RT–PCR. Six individuals served as healthy controls that did not encounter any influenza virus infection since more than 6 months from the date of sample collection, and were also real time RT–PCR negative for the viral infection.

The ELISA wells were coated with 1:2 diluted clinical specimens in coating buffer overnight at 4°C, followed by washing with PBST (1× PBS with 0.05% Tween20) and PBS. The wells were blocked with 2% skimmed milk in PBST for 1 h at 37°C and incubated with C5 phage-scFv antibody at 37°C for 2 h. The wells were washed with PBST and PBS followed by detection with the anti-M13 phage antibody B62-FE2 against PVIII coat protein of the M13 phage as described before. Following the addition of TMB substrate, the reaction was stopped and the absorbance was measured at 450 nm in an ELISA plate reader. Each sample was tested in duplicate and the maximum absorbance was measured by using the P3 peptide in place of the test samples. The samples from healthy controls served as negative controls for the test. The sensitivity and specificity of the test (26) were calculated by the following formulae, where determination of the true positive or true negative samples was done by real time RT–PCR analysis.

$$\text{Sensitivity} = [\text{True positive divided by sum of true positive and false negative}] \times 100$$

$$\text{Specificity} = [\text{True negative divided by sum of true negative and false positive}] \times 100$$

Results were expressed as sensitivity and specificity of the test in comparison to real time RT–PCR test for diagnosis of influenza A virus infection.

## Results

A total of 6- to -11-week-old Balb/c mice were immunized via intra-muscular route with pandemic influenza A H1N1 (2009) virus. A relatively high immune response was observed after booster doses of pandemic influenza A H1N1 (2009) virus as confirmed by ELISA with immunized mice sera. The absorbance measured for the anti-HA antibodies before the first virus administration was 0.620, before fifth immunization dose was 3.129 (**Table 2**) and exceeded the detectable range post 48 h of the fifth immunization. The increase in the antibody titer upon each immunization is represented in the **Figure 1**.

### Amplification and Cloning of the Antibody Genes

After achieving a considerable antibody titer against the HA antigen, spleen was collected from two of the hyper-immunized mice.

**TABLE 2 | Absorbance values observed in ELISA for estimation of anti-HA antibodies in mice sera, after each immunization with influenza A/New Caledonia/20/99(H1N1) virus**.

| S.No. | Day | $OD_{450}$ |
|---|---|---|
| 1 | 1 | 0.620 |
| 2 | 20 | 1.507 |
| 3 | 35 | 2.214 |
| 4 | 42 | 2.956 |
| 5 | 51 | 3.129 |

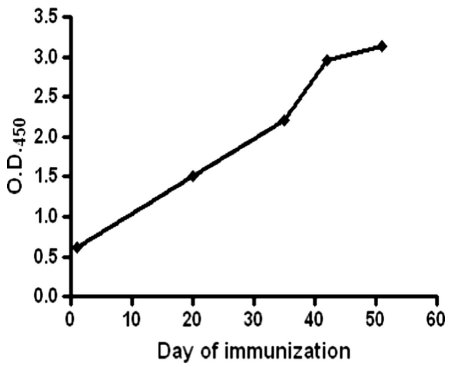


**FIGURE 1 | Graphical representation of the mean antibody titers in sera of immunized mice before each immunization**. High anti-HA antibody levels were detected in the hyper-immunized animals after 51 days of the immunization schedule.

A total of $7.5 \times 10^6$ viable B cells were obtained after preparation of single cell suspension of hyper-immunized mice's spleen. Three and a half million B cells were used for total RNA isolation. One microgram of the mRNA, which was isolated from the total cellular RNA, was subjected to synthesis of cDNA to be utilized as template for amplification of the antibody genes. The $V_L$ and $V_H$ genes were individually amplified using a commercially available mouse IgG primer set. DNA bands at expected sizes (as mentioned in the manufacturer's protocol) of 400 bp for $V_H$ and ~380 bp for $V_L$ were observed in 10 out of 11 degenerate forward primers for $V_H$ gene DNA and 9 out of 11 primers for $V_L$ gene DNA (**Figure 2**). All the amplicons of $V_L$ and $V_H$ gene were individually purified from the gel. First, the $V_L$ gene pool was cloned in the pSEX81 phagemid vector followed by cloning of the $V_H$ gene pool. Digestion with the restriction enzymes flanking the scFv cassette (*Nco*I and *Not*I) confirmed the insertion of both the genes and thus cloning of the complete scFv pool in the phagemid vector (**Figure 3**).

## Phage Rescue

The scFv displaying recombinant phages were rescued from pSEX81-scFv transformed *E. coli* XL1-Blue cells after infection with the hyperphage (M13K07ΔPIII). After overnight incubation at 34°C/220 rpm, the culture showed uniform turbidity. Different dilutions of the precipitated phage preparation were titrated against various dilutions of the tracing antibody for optimization. A dilution of 1:2 of the rescued phage and 1:200 of the tracing antibody were found optimum for detection of the recombinant phages in ELISA.

The HA-specific recombinant phages were selected by the bio-panning procedure. The phage yield was observed to show a marked increase after the sixth round of bio-panning (**Figure 4**) against the influenza A/New Caledonia/20/99 virus strain.

## Cross-Reactivity and Peptide ELISA

The influenza A/New Caledonia/20/99-bio-panned phage preparation was tested against the pandemic H1N1/09 and seasonal H3N2 viruses, after fifth and sixth rounds of bio-panning and it was observed that there was no considerable increase in the total phage yield against the non-specific viral strains, although the reactivity levels of the recombinant scFv-phage preparation, after the fifth round, were similar against the two H1 sub-types (**Figure 5**). Of the 56 clones, five (A11, C5, C8, E9 and G6) showed high reactivity against the A/New Caledonia/20/99 (data not shown). Of these five scFv clones, three clones (A11, C5 and E9) cross-reacted well with A/California/07/2009 (H1N1)-like virus, and one (C5) cross-reacted with A/Udorn/307/72(H3N2) virus (**Figure 6**).The high reactivity rAb clones were further analyzed by peptide ELISA. The identification of the epitopes, being recognized by the scFv clones was done against the previously characterized peptides (27). The phage-scFv antibodies, A11, C8 and G6, did not bind to any of the peptides tested, while C5 and E9 showed binding activities with the peptide P2, and C5 phage-scFv antibody showed variable binding activities with three peptides P3, P4, and P5 (**Figure 7**). The peptide P1 was not recognized by any of the two phage-scFv anti-HA rAbs.

## ELISA for Diagnosis of Influenza A Virus Infection

The ELISA test was developed using the C5 phage-scFv antibody, as it was found to react with all the three HA, i.e., seasonal H1, pandemic H1, and H3 antigens. The peptide P3 was taken as the positive control and as a measure of maximum absorbance. The mean absorbance value of the negative specimens was taken as cut-off to determine the sample positivity. Among the 47 true positive specimens, the rAb-based ELISA test detected influenza A virus in 38 samples, while all the six true negative controls were observed to be negative by the ELISA test. Thus, the ELISA test developed herein showed 83.9% sensitivity and 100% specificity.

# Discussion

Influenza A viruses pose considerable economic burden both on the society and individuals in terms of consumption of health care resources and lost productivity. The occurrence of frequent flu outbreaks leads to large-scale socio-economic losses and calls for improvement in the production of reagents, in terms of large scale production, uniform quality, low cost, and stability for effective diagnosis and management of the disease. With the advent of upgraded technologies, rAbs have provided great advantages against the conventional monoclonal antibodies (28, 29). Once developed, rAbs are economically one of the most feasible diagnostic and therapeutic agents and allow mass scale production. A rAb can be developed by various procedures, for e.g., by conventional recombinant expression in prokaryotic expression system or by recombinant methods, such as, phage-display technology.

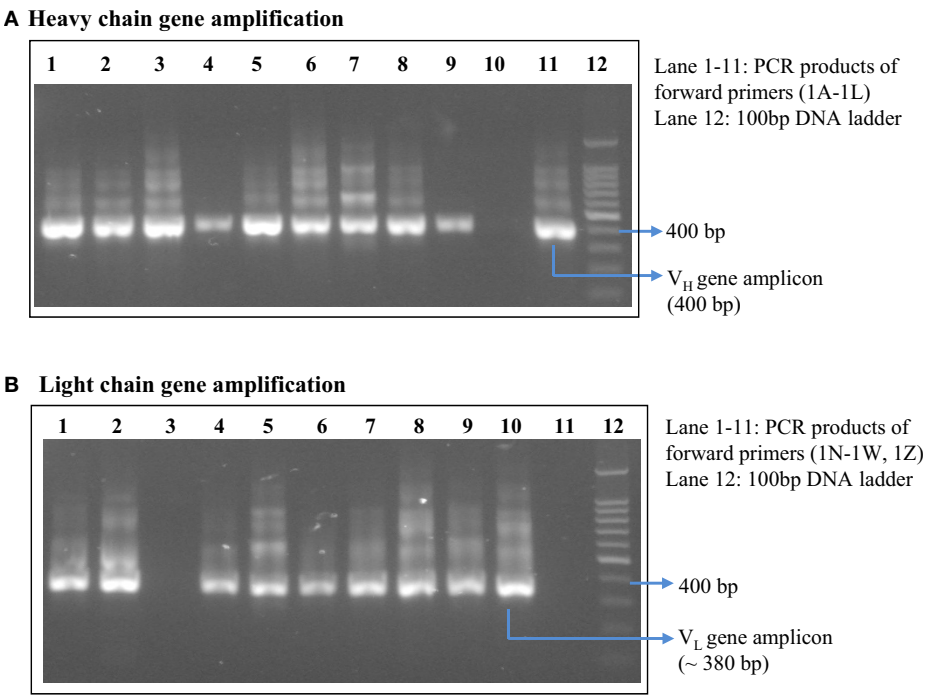


**FIGURE 2 | Amplified light chain DNA (~380 bp) (B) and heavy chain DNA (400 bp) (A) using mouse IgG library primer set**. Lanes 1–11 represent PCR products from 11 different degenerate forward primers supplied with the kit.

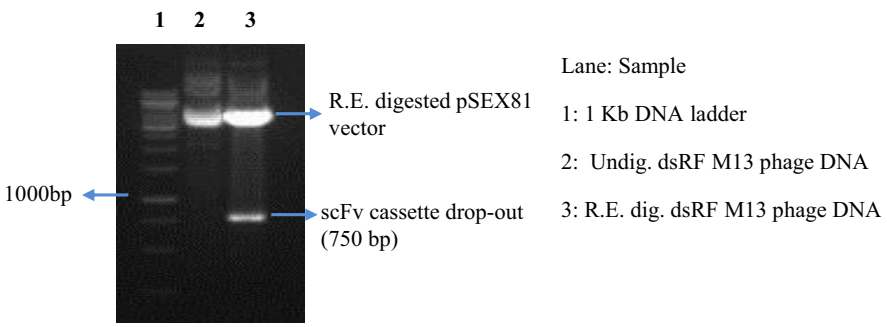


**FIGURE 3 | Cloning of the complete scFv cassette in the pSEX81 phagemid vector**. The phage-display library of the murine scFv gene pool was constructed by insertion of the antibody light and heavy chain gene DNA into the phagemid pSEX81 vector. Lane 3 represents the drop-out of the scFv DNA upon restriction digestion with the restriction enzymes, *Nco*I and *Not*I, flanking the scFv cassette.

Phage-display technology is a powerful technique (30), which allows easier and faster production of antibodies. The resulting molecules exhibit high antigen specificity and can be produced in large quantities, thus avoiding the use of conventional time-consuming methods like hybridoma technology. Therefore, in the present study, we aimed toward development of a recombinant antibody-based economical test for a cheaper yet effective diagnosis of human influenza A virus infection.

An antibody-phage-display library was constructed from B cells of Balb/c mice hyper-immunized with pandemic influenza A H1N1 (2009) virus. The $V_H$ and $V_L$ chain genes were amplified using commercially available degenerate primer sets. The sequences of the mouse IgG library primers were finalized after analysis of the various antibody sequences available in the Kabat-Wu data book (31), so as to enable amplification of as much possible antibody variable regions. Since, the desired template sequences were not known; similar sequences were grouped for choosing putative primer sequences from each group, which were then compared against all database sequences for selection of best-fitting primer sequences. The process was repeated until all the database sequences were covered (31).

In order to diminish a bias against particular sequences, the amplification of $V_H$ and $V_L$ genes was done by two polymerase chain reactions using two separate mouse IgG library primer sets. The first set provided an unbiased amplification, while the second set was necessary for introduction of restriction endonucleases sites to allow cloning of the amplified immunoglobulin gene fragments into pSEX81 phagemid vector. The recognition

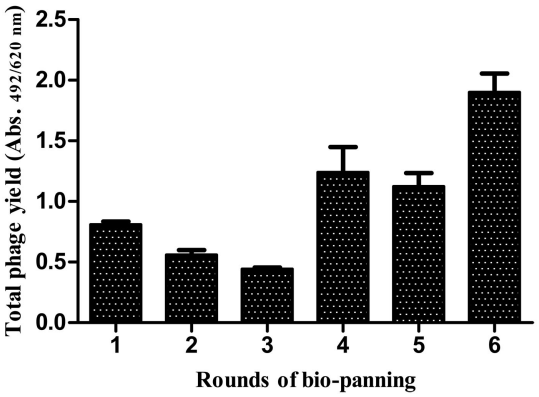


**FIGURE 4 | Affinity selection of phage-bound anti-HA scFv antibodies from the antibody library**. A considerable rise in the specific scFv antibodies was observed after the sixth round of bio-panning against the A/New Caledonia/20/99(H1N1) virus.

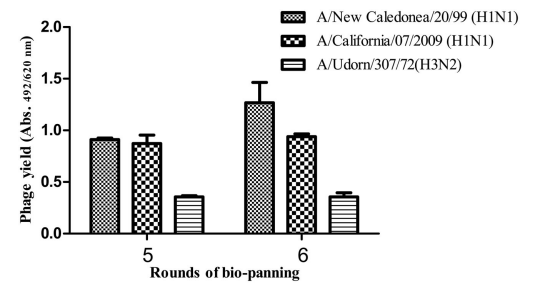


**FIGURE 5 | Binding activity of the polyclonal rAb preparation against the three viral strains**. The total phage yield after the last two rounds of bio-panning was analyzed against the A/New Caledonia/20/99(H1N1), A/California/07/2009(H1N1)-like and A/Udorn/307/72(H3N2) viruses by ELISA.

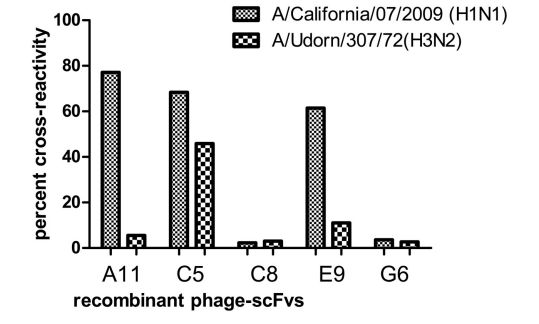


**FIGURE 6 | Assessment of cross-reactivity of the relatively high affinity anti-HA (A/New Caledonia/20/99(H1N1) virus) monoclonal rAbs against the other influenza A virus sub-types, A/California/07/2009 (H1N1)-like and A/Udorn/307/72(H3N2) viruses**. The ELISA absorbance value for each of the cross-reactive strains was divided by that of the specific strain and multiplied by 100 to generate the percent cross-reactivity.

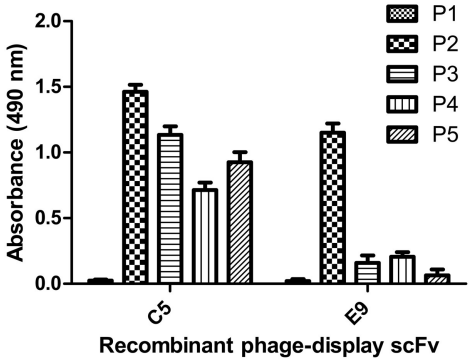


**FIGURE 7 | Peptide ELISA**. Different peptide sequences (representing linear HA epitopes) were synthesized and tested against the monoclonal phage-display antibodies for analysis of the binding activity. The C5 antibody, which was found to react with all the four HA peptides was used for development of the diagnostic ELISA test.

sequences of *Nco*I (5′) and *Hin*dIII (3′) were added to the amplified heavy chain gene fragments and *Mlu*I (5′) and *Not*I (3′) to the amplified light chain gene fragments. The choice of restriction enzymes was based on various advantages offered by them, viz. (i) low probability to cut within mouse $V_H$ or $V_L$ coding regions, (ii) optimal cloning efficiency by production of overlaps of four nucleotides or more, (iii) methylation independence, and (iv) more than 90% efficiency in double digestion reactions (32).

DNA bands at expected sizes of ~400 and ~380 bp were observed after PCR amplification of $V_H$ and $V_L$ genes, respectively, which were individually purified for cloning in pSEX81 phagemid vector. The complete scFv cassette (750 bp) containing phagemid clones were transformed in *E. coli* XL1-Blue cells and subjected to rescue of recombinant phages expressing the pIII-scFv fusion proteins on their surface. The rescued phage antibody library was subjected to selection of the anti-HA antibodies by bio-panning, which is an affinity-based selection process. The antibody with the desired specificity is selected from a recombinant heterogeneous antibody repertoire and enriched over a million fold by selection against the specific antigen (33). The rescued phages were quantified after each round of bio-panning and a marked increase was observed after sixth round of bio-panning. The recombinant scFv-displaying phage preparations, bio-panned against the influenza A/Caledonia/20/99 virus, were analyzed for cross-reactivity after fifth and sixth rounds against the A/California/07/2009 (H1N1)-like or A/Udorn/307/72(H3N2) viruses. We observed that the recombinant phages reacted to similar levels against the H1N1 strains, after the fifth round of bio-panning. However, after the sixth round of the affinity selection, an insignificant increase in the phage yield was observed against the pH1N1/2009 virus. The percentage of cross-reactivity of the recombinant phages for the H3N2 strain of the virus was very low (~40%), which can be explained by the dissimilarity of the H3 from the H1 sub-type. We chose to continue our study with the phage-bound antibodies, as the conversion of scFv-phage antibody to its soluble form leads to alteration in the antibody specificity (34). Further analysis of the single phage-scFv clones showed that six clones had relatively high reactivity against the specific influenza virus sub-type (A/Caledonia/20/99 virus), of which the six clones (A11, C5 and E9) and one clone (C5) showed cross-reactivity with A/California/07/2009(H1N1)-like virus, and A/Udorn/307/72(H3N2) virus respectively. Since, the clones were

selected via a rigorous procedure of affinity selection and showed cross-reactivity among the other HA sub-types, we attempted to test the clones against some of the already validated conserved linear epitopes (27). The C5 scFv-phage clone was found to react with three different peptides corresponding to three different HA sub-types, with variable reactivity, though. Therefore, the C5 scFv-phage antibody was employed to develop an ELISA test for diagnosis of the human influenza A virus infection in clinical specimens. The non-reactivity of the other three phage-scFv rAbs, i.e., A11, C8, and G6 antibodies, may be due to their specificities against the epitopes other than the ones tested in the experiment. Moreover, in peptide ELISA, only linear epitopes could be tested, therefore, it is likely that A11, C8, or G6 would be specific against conformational epitopes. The most widely and the recommended real time RT–PCR test imposes restriction due to its high cost, however, the ELISA test proposed in the current study may be performed in any laboratory with basic facilities/manpower/expertise. The sample size tested in the current study showed 83.9% sensitivity and 100% specificity for the ELISA test, however, a higher sensitivity is crucial for application of the test for detection of influenza A virus in the field specimens. In this regard, the work is in progress in our laboratory by testing of higher number of routinely collected human clinical samples.

## Conclusion

Presently, the real time RT–PCR test is the most widely used technique to diagnose flu (6). However, in the developing countries, the high cost of such test poses limitations in better management and efficient surveillance of the disease. Therefore, we attempted to develop the diagnostic ELISA test as a low cost alternative for the diagnosis of influenza virus infections. The number of samples tested in the present study yielded 83.9% sensitivity and 100% specificity of the test. However, further validation of the results by testing a larger number of samples would be required, which would further help in determination of the efficiency of the ELISA test. Following such validation, the ELISA test developed by us holds significance, as it can be used as an initial screening method for the diagnosis of influenza virus infection in human specimens.

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# Bad News: Analysis of the Quality of Information on Influenza Prevention Returned by Google in English and Italian

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Information available to the public influences the approach of the population toward vaccination against influenza compared with other preventative approaches. In this study, we have analyzed the first 200 websites returned by searching Google on two topics (prevention of influenza and influenza vaccine), in English and Italian. For all the four searches above, websites were classified according to their typology (government, commercial, professional, portals, etc.) and for their trustworthiness as defined by the Journal of the American Medical Association (JAMA) score, which assesses whether they provide some basic elements of information quality (IQ): authorship, currency, disclosure, and references. The type of information described was also assessed to add another dimension of IQ. Websites on influenza prevention were classified according to the type of preventative approach mentioned (vaccine, lifestyle, hygiene, complementary medicine, etc.), whether the approaches were in agreement with evidence-based medicine (EBM) or not. Websites on influenza vaccination were classified as pro- or anti-vaccine, or neutral. The great majority of websites described EBM approaches to influenza prevention and had a pro-vaccine orientation. Government websites mainly pointed at EBM preventative approaches and had a pro-vaccine orientation, while there was a higher proportion of commercial websites among those which promote non-EBM approaches. Although the JAMA score was lower in commercial websites, it did not correlate with the preventative approaches suggested or the orientation toward vaccines. For each of the four search engine result pages (SERP), only one website displayed the health-of-the-net (HON) seal. In the SERP on vaccines, journalistic websites were the most abundant category and ranked higher than average in both languages. Analysis using natural language processing showed that journalistic websites were mostly reporting news about two specific topics (different in the two languages). While the ranking by Google favors EBM approaches and, in English, does not promote commercial websites, in both languages it gives a great advantage to news. Thus, the type of news published during the influenza season probably has a key importance in orienting the public opinion due to its high visibility. This raises important questions on the relationships between health IQ, trustworthiness, and newsworthiness.

**Keywords: internet, information quality, Google, websites, influenza, vaccine, prevention, information**

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# INTRODUCTION

## Prevention of Influenza

Influenza is a highly contagious viral respiratory disease with a significant burden on public health (1, 2). Influenza causes symptoms of fever, muscle pain, headache, cough, and, in more vulnerable patients, it can cause exacerbations of respiratory disease, which can sometimes be life threatening (1). There are not many effective treatment options available, so interventions focus on prevention, with a number of interventions identified by the Cochrane Library to have a sufficient enough evidence base (1–4).

Vaccination is the most recommended preventative measure. Unlike other vaccines, the influenza vaccine provides a modest protection for adults, with children over the age of two showing far better benefits than adults (1, 2) and elderly people showing less benefit (1). Most government agencies often recommend vaccination only in populations at risk of developing serious complication from influenza, such as the over 65 age group, people who are immunosuppressed, those who have underlying health conditions such as cardiovascular or respiratory diseases, pregnant women, and healthcare workers.

Hand washing is also effective in preventing the spread of influenza; this was again particularly true for children (3). Surgical masks as physical barriers were also found to be an effective preventative intervention (3). Antiviral drugs such as Tamiflu can have a prophylactic effect (4, 5), although possible side effects and the obligation to not medicate unnecessarily make this intervention something that should only be considered by those at high risk of infection.

Vitamin C supplementation is also popular as a preventative treatment for influenza, based on publications by Linus Pauling in the 70s (6). A Cochrane review on vitamin C in the prevention of common cold, which is caused by respiratory viruses including influenza, could not find evidence of a reduced incidence of the disease although there was a reduction in its duration (7). There is also no evidence for a preventative effect on pneumonia, another complication of influenza (8).

Other interventions such as certain homeopathic remedies and increased fluid intake showed no evidence of preventative benefits (9, 10).

## Quality of Health Information and Impact on Public Health

Statistics show that up to 59% of the total adult population has at some point searched for health information online, with this proportion set to continually rise (11). Patients can become quite reliant on this ease of access, choosing to refer to the Internet whenever they have a query, with a large number eventually making a decision based on their search (12). This is further helped by the anonymous nature of the Internet, which provides a certain degree of confidentiality to the patient. Thus, the Internet has allowed patients to take a more dynamic role when seeking health information and maintaining their own health (11).

While the benefits of the Internet are clear, there are numerous concerns regarding the quality of information found online and the reliability of sources available. The Internet remains a largely unregulated entity, and while tightly regulated websites do exist, any individual can set up a website and broadcast information that is potentially unreliable or inaccurate, which may harm patients or professionals who choose to act on that information (12, 13).

The information that can be retrieved using search engines is varied and it is not just made of "standalone" websites. In fact, most of the websites in a typical search engine result page (SERP) are not independent sources of information, but are owned by magazines, newspapers, or TV news channels, and just reproduce what is reported by other types or media. A significant proportion of websites are government health agencies, patient advocacy groups, or pharmaceutical companies, posting on their websites information that is also available in their printed brochures. What is important in the use of a search engine is the proportion of these different types of websites returned in the SERP as well as their ranking, because most users will often look only at the first websites in the list (14, 15).

## Assessing Health IQ

The aim of this study was to assess the quality of the information returned by Google on the prevention of influenza in English and in Italian.

Information quality (IQ) has several dimensions (16, 17). Some of them, such as accessibility or readability (e.g., it is written in a clear language or can be accessed without a paywall), are difficult to transfer some of these concept to health information. We analyzed two dimension of IQ that seems more directly relevant. The first is the trustworthiness as defined by the Journal of the American Medical Association (JAMA) benchmarks, a tool to help users seeking to evaluate the reliability of a website (18). The benchmark consists of four criteria: authorship, attribution (referenced sources), currency (articles dated), and ownership disclosure (including financial interests). Websites that fulfill three or more of these criteria are deemed reliable and more likely to contain higher quality information (18). The tool has since become a widely established method for assessing quality when evaluating health information online. The second dimension is accuracy. Accuracy has been described when "the recorded value is in conformity with the actual value" (19), and is therefore equivalent to correctness (16). In our context, this could be interpreted as "scientifically sound" or "evidence-based." For instance, a website describing influenza as an infection by a virus would be accurate whereas one describing it as a disease due to malnutrition, rather than a virus, would be inaccurate.

One approach used to assess scientific accuracy is to have websites scored by medically trained reviewers (20). Evidence-based medicine (21) is now widely used by regulatory agencies to approve new drugs and as a rational approach in the identification of effective treatments to be reimbursed by health insurance systems or offered by public health services. In a previous study, using websites on migraine as an example, we have used as an indicator of scientific accuracy whether or not websites were pointing the reader toward treatment options that were evidence based (22).

In this study, we have followed a similar methodology, classifying the type of websites returned by a SERP on "preventing

the flu" in terms of class of website (commercial, government agencies, news, etc.) and type of intervention described (vaccine, approved drugs, complementary-alternative medicine, nutritional approach, lifestyle, hygiene). The websites were also scored for each of the four components of the JAMA criteria (authorship, attribution, currency, disclosure). We searched Google for the expression "preventing flu" (the layperson expression for influenza in English) or the Italian equivalent "prevenzione influenza." We analyzed the first 200 websites listed in the SERP to obtain a representative sample of the information available on the web. Because most people will only look at the first 10 websites or less (14, 15), we also performed a subgroup analysis on the top 10 results in the Google SERP.

Finally, we extended the study to the information on influenza vaccine, analyzing the first 200 websites returned by a search on "flu jab" (English) or "vaccino antinfluenzale" (Italian) and scoring them as above except that, instead of intervention type, we classified them according to their stance on vaccination: pro, anti, or neutral.

We then performed a hierarchical cluster analysis to identify website patterns and used natural language processing (NLP) software to identify key new topics.

The results of the analysis illustrate the different behavior of the differences in IQ across the different classes of websites and the impact of the news on the health information found through Google.

## MATERIALS AND METHODS

A search was conducted in December 2014 for information relating to the prevention of influenza infection using the Mozilla Firefox web browser and the first 200 search results were downloaded onto a spreadsheet using the SEOquake software toolset (Semrush Inc., Trevose, PA, USA).

The Google[1] search engine was chosen due to its popularity among web users. The search was carried out using the "private mode" function of the browser, as this prevented the possible interference of past searches on current results, although we are aware that the IP address revealed our (UK) location within the University campus and that may influence the results obtained. The phrase "preventing the flu" was selected as the search input as it was deemed an appropriate phrase likely to be used by the general public in British English. In fact, it has been shown that using technical language is more likely to return results of higher quality (23, 24). The search in Italian was done on Google, limiting the language of the results to Italian and using the search term "vaccino antinfluenzale." We did not take into consideration the websites labeled as "Google ads" that appear at the top of the SERP.

The websites were then visited individually by two researchers and classified as described below. Whenever a website was classified differently by the two researchers, the website was revisited and discussed to reach a consensus. Websites that were not accessible since they were downloaded or that were accessible only via a paywall were excluded. Irrelevant websites (e.g., on swine flu, advertisement for conferences, polls, off topic) were also excluded. The spreadsheet with the list of websites and the way they were classified is available in Tables S1–S4 in Supplementary Material, to allow reader to reanalyze or reclassify according to different criteria.

### Classification of Websites

The workflow for the study is described in **Figure 1**. Websites were classified in accordance to their affiliation as government, commercial, journalistic, professional, portals, non-profit organizations, or others as indicated in **Table 1**. Although in previous studies, we did not classify governmental website separately, we felt it was useful to do so here because of the large number of such websites returned by Google for this topic. The inter-rater agreement between the two markers was 96% for the search on flu prevention in English, 90% for the search on the vaccine in English, 97% for prevention in Italian and 98% for the vaccine in Italian.

Websites on prevention of influenza, in both languages, were then analyzed, and any interventions recommended were recorded. These recommendations were assigned to one of the following "types of intervention" similarly to our previously study (22): vaccine (also known as "Flu jab"), approved drug, lifestyle, hygiene, supplements, nutrition, complementary and alternative medicine (CAM) herbal products, CAM practices, and devices. Only when an intervention was mentioned in a neutral or positive way the website was classified in that particular group (i.e., an anti-vaccine website that mentioned the vaccine only to

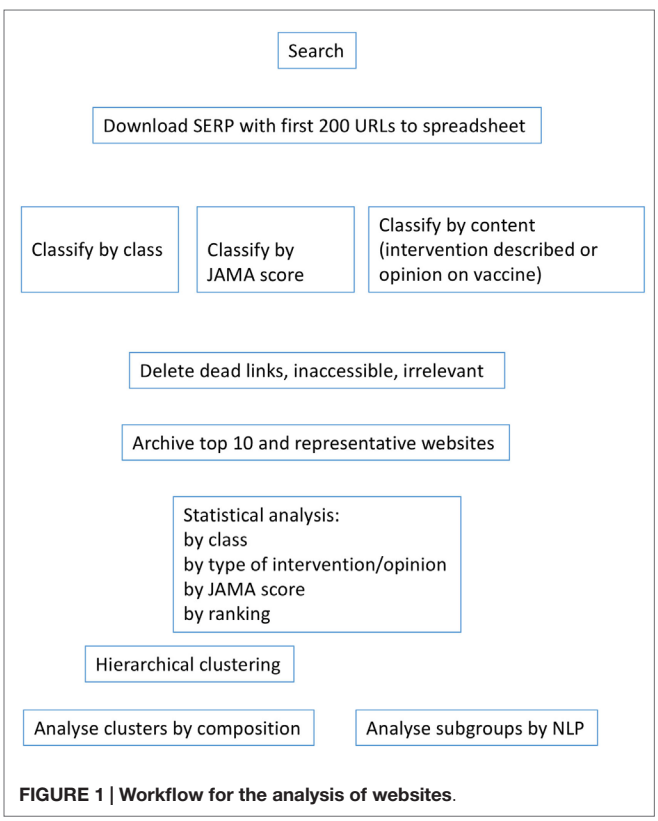


**FIGURE 1 | Workflow for the analysis of websites.**

[1] www.google.com

**TABLE 1 | Website categories, descriptions and examples.**

| Purpose | Description | Example |
|---|---|---|
| Government (G) | A website created, managed, and regulated by an official governmental body | cdc.gov<br>flu.gov<br>nhs.uk |
| Commercial (C) | A website specifically created for commercial purposes and the sale of products or services with the aim of making a profit | tamiflu.com<br>myprotein.com |
| Journalistic (J) | A website created for the distribution of news and information. Covers general news mediums as well as health related ones. Can also be for entertainment purposes | cnn.com<br>theguardian.com<br>menshealth.com |
| Professional (P) | A websites created by health professionals, experts, and professional organizations | mayoclinic.com<br>thecochranelibrary.com |
| Health Portal (HP) | A webpage with a search function that accesses information and articles from the site for a range of health topics | webmd.com<br>mcmasteroptimalaging.org |
| Non-profit Organization (NPO) | An organization with charitable/supportive/educational services that are not established for the purpose of profit | redcross.org<br>bhf.org.uk |
| Other (O) | Websites that do not fit the criteria for other categories, such as personal blogs and social networking sites | twitter.com<br>wikihow.com |

**TABLE 2 | Descriptions of types of preventative intervention.**

| Intervention | Examples |
|---|---|
| **Flu vaccine** | |
| Approved Drug | A pharmaceutical therapy that has been clinically approved by a regulatory agency (e.g., FDA, EMA, MHRA) for use in preventing influenza |
| Lifestyle | Lifestyle factors and changes. E.g., exercise, staying warm, and good sleep patterns |
| Hygiene | Washing hands, keeping distance from infected individuals |
| Supplements | Specific dietary additions that are usually taken in higher concentrations than what is normally found in food. E.g., vitamins, antioxidants, metals |
| Nutrition | Foods and meals |
| CAM Herbs | Herbal substances (e.g., Echinacea) |
| CAM Practices | Alternative medicine practices such as massages |
| Devices | E.g., facemasks |

recommend not to use it would not be classified as "vaccine"). **Table 2** details the criteria used for the classification.

Websites returned from the two specific searches on the vaccine (English and Italian) were also classified as positive (recommending the vaccination or reporting a recommendation), negative (recommending not to use the vaccine or specific anti-vaccine sites), or neutral (just reporting the opening hours to have a vaccination, news reporting of the efficacy/weakness of the vaccine or incidents attributed to vaccination).

Finally, each website was assigned a JAMA score as described previously (22, 25). Briefly, a score of 1 was assigned for each of the following four informations present in the webpage: (1) authorship (name of the author of the text); (2) attribution (references provided to back up statements); (3) currency (indication of the date of publication and/or update); and (4) disclosure (website describes ownership and commercial interests). These scores where then added up giving a JAMA score between 0 and 4.

If the information was not available on the initial website page, then the three-click rule was used. The three-click rule is an unofficial website navigation rule that suggests information should be accessible within three clicks (26) In previous studies, a website scoring a mean JAMA score of 3 or above has been suggested to be of high quality (27, 28).

## Statistical Analysis

The Kruskal–Wallis test was used for multiple comparisons of non-parametric variables, followed by Dunn's test, using GraphPad Prism software (GraphPad Prism Software Inc., La Jolla, USA). The Mann–Whitney test was used where there were two independent groups. When indicated, contingency tables were analyzed using a Chi-square test for non-parametric data. Hierarchical cluster analysis was performed using the Genesis software (University of Graz, Austria; version 1.7.6 for Mac OSX).

Because websites URLs are not permanent, to ensure that the reader will be able to see examples of the search results, the top 20 URLs returned in each of the Google SERPs as well as examples of some types of websites were archived. For this purpose, we used WebCite®, an on-demand archiving system for web references and the archived URL is provided next to the original URL. For webpages that could not be archived, either because the link to the site in question was already dead or the website refused connections by crawling robots, the archive link is indicated as not available (n/a).

## Natural Language Processing

Four corpora were created from the lists of URLs in each SERP using WebBootCaT, an online tool for bootstrapping text corpora from Internet, and analysis of natural language was performed using the corpus analysis software Sketch Engine[2] (29). Bi-gram and tri-gram (repeated 2 and 3 word sequences in the text) frequency lists were compiled for each corpus, with obvious words (vaccine or influenza and their derivate in both languages, and common words, also known as "stop" words) not considered.

# RESULTS

## Searching Google for Influenza Prevention

**Figure 2** shows the types of websites returned by Google when searching for influenza prevention in English or Italian. For each search, we analyzed the overall SERP (blue bars) or the top 10 websites only (orange bars). For the search on prevention in English, the most represented class of websites were journalistic

[2]www.sketchengine.co.uk

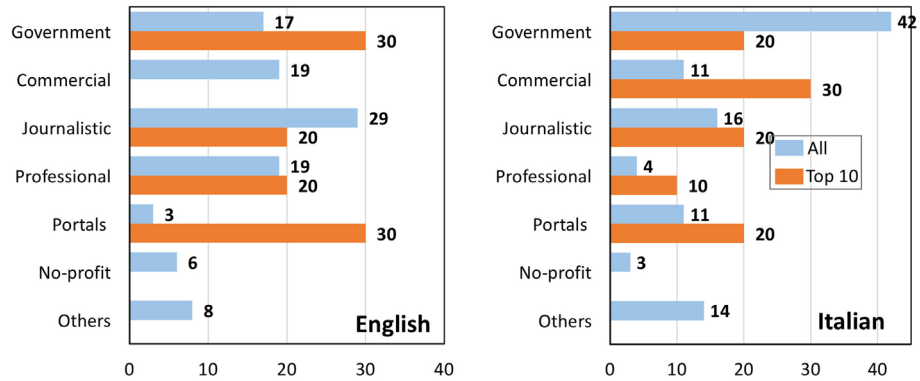


**FIGURE 2 | Percentage of different classes of websites from a search on influenza prevention in English (left) or Italian (right)**. Blue bars, all websites; orange bars, top 10 results returned by Google.

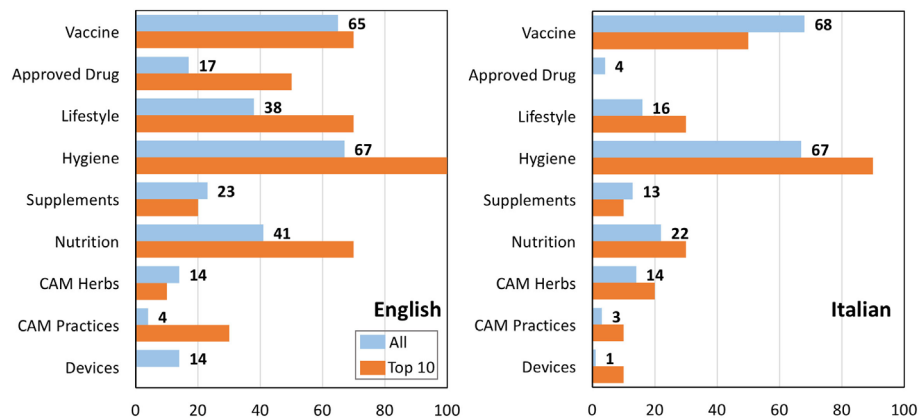


**FIGURE 3 | Indication provided by various websites in a search on influenza prevention in English (left) or Italian (right)**. Data are expressed as the percentage of websites describing specific interventions (the total is >100% as many websites described more than one preventative approach). Blue bars, all websites; orange bars, top 10 results returned by Google.

(29%), followed by professional, commercial, and government (all around 18%). However, if we look at the top 10 websites in both searches, there are no commercial websites and government websites are prevalent, along with portals (each being 30% in top-10). It should be noted that the high percentage of portals is due to the presence of three www.webmd.com pages. The distribution of the websites in Italian was very different from that in English. Government websites are by far the prevalent ones, with 42% (compared with 17% in English), followed by journalistic websites with 16%. Another striking difference in the search in Italian was that 3 commercial websites were present in the first 10 hits returned by Google, three times their frequency in the total of the 163 websites ($P = 0.03$ by one-tailed Chi-square test). Of these three websites, one was a company selling CAM products, supplements, and books on alternative medicine[3]; one was selling appointments with a naturopathic doctor, a "mini-market bio" and online sale of CAM herbs[4]; the third was a website from Vicks, a company selling over-the-counter products for cough and cold.[5]

An analysis of the indication provided by website on influenza prevention is shown in **Figure 3**. In both languages, the majority of the websites pointed at vaccination and hygiene (65–68%), two evidence-based approaches. Supplements or CAM herbs occurred less frequently, while two other non-evidence-based medicine (EBM) approaches, nutrition and lifestyle, were present in higher percentage in English when compared to Italian. There was no evidence for any of the prevention approaches being differentially represented in the top 10 searches.

[3]http://www.guna.it/prevenzione-invernale (archived in: http://www.webcitation.org/6Z9XJAxUH)

[4]http://www.lucaavoledo.it/2011/10/prevenzione-naturale-di-influenza.html (archived in: http://www.webcitation.org/6Z9Xgb9Mr)

[5]http://www.vicks.it/saperne-di-piu/articoli-prevenzione/articoli/raffreddore-influenza-bambini/ (archived in: http://www.webcitation.org/6Z9a82Vfi)

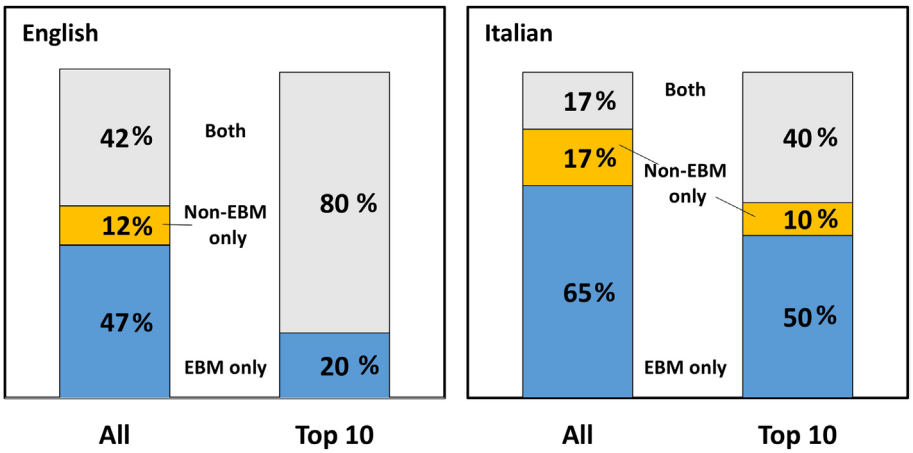


**FIGURE 4 | Preventative approaches**. Percentage of websites on influenza prevention describing only EBM approaches (blue), only non-EBM (yellow) or both (gray) in English (left) or Italian (right). Both the whole search and the top 10 websites are shown.

We then attempted to analyze how many websites described only EBM-based preventative approaches (i.e., one or more of the following: vaccine, hygiene, approved drugs), only non-EBM (lifestyle, nutrition, CAM, supplements), or both. We did not include in this analysis the seven websites on devices as it was unclear which of these are EBM based. The results are shown in **Figure 4**. In the SERP in English, the majority of websites (almost 90%) described either only EBM, or both EBM and non-EBM approaches, and only 12% described only non-EBM ones. In the top 10 websites, 8 mentioned both EBM and non-EBM option and 2[6] only EBM approaches. Therefore, Google ranking promoted the websites describing both EBM and non-EBM approaches, as these were 40% in the overall search and 80% in the top 10 ($P < 0.05$ by a two-tailed Chi-square test).

In a search in Italian, also shown in **Figure 4**, the overall pattern was similar except that the number of websites describing only EBM approaches was significantly higher than in English (101/163 in Italian vs. 85/185 in English, $P = 0.002$ by Chi-square test). Looking at the top 10 results in Italian, 4 mentioned both EBM and non-EBM option, 5 only EBM and 1 only non-EBM options. The most striking difference between Italian and English top websites was that the ones in Italian were less "neutral" (or more "partisan") in that 60% (compared to 20% in English) only described one type of approach (EBM only or non-EBM only).

These differences can, at least in part, be explained by an analysis of the typologies of websites in the three categories shown in **Figure 5**. In English (left panel), the most striking finding was that non-EBM-only websites did not have any government website but had a significantly higher proportion of commercial websites that represented 62% of this group, vs. 13 and 14% only in the other two groups. ($P < 0.0001$ vs. both groups by two-tailed Chi-square). In Italian (also in **Figure 5**) the large number of government websites accounts for the high percentage of websites reporting EBM-only approaches noted in **Figure 4**. A full breakdown of the different preventative approaches by class of websites is provided in Figures S1 and S2 in Supplementary Material for English and Italian, respectively.

When we analyzed the JAMA score of the websites in English, there was no difference among the three subgroups of types of intervention (mean ± SD was: EBM-only, 1.8 ± 1.0; non-EBM-only, 1.7 ± 0.9; both, 1.8 ± 1.0). However, in Italian, EBM-only websites had a significantly higher JAMA score (3.1 ± 1.2, $n = 101$) than websites mentioning non-EBM only (1.7 ± 1.1, $n = 27$) or both (1.8 ± 0.9, $n = 35$); $P < 0.0001$ comparing EBM only with any of the other two groups.

In order to be able to visualize patterns, we performed a cluster analysis of the various websites according to the preventative intervention they describe (**Figure 6**). In English (**Figure 6**, left), we evidenced four patterns. The first cluster ("a") is made by 22 websites pointing exclusively at the vaccine. These were mainly journalistic sites (45%), followed by professional (18%), commercial (14%), government, and others (9%). Two clusters ("b" and "c") are noticeable as they do not mention vaccines at all. In cluster "b," of the 18 websites, 61% are commercial, and 33% journalistic. In this cluster, vaccination is not mentioned in favor of supplements, nutrition, and complementary medicine. This confirms the earlier conclusion that commercial websites are the less likely to point to vaccination as an option. Cluster "d" also does not recommend vaccines but favors mostly hygiene as a preventative approach. In this cluster, most of the 21 websites are also commercial (29%) followed by professional (22%), journalism (21%), and government (17%).

A cluster analysis of Italian websites on prevention (**Figure 6**, right) identified two major EBM-only clusters. The first (cluster "a"), recommending both the vaccine and hygiene measures, included 70 websites, of which 81% were government websites and none was a commercial website. Cluster "b" (vaccination only) had 19 websites of which

[6]www.cdc.gov and www.mayoclinic.org

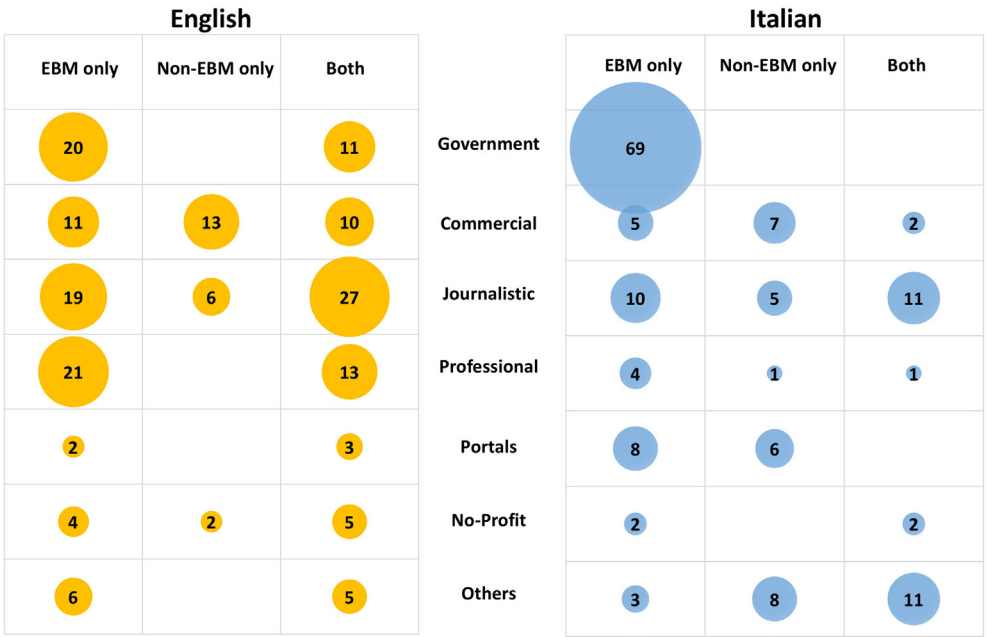


**FIGURE 5 | Preventative approaches described by different types of websites**. Number of websites in different classes describing only EBM, only non-EBM, or both preventative approaches. Left panel, English; right panel, Italian.

37% were government ones, confirming that these websites preferentially mention both main EBM preventative measures, hygiene, and vaccination.

## Searching Google for Influenza Vaccine

**Figure 7** shows the types of websites returned by Google when searching for influenza vaccine in English or Italian. For each search, we analyzed the overall SERP (blue bars) or the top 10 websites only (orange bars). It can be seen that journalistic websites are the most numerous (30% in English, 45% in Italian) and have a high visibility in the top 10 results for both languages. Government websites follow with 23–27%.

When these websites were analyzed for the recommendations made, either pro-vaccine, anti-vaccine, or neutral, the majority of websites in both languages were pro-vaccination or gave a neutral information, the latter prevailing in Italian websites (**Figure 8**). A similar distribution was observed in the top 10 results with the exception that, in Italian, an anti-vaccine website made it to the top 10. This was an article by a vaccine-skeptic doctor published in the online comments section of the newspaper "il fatto quotidiano."[7] As shown in **Figure 9**, a government affiliation was associated mainly with pro-vaccine statements, with a few giving neutral information (such as opening hour of a surgery). Although there were few anti-vaccine websites in English, we can notice a significantly higher ($P = 0.04$) proportion of "other" websites (that include blogs and personal websites) (25% of the anti-vaccine group vs. 8% in the overall SERP). The other striking observation, more evident in Italian, was the high proportion of journalistic websites providing information that we classified as "neutral."

While the proportion of journalistic websites was the same in the two searches in English (30%, both for prevention and the vaccine search – **Figures 1** and **7**), in Italian, they were significantly ($P < 0.0001$) more frequent in the search of the vaccine (45%) than in that on prevention (16%).

When we analyzed the JAMA score from the websites returned from the vaccine search according to class of websites, there was a trend, in both languages, for anti-vaccine websites to score higher than pro-vaccine websites, but this was not statistically significant (English JAMA scores were: pro-vaccine, 2.3 ± 1.3; anti-vaccine, 3.1 ± 1.2; neutral, 2.8 ± 1.3. Italian JAMA scores were: pro-vaccine 2.2 ± 1.1, anti-vaccine, 2.5 ± 1.4; neutral, 2.2 ± 0.8). When the JAMA score for all four searches was analyzed by class of websites (Tables S5 and S6 in Supplementary Material), in both languages, commercial websites had a significantly lower JAMA score, a pattern that had been observed in previous studies (22, 25). Journalism websites, on the contrary, had a significantly higher score, which was also found previously (22).

## Impact of News on the Websites Returned by Google

Because the results reported above show a large proportion of journalistic websites and their high ranking in a Google search on influenza vaccine, we used a NLP technique to analyze the two corpora on journalistic websites returned in both languages. In English, a corpus analysis of the 50 journalistic websites revealed

[7] http://www.ilfattoquotidiano.it/2014/12/09/vaccino-antinfluenzale-dati-mortalita-fede/1258733/ (archived in: http://www.webcitation.org/6Z8VM2Ipp)

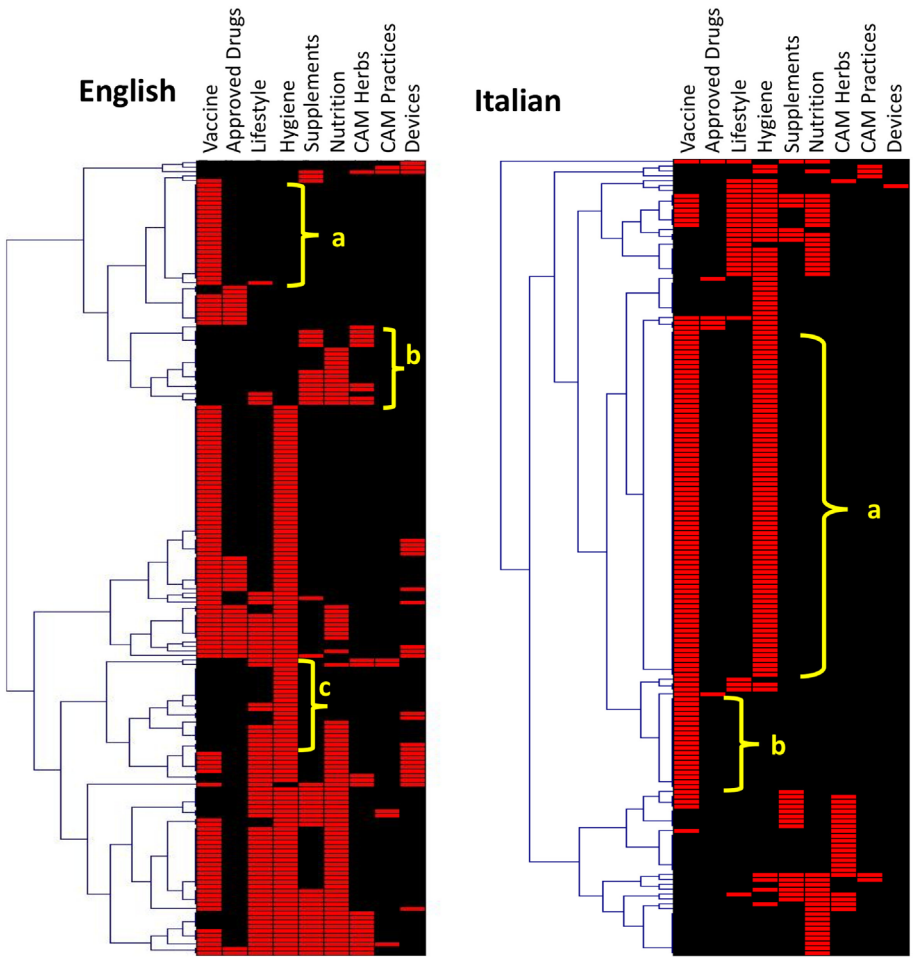


**FIGURE 6 | Hierarchical cluster analysis of the websites on influenza prevention returned by Google**. Type of preventative intervention mentioned by websites on influenza prevention in English (left) or Italian (right). The clusters indicated are discussed in the text.

38 occurrences of the string "Public Health England" and 15 of "Center for Disease Control" (full frequency list of tri-grams is shown in Data Sheet S2 in Supplementary Material, sheet 1). A similar search in the whole corpus represented by the search on "flu jab" revealed many more occurrences of these strings. Analyzing the context where this expression was used (full concordance for the string "Public Health England is shown in Data Sheet S2 in Supplementary Material, sheet 2) and further manual scrutiny revealed many websites were reporting the same news, namely, the very low efficacy of the vaccine in the 2014/2015 season. Thirteen were based on a press release from Public Health England reporting that the vaccine used in the UK was only 3% effective (5–6 January 2015). Similar news was published in various English speaking countries. Four (5–10 December 2014, one journalistic website, two portals, one blog an anti-vaccine no profit website) were based on a news release from the Center for Disease Control reporting a low efficacy of the vaccine. Five such journalistic websites were in the top 10 results of the Google SERP.

We then analyzed the 83 journalistic websites from the search on the vaccine in Italian. Calculating the ngram frequencies in this corpus, and eliminating common words and all those derived from the words vaccine, vaccination, and influenza, the second bi-gram in the list was "morti sospette" (suspicious deaths), occurring 78 times in this corpus (full word list of bi-grams is shown in Data Sheet S2 in Supplementary Material, sheet 3). Analyzing the context where this expression was used (see the concordance in supplementary material; Data Sheet S2 in Supplementary Material, sheet 4), it was clear that this referred to a series of news articles reporting the news that the influenza vaccine could have caused the death of up to 13 elderly people, a connection then ruled out by the Italian and European public health authorities. Two such news websites were in the top 10 websites, one from the magazine "Altroconsumo" and one from the financial newspaper "il Sole 24 Ore."[8]

[8] http://www.altroconsumo.it/salute/influenza/news/vaccino-antinfluenzale-ritirato-fluad-novartis (archived in: http://www.webcitation.org/6Z8YgS4gz); http://www.ilsole24ore.com/art/notizie/2014-11-29/vaccini-antinfluenzali-morti-sospette-salgono-12-ministro-lorenzin-primi-test-negativi-220813.shtml?uuid=ABiFgtJC (archived in: http://www.webcitation.org/6bjY7VCjs).

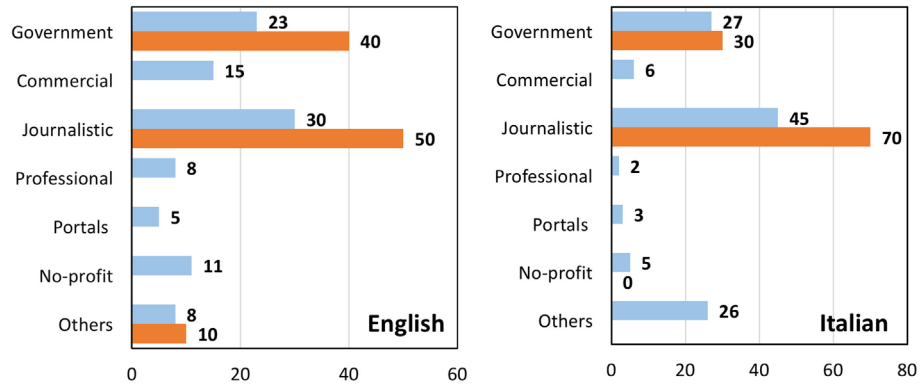


**FIGURE 7 | Percentage of different classes of websites from a search on influenza vaccine in English (left) or Italian (right)**. Blue bars, all websites; orange bars, top 10 results returned by Google.

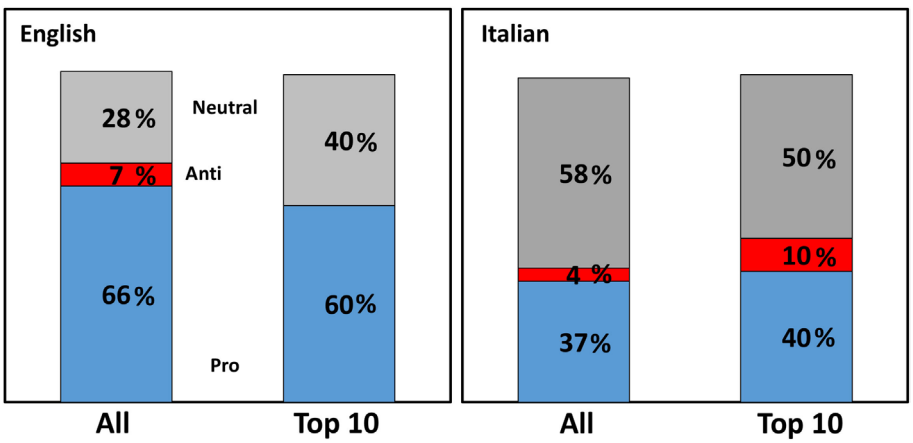


**FIGURE 8 | Orientation of websites from a search on influenza vaccine**. Percentage of websites on influenza vaccine having a pro-vaccine (blue), anti-vaccine (red), or neutral (gray) orientation, in English (left) or Italian (right). Both the whole search and the top 10 websites are shown.

# DISCUSSION

## Typology of Websites

The present study analyzed four SERPs with 200 websites, each on the prevention of influenza or its vaccine. The relatively large number of websites provides a good sample for the analysis of the composition of the SERP in terms of typology of websites and their orientation. In addition, we looked at the composition of the first ten websites and compared them with that of the whole 200-websites SERP to gain information on the visibility of websites. We are aware that this is a sort of reverse engineered search engine optimization, because the algorithm used by Google is not public. However, website visibility is important because users, in most cases, do not go beyond the first results in the SERP (14, 15).

We found that, at least in English, contrary to a common prejudice that Google might privilege market-oriented or promotional sources, commercial websites are ranking low and are not present in the top 10 websites in either the SERP on influenza prevention or that on the vaccine. This is in agreement with findings from our previous studies on IQ on two Google searches on diabetic neuropathy or migraine where commercial websites, that accounted for 20 or 30% of the 200-website SERP, respectively, were absent in the top 10 results (22, 25). The low ranking of commercial websites is aligned with users' behavior as studies on trust in online advice have shown that, both in financial or health matters, trust is negatively influenced by the perception of commercial intentions (30, 31). This was not true in Italian where three commercial websites were in the top 10.

## Orientation of Websites

Another concern often voiced in studies on health IQ is that search engines could give high visibility to non-scientific websites or point users to low-quality information. Google ranks websites by multiple criteria and it is not clear how much of the content is taken into account. We used the type of intervention suggested as an indicator as to whether the information provided was in

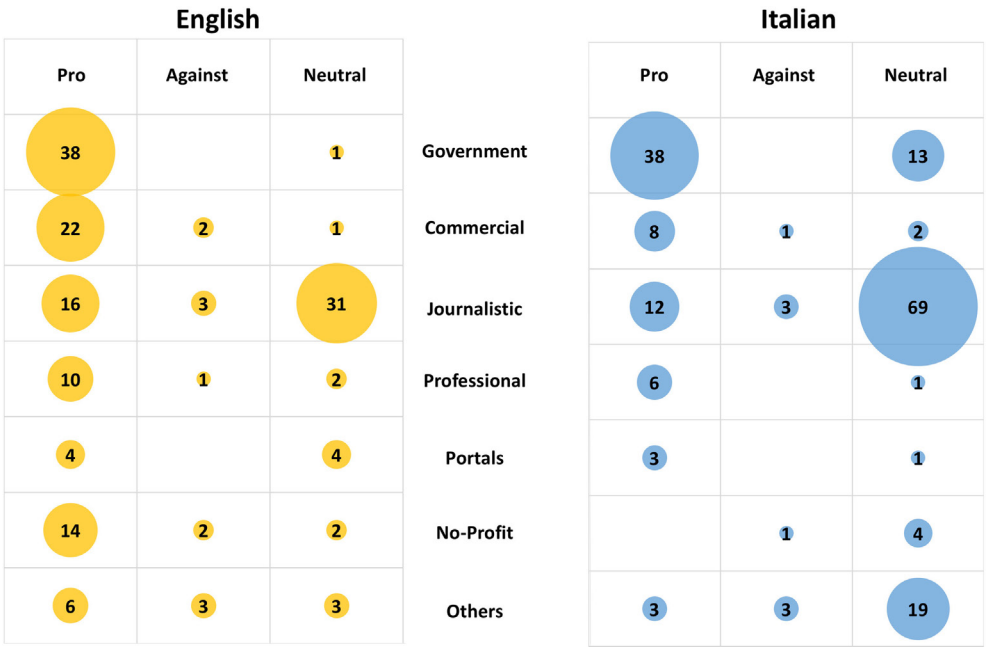


**FIGURE 9 | Orientation on influenza vaccine by different types of websites**. Number of websites in different classes having a pro-vaccine, anti-vaccine, or neutral orientation. Left panel, English; right panel, Italian.

accordance with the principles of EBM. For this, we relied on the conservative indications provided by the Cochrane collaboration or the UK National Institute for Health and Care Excellence. We are aware of the potential problems and the fact that this could underestimate the potential of some treatments, but these organizations are often seen as setting the "gold standard."

Google gives a fair balance of websites favoring EBM-informed approaches. This is particularly evident in English where the non-EBM-only prevention website ranking highest was 39$^{th}$, although from a different perspective, the fact that 80% of the top 10 websites described both EBM- and non-EBM approaches, which could be a problem if people thought it would be easier to eat tomatoes rather than taking the vaccine or washing their hands. In Italian, the trend was similar with 17% of websites pointing to non-EBM-only preventative approaches, but here the first such websites ranked 6th and the next 12$^{th}$, and thus had better visibility.

Likewise, in English, anti-vaccination websites do not show in the first 10 results, the first anti-vaccine website ranking 62nd. However, in Italian, although there were less anti-vaccine websites, one such website was 1st in the ranking and the next one 33rd.

In general, in Italian, non-EBM-only websites and anti-vaccine websites are more likely to rank higher than in a search in English, as summarized in **Figure 10**. The reason for this is not clear because the Google ranking is based on an automated evaluation of IQ by some intrinsic characteristics of websites, without an assessment of its content, such as readability, structure, links, and the same considerations we made above on commercial websites in Italian apply.

It should also be noted that our observation that anti-vaccine websites in English are ranked low by Google is in contrast with previous reports that 2–10 of the top ten websites resulting from a search on "vaccination" or "immunization" were anti-vaccine websites (32, 33). One explanation could be that those studies were on searches on vaccination in general, and most of the anti-vaccine websites focus on those vaccines, such as the MMR vaccine, which are made compulsory in many countries, while the influenza vaccine is only recommended to populations at risk, and therefore, raises less opposition.

It is also interesting that commercial websites are frequently recommending non-EBM treatments, but this was probably to be expected as pharmaceutical companies do not sell or advertise their drugs on the internet but rely on prescriptions or doctors' recommendation. Likewise, the generally pro-vaccine information of government websites is not surprising as many of these websites were in fact set up to promote a vaccination campaign. This seems to be an effective measure as a Canadian study on 250 pregnant women found that those who relied on government agencies' websites were more likely to be vaccinated than those relying on websites from mainstream media (34).

## Indicators of Trustworthiness

The analysis of the JAMA score indicated that this is not predictive of whether a website is describing EBP-approaches or not (except in Italian), or whether they are pro- or anti-vaccine. On the other hand, we confirmed our earlier observations that commercial websites have a lower JAMA score (22, 25). It is interesting to hypothesize that the lack of some quality indicators

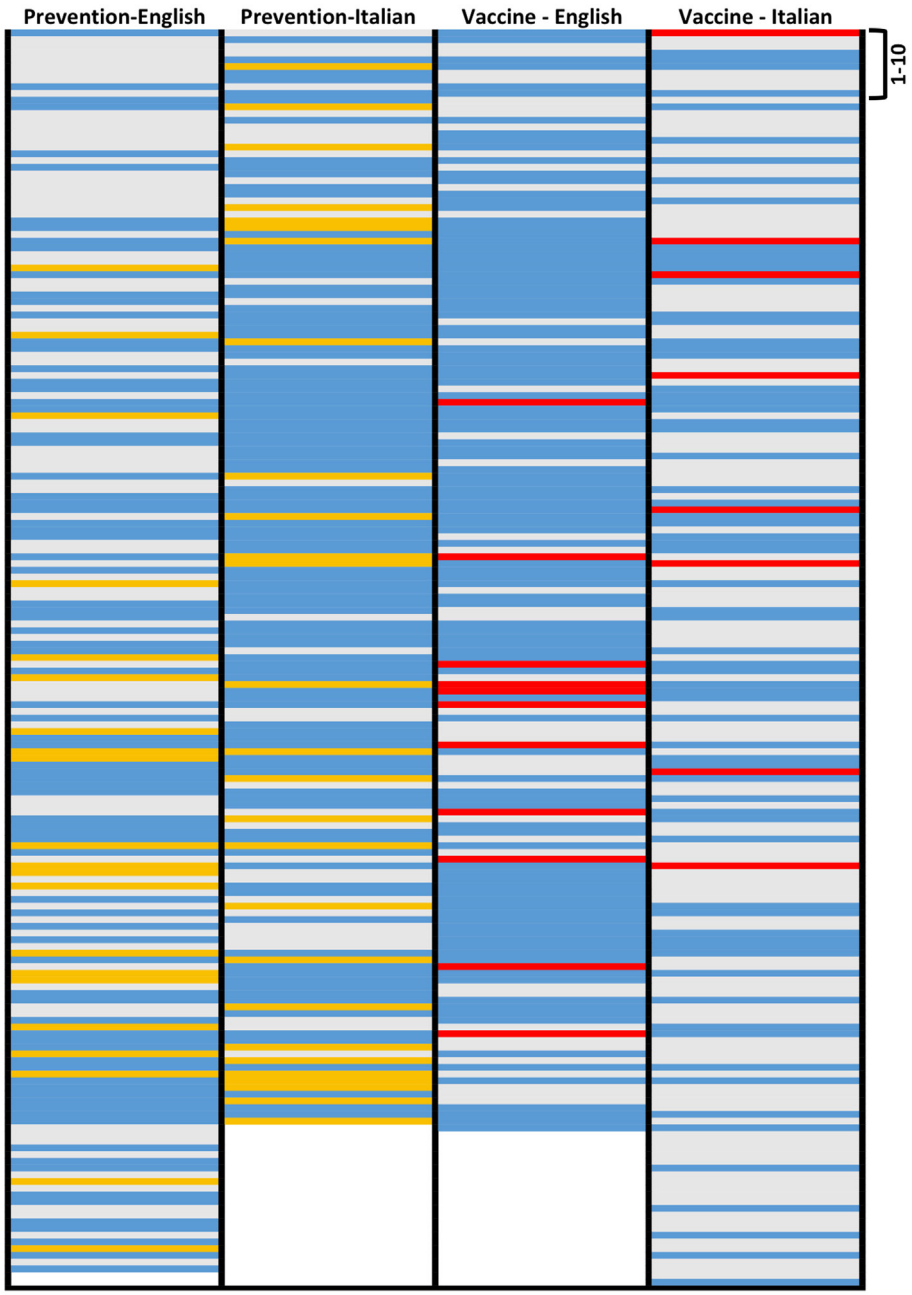


**FIGURE 10 | Graphical representation of the ranking of websites with different orientation in English and Italian**. Left two columns (search on influenza prevention): only EBM approaches, blue; only non-EBM, yellow; both, gray. Right columns (search on vaccine): pro-vaccine, blue; anti-vaccine, red; neutral, gray.

of a document, such as date, author, references – all components of the JAMA score, may contribute to the low ranking of commercial websites by Google.

Another established criterion for health IQ is having the website approved by the Health-On-The-Net foundation and displaying the HON seal of approval. However, crawling the websites of our four searches using NLP software we searched for text references to the HON seal, and we only found 4 websites containing text references to HONcode (one for each of the four SERPs) l.[9] Probably the fact that the HON seal is something that a webmasters need to apply for and has a cost, which is not clarified

[9]Prevention in English, http://www.everydayhealth.com/cold-and-flu-pictures/ways-to-prevent-colds-and-flu.aspx; prevention in Italian, http://www.abcsalute.it/blog/influenza-americana-inverno-2014-cose-e-come-curarla/; vaccine in English, http://www.everydayhealth.com/drugs/influenza-virus-vaccine-live-trivalent-http://www.webcitation.org/6buN57wQn; vaccine in Italian, http://www.farmacoecura.it/influenza/influenza-2008-2009-vaccino-prevenzione/)

on the HON website, may limit its diffusion. In addition, at list in our search, it is not displayed by websites from government agencies, public healthcare services or Universities.

## News

The impact on news websites deserves more discussion. First of all, they give a more "balanced" view and this is the class of websites that is more likely to describe both EBM and non-EBM approaches, in both languages (**Figure 5**) and to describe the influenza vaccine in a "neutral" way (with the caveat discussed below). This has also been noted in a Dutch qualitative study similarly reported that news websites are more objective and non-judgmental while a critical view of vaccination was more common in social media (35). On the other hand, journalism websites are not very well represented among those recommending the vaccine when searching for preventative strategies.

The textual analysis of the journalistic websites shows that, for the past winter, they all gave the same type of news, different in the two languages. This is probably due to the fact that newspapers are nowadays understaffed and tend to just re-post press releases rather than having articles written by their own scientific journalists. English websites mainly reported data from government agencies describing the low efficacy of that year's vaccine. Although most newspapers reminded the reader that, despite the concerns reported, the government still recommended vaccination for those at risk, the titles had a negative tone (Sky News: "flu jab found to work in just 3% of cases"[10]; Daily Mail, "Flu jab is a waste of time for 97% of patients"[11]).

The news websites in Italian were even more peculiar. On 28th of November, 2014, all main newspapers published in the front-page headline that three elderly people died within 2 days from having been vaccinated (e.g., il Corriere della Sera and il Giornale[12]) and panic ensued. The health authorities suspended two batches of vaccine and the number of "suspicious deaths" reached 13, as reported in the front page of il Sole 24 Ore.[13] The emergency did not last long and a week later newspapers reported (with much less emphasis) that the health authorities have concluded that those deaths were not associated with the vaccine, which was confirmed on 3 December 2014 by the Pharmacovigilance Risk Assessment Committee of the European Medicines Agency (EMA).[14]

As early as the 29th of November, Dr. Cirielli, president of the Società Italiana di Medicina Generale (Simg) noted that every day in Italy 1,600 over-65s die; as the uptake rate for the influenza vaccine in that population is over 50%, 400 people die every day "after having been vaccinated."[15] Thus, it would require a highly powered study to attribute three deaths to the vaccination.

It is nuclear what have sensitized the public opinion to report those deaths. One interesting coincidence is that three days earlier, the 25th of November, most newspapers reported that judge Di Leo of the Tribunale del Lavoro di Milano (employment tribunal), based on an 18-page report from forensic doctor Alberto Tornatore, concluded that a hexavalent vaccine (against, among others, *Haemophilus influenzae*) had probably caused autism in a child and sentenced the Italian government to pay monthly compensation.[16]

Thus, in each language, a language-specific "bad news" made up a very significant portion of the journalistic websites on the influenza vaccine. There was a small overlap between the two SERP on vaccine in that two Italian websites reported the data of low efficacy released by the CDC[17] and two websites in English reported the news of the suspected accident in Italy.[18]

Many studies have investigated the quality of health information available on the web, often with a preconception that either the uncontrolled nature of the web will allow posting of low quality information, misinformation or disinformation. In this perspective, the layperson not being able to discriminate between low-quality and high-quality sites, there is a concern that the use of the Internet by the patient may cause harm by promoting potentially unsafe treatments (36), although others found little evidence of this in the medical literature (37).

# CONCLUSION AND LIMITATIONS

In drawing conclusions, one should be aware of the limitation of this study. Search results vary with time; different search terms or health topics will give different results; searching from a different location (as defined by the IP address) will give different results. Although some findings were in agreement with previous studies from our group performed in the last three years across different searches (such as commercial websites ranking lower and having a lower JAMA score), other findings may not be generalized.

The other finding that would be worth confirming further is that most websites returned are not "standalone websites" but are in fact documents and publications from professional organizations,

[10] http://news.sky.com/story/1422308/flu-jab-found-to-work-in-just-3-percent-of-cases (archived in: http://www.webcitation.org/6bvdaI4M9)

[11] http://www.dailymail.co.uk/news/article-2941896/Flu-jab-waste-time-97-patients-Vaccine-developed-year-ago-no-longer-matches-virus-mutated-much.html (archived in: http://www.webcitation.org/6Z9hZ28b2

[12] http://archiviostorico.corriere.it/2014/novembre/28/Vaccini_morti_sospette_caos_co_0_20141128_e7fda2a2-76d2-11e4-b904-b99b9498524a.shtml (archived in: http://www.webcitation.org/6bsuHeVY2); http://www.ilgiornale.it/news/politica/serie-morti-vaccino-panico-regioni-nel-caos-1071444.html (archived in: http://www.webcitation.org/6bsugIYSo)

[13] http://www.ilsole24ore.com/art/notizie/2014-11-29/vaccini-antinfluenzali-morti-sospette-salgono-12-ministro-lorenzin-primi-test-negativi-220813.shtml?uuid=AbiFgtJC (archived in: http://www.webcitation.org/6bsusggIm)

[14] http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2014/12/news_detail_002228.jsp&mid=WC0b01ac058004d5c1; http://www.webcitation.org/6bu31e9mg (archived in: http://www.webcitation.org/6bvfp65Vq)

[15] http://www.quotidianosanita.it/scienza-e-farmaci/articolo.php?articolo_id=24648; archived in: http://www.webcitation.org/6bu3d4t4R

[16] http://www.repubblica.it/salute/medicina/2014/11/25/news/il_tribunale_bimbo_autistico_per_colpa_del_vaccino-101357013/ (archived in: http://www.webcitation.org/6bswRLubo)

[17] http://www.assis.it/cdc-questanno-il-vaccino-antinfluenzale-e-pressoche-inefficace/ (archived in: http://www.webcitation.org/6bssq8yTV); http://www.terranuova.it/Medicina-Naturale/Vaccino-antinfluenzale-ora-si-scopre-che-e-anche-inefficace (archived in: http://www.webcitation.org/6bstEgxJV)

[18] http://time.com/3610872/italy-flu-vaccine-deaths/ (archived in: http://www.webcitation.org/6bstODqvC); http://blogs.wsj.com/pharmalot/2014/12/01/italy-investigates-novartis-flu-vaccine-after-12-deaths-are-reported/ (archived in: http://www.webcitation.org/6bstTpbD1)

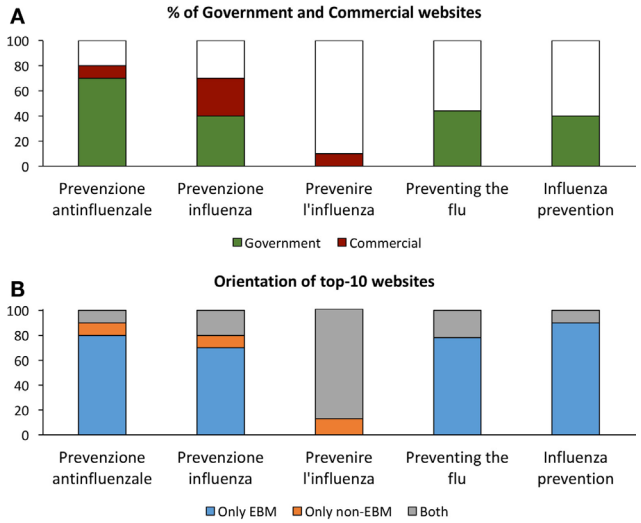


**FIGURE 11 | Percentage of government and commercial websites and their orientation in the top 10 results searching different terms.** Searches for the indicated terms, the first three in Italian, the last two in English, were performed on 15/11/2015 and the first 10 fits analyzed. **(A)** Percentage of commercial or government websites. **(B)** Percentage of websites on prevention describing only EBM approaches (blue), only non-EBM (yellow), or both (gray) in Italian (left) or English (right).

newspapers, government agencies, and commercial companies. If this was generalized, then the idea is that the Internet is a source of uncontrolled information where anyone can make any statement.

Another important finding was that, for relatively hot topics, Google returns a high proportion of journalistic websites from TV channels or print journals. This raises the interesting theoretical point on whether "newsworthiness" should be considered an indicator of IQ like trustworthiness or if it is a confounder, as well as the role of journalist as the gatekeeper. When we discuss health IQ, we think of information as "knowledge obtained from investigation, study, or instruction" but the word also defines "intelligence or news" (Merriam-Webster Online Dictionary). It is not clear whether the ways we assess IQ are the same for the two definitions of "information." Also, we do not know which ones of the top 10 websites a user will actually read and whether they will give the same weight to an article in the press or a different type of information. This would be an important follow up of this study.

It would be important to know whether the different pattern in Italian vs. English is observed with other search terms, and to what extent this happens with other non-English languages. It is also curious that only "bad news" received so much attention. An earlier study on news on medical research in the press had concluded that "Newspapers underreported randomized trials, emphasized bad news from observational studies, and ignored research from developing countries" (38).

As suggested in the peer review process, we performed a search on the prevention of influenza comparing different search terms. In English, we searched for "preventing the flu" and "influenza prevention"; in Italian for "prevenire l'influenza," "prevenzione influenza" and "prevenzione antinfluenzale" and analyzed the top 10 websites. We investigated: (1) the percentage of commercial websites and (2) the orientation (EBM vs. non-EBM). The results are shown in **Figure 11** (raw data are available in the Supplementary Material as Data Sheet S3 in Supplementary Material). Because this search was performed several months later, it cannot be compared to the ones above. However, the results confirm the main messages of the study, that is, that a search in Italian gives more visibility to commercial websites and to non-EBM websites than those in English. In fact, a search in Italian for "prevenire l'influenza" (which is a less formal language than without the other search terms without the article) returns no government websites and is more oriented toward non-EBM terminology as compared to the results with other search terms. This probably should suggest government agencies to ensure that informal language as it may well be that official government in Italian are written in a more technical language than their correspondent texts in English and should carefully consider this point when performing search engine optimization. The results also suggest performing future research on the impact of the language style on the quality of health information returned by Google.

Finally, it is important to monitor the impact of web health information on public health. The output of search engines can affect knowledge and attitude about vaccination significantly, (39) and thus might impact on the uptake of vaccination (40). It would be interesting to have a follow-up epidemiological study to assess how the health information available in the press has influenced the vaccine uptake in the next year.

## AUTHOR CONTRIBUTIONS

PG, AM: designed research, analysed the data, and wrote paper. RE: designed research, wrote paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2015.00616.

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