B Cell Responses to Influenza Infection and Vaccination

Christopher Chiu, Ali H. Ellebedy, Jens Wrammert and Rafi Ahmed

Abstract Although vaccines against influenza are widely available, control of the disease remains elusive. In part, this is due to the inability of current vaccines to induce durable, broadly protective immune responses. Prevention of influenza depends primarily on effective antibody responses that block virus entry. Following infection, high-affinity IgA antibodies are generated in the respiratory tract that lead to immune exclusion, while IgG prevents systemic spread. These are effective and long-lasting but also exert immune pressure. Mutation of the antigenic determinants of influenza therefore rapidly leads to emergence of novel variants that evade previously generated protective responses. Not only do vaccines suffer from this strain-specific limitation, but also they are suboptimal in their ability to induce durable immunity. However, recent evidence has demonstrated the possibility of inducing broadly cross-reactive antibody responses. Further understanding of the ways in which high-titer, long-lived antibody responses directed against such cross-reactive epitopes can be induced would lead to the development of novel vaccines that may remove the requirement for recurrent vaccination.

C. Chiu
Centre for Respiratory Infection, National Heart and Lung Institute, Imperial College London, London W2 1PG, UK

A.H. Ellebedy · R. Ahmed
Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA

A.H. Ellebedy · R. Ahmed (✉)
Department of Microbiology and Immunology, Emory University School of Medicine, G211, 1510 Clifton Road, Atlanta, GA 30322, USA
e-mail: rahmed@emory.edu

J. Wrammert
Department of Pediatric Infectious Disease, Department of Hematology and Oncology, Emory University School of Medicine, Atlanta, GA 30322, USA

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1 Introduction

Despite the widespread availability of influenza vaccines and their place in the vaccination programs of most high-income nations, control of influenza infection remains a major unresolved health issue. As well as the logistic difficulties associated with vaccine delivery and uptake, the fundamental biology of influenza virus raises hurdles that current vaccination strategies are unable to overcome. Furthermore, our understanding of the mechanisms underlying the generation of durable immunity is still incomplete. Until it becomes possible to manufacture vaccines that induce immune responses that match or even improve upon those following natural infection, it is likely that influenza will continue as one of the foremost causes of morbidity and mortality worldwide.

Protection from infection depends on the induction of effective immunological memory. While cellular immunity may have the capacity to clear influenza virus and reduce disease severity, B cells and antibodies are the major mechanism by which prevention of re-infection occurs. However, the propensity for influenza to escape via mutation under immune pressure means that this protection is short-lived and strain-specific. Current vaccines not only suffer from this limitation but also exhibit limited durability of protection even in the absence of strain variation. Annual re-vaccination is therefore required. However, recent evidence suggests that antibodies can be generated that have the capacity to recognize multiple influenza strains. More detailed understanding of how high-affinity long-lasting antibodies against influenza can be generated in the appropriate compartments and the ways in which broadly cross-reactive antibodies can be encouraged may assist in the development of the so-called universal influenza vaccine.

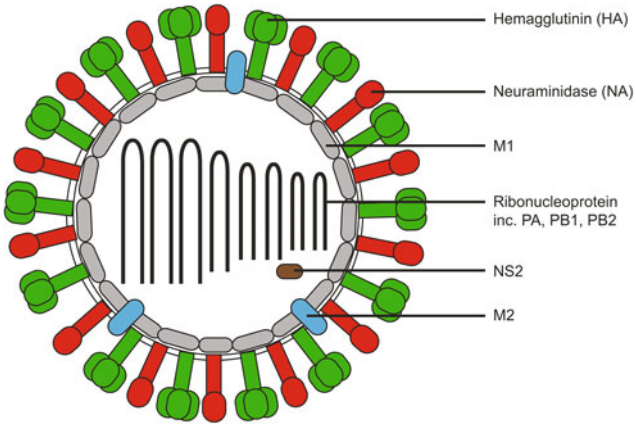

Fig. 1 Schematic of the influenza virion. Influenza is an enveloped RNA virus. Its major surface proteins are hemagglutinin (shown in *red*) and neuraminidase (*green*) with the M2 protein (*blue*) forming a transmembrane ion channel. The capsid is composed of the M1 protein (*gray*) and contains a few copies of NS2 (*brown*) and the eight segments of negative-sense RNA complexed as ribonucleoprotein with NP, PB1, PB2, and PA

2 Targets of Humoral Immunity in Influenza

Influenza belongs to the family *Orthomyxoviridae* and consists of an enveloped capsid containing an eight-segment RNA genome (Fig. 1) (Fields et al. 2007). Two major glycoproteins are expressed on the surface of influenza, hemagglutinin (HA), and neuraminidase (NA). Both are targets of humoral immunity. HA binds to sialic acid, facilitating viral attachment and entry following membrane fusion in the late endosome (Skehel and Wiley 2000). NA cleaves sialic acid and allows virus particles to escape the host cell in which they have been generated (Mitnaul et al. 2000). HA-specific antibodies are therefore capable of preventing infection of cells and are believed to be the primary method by which prevention of infection occurs.

The HA polypeptide, which complexes as a homotrimer, is composed of a globular head (HA1 domain) and a stalk (largely comprised of the HA2 domain) linked to the transmembrane region (Fig. 2). Most anti-HA antibodies are directed against the head and exert immune pressure so that viruses with mutations in this area capable of evading antibody recognition are selected. Over time, at least 18 influenza A HA subtypes have arisen, which are classified into two phylogenetic groups: group 1 includes subtypes expressing H1, H2, and H5, while group 2 contains H3 and H7. Similarly, there are 9 NA subtypes in influenza A, again falling in two groups. The NA head forms a homotetramer, and the stalk is joined to a short cytoplasmic tail via the hydrophobic transmembrane region.

Since RNA polymerase causes rapid mutation within both HA and NA, antigenic drift can quickly give rise to new strains, thus causing epidemics (Yewdell et al. 1979). In addition, the shuffling of the influenza genome allows wholesale re-assortment of its segments so that antigenic shift may occur. New strains may

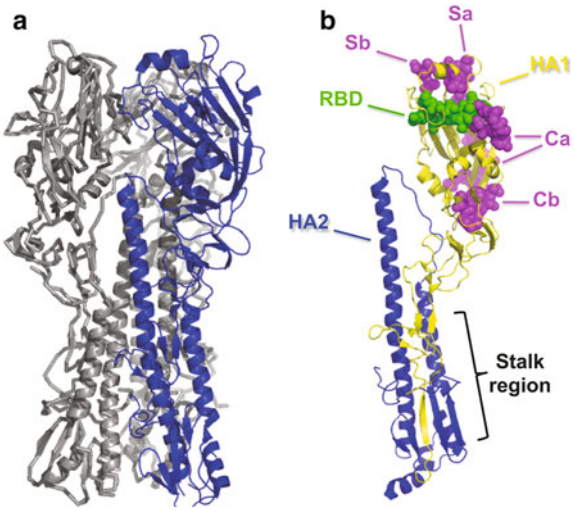

Fig. 2 A structural view of the H1 hemagglutinin from A/PR8/34 (H1N1). **a** Depiction of hemagglutinin as a trimer (with one monomer colored *blue*) and **b** the monomer. Each monomer is composed of two subunits: HA1 (*yellow*) and HA2 (*blue*). Within the HA1 subunit, well-defined neutralizing epitopes within the globular head region (Sa, Sb, Ca, and Cb) are shown in magenta (Caton et al. 1982). The receptor-binding domain (*RBD*) is shown in *green*. The HA stalk domain is formed by the N- and C-terminal domains of HA1 plus the ectodomain part of HA2

therefore arise expressing novel HA and NA variants to which existing antibodies are unable to react on a population level, thus giving rise to influenza pandemics. The humoral response to influenza in most individuals is therefore characterized by a complex mixture of responses originating from encounters with previous as well as contemporary influenza strains.

3 Humoral Correlates of Protection in Influenza

Antibodies alone have long been known to have the capacity to prevent influenza infection. This may be seen after parenteral or intranasal administration of antibodies in animal models (Henle et al. 1941; Gerhard et al. 1997) or in the neonatal period following transfer of maternal IgG (Zinkernagel 2001; Zaman et al. 2008). Recent studies during the influenza A(H1N1)2009 pandemic have also implicated their protective role against fulminant disease (Guihot et al. 2014). They are therefore the most frequently used correlate of protection and are the primary mechanism by which most currently available vaccines aim to mediate protection. Vaccine development has relied largely on antibody-mediated surrogate correlates of protection to assist in the licensing of new products and a number of techniques have been used to define responses of adequate magnitude in this regard.

At the simplest level, enzyme-linked immunosorbent assay (ELISA) may be used to measure antibodies that possess virus-binding capacity (Grund et al. 2011). However, ELISA cannot assess the functional efficacy of antibodies. Historically, the most frequently used measure of serum antibody function has therefore been the hemagglutination inhibition (HAI) assay (Black et al. 2011; Ohmit et al. 2011). This relies on the sialic acid-binding capacity of influenza virions, which causes the agglutination of erythrocytes. Antibodies that interfere with the receptor-binding site of HA can therefore inhibit hemagglutination, and their titers may be used to correlate with likelihood of protection. HAI assays are inexpensive, are easy to carry out, and have been validated extensively in studies of seasonal influenza. Seroconversion is conventionally defined as a >four-fold increase while seroprotection is expressed as an HAI titer >1:40. These definitions are used for influenza vaccine licensing. However, the minimum seroprotection level only equates to around 50 % reduction in infection risk on the basis of experimental challenge and epidemiological studies in adults. Furthermore, prevention of hemagglutination measures only one of a number of mechanisms by which antibody can act and fails to assess Fc-mediated activity among others.

Microneutralization assays can examine neutralization of virus infectivity in cell culture by plaque reduction or other methods and have been found both to be more sensitive and more direct a measure of functional activity (Grund et al. 2011). However, these are less well validated with regard to their use as measures of vaccine efficacy. Standardization of these assay protocols has been problematic and inter-laboratory reproducibility remains an issue (Stephenson et al. 2009).

4 The Generation of Antigen-Specific B Cells and Antibodies in Influenza Infection

Studies in the late 1960s and 1970s defined the durability and subtype specificity of the immunity generated by influenza infection (Couch and Kasel 1983). Classic experiments involving experimental human challenge infections demonstrated homotypic immunity that lasted many years and these were shown to correlate with antibody levels. Studies primarily in mouse models have since sought to elucidate the mechanisms underlying the protective immunity provided by B cells.

Influenza infection occurs principally in the respiratory tract due to tropism conferred by the HA–sialic acid interaction (Thompson et al. 2006). Systemic spread is unusual except in cases of severe infection, highly pathogenic strains, or failure of immune control (Yen et al. 2009; Choi et al. 2012). In the respiratory tract, “natural” IgM antibodies, which are polyspecific and produced by a small subset of B cells, B-1 cells, may play a role in preventing influenza infection independent of B cell receptor ligation (Choi and Baumgarth 2008). However, priming of naïve B cells is believed to occur when they migrate through regional lymphoid tissues where dendritic cells present influenza antigens transported there

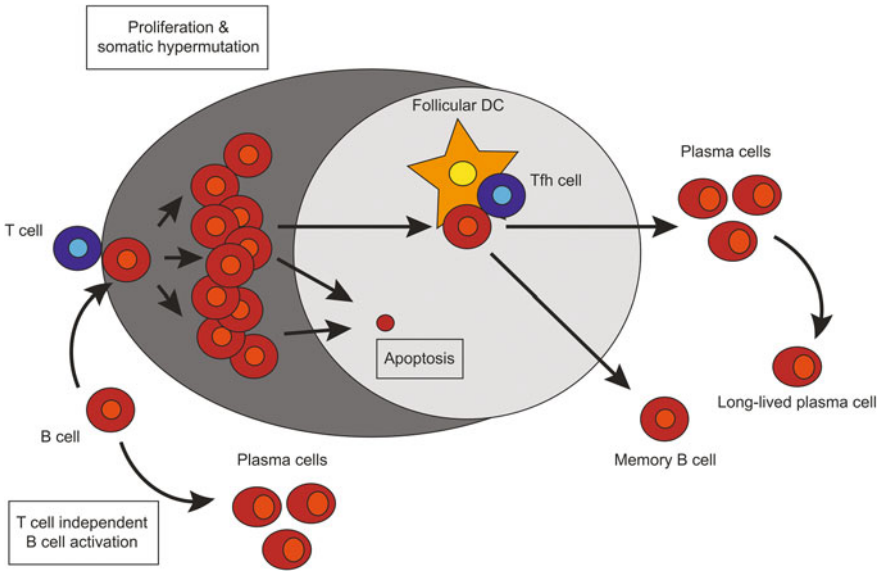

Fig. 3 Generation of antibody-secreting and memory B cells in the germinal center. B cells are activated following encounter with professional antigen-presenting cells displaying viral antigen. Cognate interactions with T follicular helper cells promote the formation of germinal centers in which somatic hypermutation, affinity maturation, and the generation of antigen-secreting cells and memory B cells occur

from the respiratory tract (Pape et al. 2007; Batista and Harwood 2009). This occurs at two main anatomical sites: draining mediastinal lymph nodes or mucosa-associated lymphoid tissue (MALT) (van Riet et al. 2012). Here, recognition of antigen by the B cell receptor induces a program of transcriptional changes that starts the differentiation process and causes the B cell to move to the edge of the lymphoid follicle (Garside et al. 1998).

If the B cell expresses the transcriptional repressor Blimp-1 at this point, it immediately proceeds toward antibody-secreting cell (ASC) differentiation with a short-lived phenotype (Shaffer et al. 2002). This leads to rapid generation of an early local antibody response but does not allow for affinity maturation. Extrafollicular responses such as these can be T cell independent via the involvement of B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL) produced by DCs (GeurtsvanKessel et al. 2009).

The classical T cell-dependent differentiation pathway, however, depends on the B cell making contact with specialized CD4+ T follicular helper (Tfh) cells that recognize cognate antigen presented by the B cell via MHC class II (Fig. 3). Tfh cells express CD40L and cytokines including IL-4, IFN-γ, and TGF-β that induce immunoglobulin class switching (McHeyzer-Williams et al. 2012). This irreversible recombination event causes the immunoglobulin isotype to switch from IgM to IgG or IgA. For the production of high-affinity ASCs and effective MBC differentiation,

Bcl-6 is up-regulated, leading to further interaction with Tfh cells in the follicular core and participation in the germinal center (GC) reaction (McHeyzer-Williams et al. 2001). Within the GC, Tfh cells and B cells interact via ICOS/ICOSL, PD-1/PD-L1, CD28/B7, and SLAM/SAP, leading to IL-21 secretion and promotion of B cell survival, somatic hypermutation, and affinity maturation (King et al. 2008). These result in the generation of high-affinity antibody-secreting plasma cells and MBCs, the latter having a long-lived phenotype but no antibody-producing capacity.

Studies in humans have primarily focused on the B cell responses in peripheral blood. Most adults will have been infected or vaccinated with influenza multiple times, and secondary responses are therefore the most commonly examined. During natural infection with influenza A(H1N1)pdm09, virus-specific plasmablasts are readily detected at a frequency of approximately 1,000 IgG-secreting cells per million PBMCs using B cell ELISpot assay at 7–10 days after symptom onset (Wrammert et al. 2011; Lee et al. 2011). The kinetics of these responses have been studied in more detail in the experimental human challenge model, where infection with seasonal influenza H1N1 A/Brisbane/59/07 led to an expansion of ASCs peaking at an average of 166 IgG+ ASCs per million PBMCs around 7 days post-inoculation, correlating with viral shedding and symptoms (Huang et al. 2014). Influenza-specific MBCs were significantly induced by 28 days post-inoculation and were all found in the class-switched IgM-negative population.

Once the infection is cleared, the majority of ASCs undergo apoptosis, while a small proportion go on to further differentiate into long-lived plasma cells (LLPCs) (Chu and Berek 2013). These travel to long-term survival niches such as the bone marrow where they persist and are responsible for ongoing production of antibody. MBCs, which may be found circulating in blood and at peripheral sites, remain poised to respond to re-encounter with antigen in order to rapidly proliferate and differentiate, once again producing ASCs capable of boosting antibody levels.

5 The Generation of Mucosal IgA+ B Cell Responses

Since influenza primarily involves the respiratory mucosa, IgA+ B cells are a major component of the response to infection. Secretory IgA is the principal isotype found at the mucosal surface and is one of the major mechanisms by which immune exclusion (i.e., early immunity capable of preventing infection itself) occurs. This has been demonstrated in IgA knockout mice, which are poorly protected against influenza infection (Asahi et al. 2002). Conversely, transfer of IgA via respiratory secretions can confer protection in naïve mice (Asahi-Ozaki et al. 2004). Furthermore, there is evidence that some cross-protective immunity is provided by antibodies of this isotype as passive intranasal transfer of anti-HA IgA confers some protection against non-homologous strains (Tamura et al. 1991).

At the draining mediastinal lymph nodes, canonical T cell-dependent B cell responses occur and the provision of Tfh signals via CD40L and TGF-β1 leads to class switching to IgA. Here, B cell differentiation and proliferation can also occur

in the absence of cognate T cell interactions and IgA+ B cell responses in particular may be less reliant on cognate T cell help. In MHC class II- and CD40-deficient animals, antibody responses to influenza can be generated in the absence of cognate signaling, with IgA+ plasma cells formed even when IgM and IgG responses are abrogated (Sangster et al. 2003). Again, extra-follicular B cell activation can occur in the absence of any T cell help through DC-derived BAFF and APRIL.

These mechanisms can also take place in the respiratory tract itself, where IgA-secreting B cells are preferentially generated in MALT. These secondary lymphoid tissues include lymphoid aggregates in the oronasopharynx (Waldeyer's ring) and bronchus-associated lymphoid tissue (BALT) (Kunisawa et al. 2008). In addition, tertiary lymphoid aggregates may also form during the course of infection. These inducible structures (iBALT) contain follicles and T cell areas, with GC reactions leading to the generation of ASCs and MBCs (Moyron-Quiroz et al. 2004, 2006). These sites may represent preferential compartments in which IgA+ GC B cells are formed, with the frequency of GC B cells peaking later here than in the draining lymph nodes (Boyden et al. 2012). In addition, GCs at mucosal sites may be present for much longer than in secondary lymphoid tissues, with their persistence potentially driven by the presence of antigen beyond the acute period (Baumgarth et al. 2008).

Respiratory mucosa also represents a site for the persistence of LLPCs that function to produce long-term mucosal antibody (Jones and Ada 1986). This may take place at several anatomical locations, with antibody-producing plasma cells found in murine nasal-associated lymphoid tissue for at least 18 months following infection with influenza (Liang et al. 2001). In addition, selective depletion of CD11c+ DCs leads to failure for iBALT to be maintained with an associated reduction of IgA and IgA+ plasma cells, suggesting that prolonged local IgA production may be dependent on iBALT persistence (GeurtsvanKessel et al. 2009).

In the gut, innate signals including TLR ligation have been shown to be important in IgA+ B cell activation and the formation of lymphoid structures, with enhancement and modulation affected by the microbiome (Pabst 2012). Similarly, in the lung, it is increasingly understood that that innate signaling is important in the generation of respiratory B cell responses. Although the respiratory microbiome is more limited than that of the gut, it nevertheless contributes bacterial components that enhance and modulate both innate and adaptive immune responses (Abt et al. 2012). Furthermore, following B cell receptor engagement, pattern recognition receptors including TLR9 and TLR10 are up-regulated on human B cells, providing additional signals that increase immunoglobulin production and are the likely basis for adjuvant effects (Bernasconi et al. 2003).

Thus, the B cell response to influenza infection is generated at several anatomical sites with multiple layers of redundancy, leading to high levels of respiratory IgA that promotes immune exclusion. Furthermore, responses in regional secondary lymphoid tissues generate systemic IgG that may prevent disseminated infection. Durable immunity occurs via T cell-dependent mechanisms and is optimized by the integration of inflammatory signals including those provided by TLRs. This results in the generation of MBCs able to rapidly respond on secondary

infection and LLPCs that survive in niches within the respiratory mucosa as well as bone marrow, thus providing long-term protection. Of note, however, these mechanisms have been primarily elucidated in animal models and studies of respiratory mucosal responses in humans remain limited.

6 Replicating the B Cell Response to Natural Infection by Vaccination

Despite this, protective humoral immune responses represent a benchmark for vaccine-induced immunity and vaccinologists have therefore sought to replicate the features of immune protection following natural infection. Two types of influenza vaccine currently exist: inactivated influenza vaccine (IIV) and live attenuated influenza vaccinelive attenuated influenza vaccine (LAIV). IIV relies primarily on the generation of HA-specific neutralizing antibodies while LAIV also induces mucosal immune responses and cellular immunity. Both types of vaccine induce highly strain-specific protection, and seasonal vaccines are therefore formulated as trivalent or quadrivalent, with current vaccines containing HAs from H1N1, H3N2, and one or two influenza B strains. If vaccine strains are well matched with circulating strains that season, vaccine efficacy is estimated to be 65–85 % while mismatch leads to reduced efficacy of as little as 40–50 % (Tricco et al. 2013). Furthermore, the durability of vaccine-induced protection is poor compared to infection with an estimated half-life for HAI titers of less than a year (Wright et al. 2008). Therefore, currently available influenza vaccines are suboptimal, sharing the same limitations with regard to subtype-specific immunity as natural infection with shorter duration of protection. This, coupled with low vaccine uptake, has led to poor coverage in most populations.

The acute response to IIV delivered intramuscularly is characterized by rapid proliferation of ASCs that arise from naïve B cells and/or MBCs with similarities to that seen following infection. Our work in healthy adults showed that this peaks around 7 days post-vaccination and is short-lived, returning to pre-vaccination levels within a week. By B cell ELISpot, the peak frequency of IgG-producing ASCs in response to the monovalent pandemic A(H1N1)2009 influenza vaccine showed substantial variability between individuals but was, on average, 520 per million PBMCs (+/−SEM 253) (Li et al. 2012). These cells exhibit an activated proliferating phenotype, with high expression of HLA-DR and Ki-67, and their frequencies correlate with the increment in serum antibody as measured by HAI, indicating that acutely generated ASCs are responsible for “seroconversion”. During infection, IgA-producing ASCs are at least as frequent as IgG due to the mucosal site of infection, but there is evidence that intramuscular vaccination is also able to partially recall these responses (Sasaki et al. 2011; Fink 2012; Li et al. 2012). However, following IIV, most ASCs secrete IgG with as little as 10 times fewer IgA+ ASCs and little or no IgM production, implying that in adults, the response to vaccination is dominated by secondary recall of IgG+ memory responses (Sasaki et al. 2011).

LAIV consists of cold-adapted influenza strains that is unable to replicate at the higher temperatures found in the lower respiratory tract. Immunity is therefore focused on the nasal mucosa, and systemic responses are modest (Cao et al. 2014). However, efficacy in children is better than IIV, suggesting the effective role of local immune responses in protection from infection (Ambrose et al. 2011). In contrast, the effectiveness of LAIV falls with increasing age and is therefore not licensed for older adults, reflecting the effect of pre-existing mucosal immunity that prevents the productive infection required for LAIV efficacy. Following LAIV, the frequency of ASCs induced is significantly smaller than following IIV and this correlates with lower vaccine-specific serum ELISA titers (Sasaki et al. 2014). However, IgA+ ASCs make up a greater proportion of the plasmablast response than IgG+ cells consistent with the route of administration. Differences are also seen in the induction of innate signals that might contribute to the response, with LAIV stimulating a later up-regulation of interferon-signaling genes than IIV (Cao et al. 2014). Interestingly, both IgG- and IgA-producing MBCs are only consistently detected in blood following TIV, suggesting that these cells when locally stimulated might remain associated with the respiratory tract (Sasaki et al. 2007).

7 Unresolved Problems Remaining with Influenza Vaccination

Despite the fact that B cell responses exhibit superficially similar characteristics to those seen following infection, the durability of vaccine-induced responses is comparatively poor. In addition, the protection conferred remains highly strain-specific. Although the more recently developed LAIV has been shown to induce both T cells and IgA-producing B cells with cross-reactive potential, epidemiological evidence has not supported the idea that these vaccines confer clinically significant cross-protection (Belshe et al. 1998; Hoft et al. 2011; Sasaki et al. 2014). Furthermore, vaccine efficacy in frankly or relatively immunosuppressed populations, such as older adults, is especially poor. Since these are high-risk groups in whom the burden of mortality is the greatest, these unresolved issues represent important areas for further study and development.

8 Overcoming Immunosenescence to Improve Protection

Clinically important antigenic drift and shift do not occur at constant rates. For example, since the emergence of the most recent pandemic influenza A strain in 2009, this has remained the predominant circulating strain of H1N1. An influenza vaccine that could confer protection beyond a single influenza season would therefore be valuable in increasing vaccine coverage. This is an even greater issue in elderly adults whose immune responses in general are blunted and in whom the

relative reduction in hospitalization conferred by influenza vaccination can be as little as 27 % (Nichol et al. 2007). Improvements in vaccine immunogenicity are therefore urgently required.

The proportion of older adults continues to increase worldwide as life spans extend and birth rates fall. Between 1975 and 2000, there was almost a doubling of individuals aged over 60 years and this increase is accelerating (http://www.un.org/esa/population/publications/worldageing19502050/). With advancing age, the frequency of ASCs decreases, with defects in GC formation, decreased capacity for affinity maturation and decreased survival of long-lived B cell subsets (Haq and McElhaney 2014). B cell responsiveness to vaccination is therefore impaired, with fewer plasmablasts generated following IIV in the elderly leading to significantly reduced increments in HAI titer compared with young adults (Sasaki et al. 2011).

A number of approaches are currently being explored in an attempt to enhance immunogenicity. The simplest strategy has been an increased dose formulation of IIV that has been shown to increase rates of seroconversion/seroprotection (Chen et al. 2011). Also already in use are adjuvanted influenza vaccines, the first of which licensed for use in older adults was the oil-in-water emulsion, MF59 (O'Hagan et al. 2007). Antibody responses to MF59-adjuvanted influenza vaccine are enhanced, allowing antigen dose-sparing and potentially increasing cross-reactive responses. This probably acts through innate myeloid cells, increasing antigen uptake and expression of chemoattractants including CCL2. More recently, alternative delivery methods have been explored including intradermal administration that may enhance vaccine-induced responses (Koutsonanos et al. 2013). Despite these approaches, the induction of long-lasting antibody and B cell responses by influenza vaccination is still suboptimal but may be improved by additional understanding of the biology of antibody generation.

9 Avenues to Overcoming Strain-Specific Immunity to Influenza

In April 2009, a novel strain (influenza A/California/04/2009) emerged with a genome re-assorted from genetic segments originating in swine, avian, and human viruses (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team 2009). This spread rapidly causing a pandemic and emphasizing that antigen shift is capable of generating strains to which there is no herd immunity in spite of widespread vaccination and experience of serial influenza HA and NA antigens. Current vaccine manufacturing techniques cannot produce vaccines against newly emergent pandemic strains quickly enough. Therefore, an ideal vaccine would be able to induce broadly cross-protective immunity so that even highly divergent viruses with pandemic potential would be recognized by preexisting immunity. However, this remains an elusive goal.

It is commonly understood that cross-protective immune responses occur by recognition of conserved antigenic regions, which are restrained by their structure

and/or function from significant divergence. Thus, most or all strains of a pathogen would possess these epitopes and an immune response directed against them would protect broadly. This is widely seen in T cell responses, where antigen processing of non-structural proteins such as nucleoprotein allows T cells to recognize conserved epitopes, although the clinical efficacy of vaccines seeking to induce these has not yet been proven (Powell et al. 2013).

Until recently, it was believed that antibodies capable of preventing influenza infection were universally directed against the globular head regions of HA and NA. These were thought to recognize highly glycosylated regions that had little restriction in terms of mutation and rapidly underwent antigenic drift. These epitopes would be easily accessible on the surface of the virion so these antibodies would be highly effective in neutralizing and opsonizing the virus but would be useless following antigenic change. However, it has become increasingly clear that relatively conserved regions in both the head and the stem of HA can represent targets for the induction of cross-reactive antibodies.

10 Broadly Cross-Reactive Stem-Binding Antibodies

In the early 1990s, Okuno et al. (1993) first described broadly cross-reactive antibodies in mice that recognized the relatively conserved HA stem. These were described as being able to neutralize virus infection but had no HAI activity, blocking virus entry rather than aggregating virus particles. However, their existence in humans remained unproven until the advent of high-throughput technologies. Such methods, including phage display and high-throughput screening of immortalized MBCs, have demonstrated broadly cross-reactive stem-binding antibodies in some individuals (Throsby et al. 2008; Ekiert et al. 2009; Sui et al. 2009; Corti et al. 2010). One monoclonal antibody (mAb) has even been found that can neutralize all known influenza A subtypes from both group 1 and group 2 (Corti et al. 2011). Usage of the heavy chain variable region gene VH1-69 is commonly over-represented and the structural mechanisms underlying this are being elucidated (Avnir et al. 2014) (Fig. 2). However, all found that broadly cross-reactive stem-binding antibodies existed at extremely low frequencies and did not exist in every individual. Their clinical relevance, especially since heterosubtypic immunity is not seen in the population, was therefore unclear.

Following natural infection with the pandemic A(H1N1)2009 virus, we were surprised to see that not only were these antibodies easily detectable but that in some patients they dominated the antibody response, making up over a third of HA-specific mAbs (Wrammert et al. 2011). These appeared to be similar antibodies to those described in other systems and demonstrated no HAI activity despite being able to bind and neutralize virus. All shared the VH1-69 gene usage and were cross-protective. Their preponderance following pandemic A(H1N1)2009 infection was attributed to the presence of the entirely novel HA head to which few memory responses were directed (Fig. 4). Instead, the relatively few MBCs that recognized

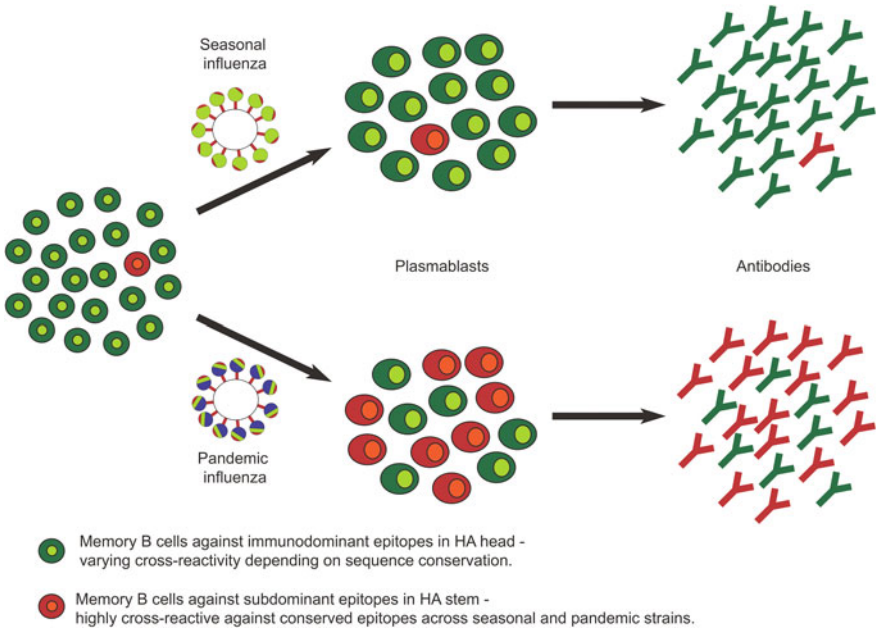

Fig. 4 The antibody response following exposure to seasonal versus pandemic influenza virus strains. All adults have influenza-specific memory B cells primarily recognizing epitopes from the HA head from recent seasonal strains (shown in *green*). Since these change relatively little year to year by antigenic drift, the same anti-head memory B cells are repeatedly boosted by infection or vaccination and come to dominate the repertoire. Memory B cells that recognize highly conserved epitopes in the head and stem (*red*) therefore remain in the minority. However, most of these previously immunodominant epitopes in the HA head are replaced in a pandemic strain (*blue*), leaving only the conserved epitopes in the stem and head. Cross-reactive memory B cells specific for these epitopes are therefore rapidly recruited into the response and come to dominate it. Naïve responses to the novel epitopes in the head are primed more slowly and therefore make a relatively small contribution to the new response. This figure was adapted from Li et al. (2012)

the HA stem (which displayed reactivity against the conserved but subdominant epitope) were preferentially stimulated and outgrew head-specific responses. In the context of seasonal influenza, this minority population would have a low probability of being recruited into the response, thus explaining the rarity of stem-binding antibodies in earlier analyses.

11 Cross-Reactive Antibody Responses to Vaccination and the "Universal" Influenza Vaccine

These antibodies were also seen following pandemic A(H1N1)2009 vaccination, albeit at lower frequencies (Li et al. 2012). Although broadly cross-reactive stem-binding antibodies were only found in a minority of individuals after immunization,

they were still found at a much higher frequency than when pre-pandemic memory B cells had been screened. Furthermore, some cross-reactive antibody responses could be seen in almost all vaccinees. On further analysis, the majority of these monoclonal antibodies were capable of recognizing HAs from a diverse panel of H1N1 influenza strains from the 1919 pandemic to the immediately preceding seasonal A/Brisbane/59/2007 strain. These did not show the cross-group recognition of H3 HAs characteristic of stem-binding antibodies but recognized relatively conserved areas of the globular head, allowing neutralization of more closely related H1N1 strains only. Thus, the antibodies following vaccination (along with more broadly cross-reactive stem-binding ones found after pandemic influenza infection) conferred cross-reactive immunity to a range of related H1N1 strains. This may explain the phenomenon whereby the preceding seasonal strain is eliminated from the population, following the emergence of a new pandemic virus (Pica et al. 2012).

The evidence that monovalent pandemic A(H1N1)2009 vaccine could induce broadly cross-reactive antibodies provided proof of principle that these responses could be achieved. Several strategies are now being explored, coupled with improvements in vaccine immunogenicity, in an attempt to develop a "universal" influenza vaccine. In animal models, successive infection or vaccination with differing strains of influenza promotes the generation of cross-reactive antibodies (Wang et al. 2010; Wei et al. 2010). This has been achieved by sequential DNA vaccination of diverse H1 or H3 strains and recapitulates the events that occur when a pandemic strain emerges, with repeated stimulation of MBCs against the HA stem potentially allowing accumulation of these responses to a protective level. Alternatively, stem-specific responses may be encouraged by vaccinating against this region alone using novel stem-only immunogens without the interference that the HA head might cause (Steel et al. 2010).

12 Conclusion

The B cell response to influenza infection represents a complex multi-stage process that requires the coordination of many signals in several anatomical locations. In natural infection, this provides enduring immunity against the infecting strain. However, the antigenic variability of influenza overcomes this and natural immunity cannot confer heterotypic protection. While current vaccines seek to replicate some features of the natural immune response, these remain suboptimal. However, the recent discovery of the nature of broadly cross-reactive antibodies and the ways in which they can be induced offers renewed possibility of a "universal" vaccine that will do away with the need for annual re-vaccination.

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