Antiviral Research 92 (2011) 15–26

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

A potential role for monoclonal antibodies in prophylactic and therapeutic treatment of influenza

Nicasio Mancini *[,1], Laura Solforosi [1], Nicola Clementi, Donata De Marco, Massimo Clementi, Roberto Burioni

Laboratorio di Microbiologia e Virologia, Università "Vita-Salute" San Raffaele, Milano, Italy

ARTICLE INFO

Article history:
Received 21 March 2011
Revised 19 May 2011
Accepted 12 July 2011
Available online 21 July 2011

Keywords:
Monoclonal antibodies
Influenza viruses
Universal epitopes

ABSTRACT

The role of humoral response in the effective control of infection by influenza viruses is well known, but the protection is usually limited to the infecting or vaccinating isolate and to few related strains. Recent studies have evidenced the existence of B-cell epitopes broadly conserved among different influenza subtypes recognized by monoclonal antibodies endowed with unprecedented broad activity. In this review, all major monoclonal antibodies directed against different influenza virus proteins are reported and their potential in the design of new anti-influenza prophylactic or therapeutic strategies is discussed.

1. Introduction

Influenza is the most recurring respiratory affection in humans, and its socio-economical impact has been often dramatic. During the 20th century, influenza A viruses have afflicted the human race with three pandemics: in 1918 caused by a virus belonging to the H1N1 subtype, in 1957 caused by a H2N2 isolate, and in 1968 caused by a H3N2 isolate (Palese, 2004). In 2009 a H1N1 reassortant strain has caused the first pandemic of the new century (Girard et al., 2010) and is now the cause, together with H3N2, of the ongoing seasonal epidemic. The 2009 pandemic proved to be not as severe as initially feared, but it drew attention to the continuous risk of a major influenza pandemic caused by a completely novel strain seriously threatening global public health. This inevitable risk, due to episodic major antigenic changes on the surface of influenza virions (antigenic shift) will always face humankind and the potentially dramatic impact of the next major pandemic is not predictable.

Apart from containment and preventive public health strategies which will not be discussed in this review, the only prophylactic or therapeutic measures now available for governments and public health agencies around the world are two classes of anti-influenza drugs (neuraminidase inhibitors and M2 blockers) and the annual vaccination campaigns. Several concerns have been raised regarding the real cost-effectiveness of the broad prophylactic use of available drugs (Burch et al., 2009a,b), which have been and are currently stockpiled by public health agencies in anticipation of a possible future pandemic. The concerns include the need of their prompt administration to be effective (Beigel and Bray, 2008), the rapid emergence of resistant isolates (Cheng et al., 2009; Ramirez-Gonzalez et al., 2011) and several associated side-effects especially in high-risk categories, such as children and pregnant women (Burioni et al., 2009a,b; Kitching et al., 2009). In addition, current influenza vaccination campaigns are based on a presumptive process: each year, a new vaccine is prepared that aims to match the strains predicted to circulate in the coming flu season. This is because the virus continuously undergoes genetic mutation to escape from the host immune response and the resulting hypervariability is particularly evident on the two major influenza surface proteins, hemagglutinin (HA) and neuraminidase (NA) (Gamblin and Skehel, 2010). This continuous variability (antigenic drift) is the molecular basis causing seasonal influenza infections (Carrat and Flahault, 2007; Webster et al., 1992), and it is the molecular factor determining the already observed mismatches between the predicted vaccinal strains and the circulating strain, therefore causing vaccine ineffectiveness (Monto et al., 2009). Moreover, vaccine ineffectiveness is highly probable in the case of the major antigenic variations associated with a future novel pandemic strain.

In order to overcome the drawbacks of the available prophylactic and therapeutic approaches, new broad-range strategies are therefore needed and, accordingly, several research strategies have been already described (Ansaldi et al., 2009; Monto et al., 2009; Nabel and Fauci, 2010; Stanekova and Vareckova, 2010; Steel et al., 2010). A pivotal role in this field will certainly be played

* Corresponding author. Address: Laboratorio di Microbiologia e Virologia, Università "Vita-Salute" San Raffaele, DIBIT2, 2° piano, via Olgettina 58, 20132 Milano, Italy. Tel.: +39 02 2643 4195; fax: +39 02 2643 2640.
E-mail address: mancini.nicasio@hsr.it (N. Mancini).
[1] These authors contributed equally to the paper.

0166-3542/$ - see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2011.07.013

by the identification of monoclonal antibodies (mAbs) capable of targeting neutralizing epitope broadly shared among different subtypes (Karlsson Hedestam et al., 2008; Nabel and Fauci, 2010). Indeed, it is known that antibodies play an important role in the natural protection against influenza viruses and that HA is the main target for virus-neutralizing Abs (Epstein et al., 1993; Gamblin and Skehel, 2010; Gerhard et al., 1997; Palladino et al., 1995). In particular, in several animal models secretory anti-HA IgA are important in protecting the upper respiratory tract mucosa in the initial phase of the infection, whereas serum IgG (the Ig isotype mostly elicited after intramuscular administration of the inactivated influenza vaccine) are crucial in preventing lung infection and in limiting its spread to other organs (Epstein and Price, 2010; Renegar et al., 2004). However, although a single influenza infection or vaccination provides lifelong immunity against the homotypic strain and a limited number of antigenically correlated strains, the host remains susceptible to infection with a novel flu variant due to HA hypervariability (Carrat and Flahault, 2007; Webster et al., 1992). Moreover, a possible paradoxical immunological consequence of the protective immunity against a limited panel of strains is that it may reduce the effectiveness of the immune response elicited against other drifted strains. This phenomenon, named "original antigenic sin", suggests that a virus causing a first infection may somehow imprint the immune system to preferentially recognize its own antigenic features; as a consequence, subsequent responses to drifted variants would be mainly limited to antibodies cross-reacting with the old strains and therefore not necessarily neutralizing the new variants (Dormitzer et al., 2011).

Overall, when considering anti-influenza immunity, three main different types of protection may be distinguished:

i. Homologous immunity: that is, immunity against a single influenza isolate. This kind of protection is the one observed after an infection or a vaccination, but it usually confers a very limited protection against other isolates.
ii. Homosubtypic immunity: that is, immunity against isolates belonging to the same subtype. This kind of immunity may be seen after a natural infection or a vaccination, but it is lost when the mutation rate among different strains increases considerably.
iii. Heterosubtypic immunity: that is, immunity against isolates belonging to different subtypes. This kind of immunity is uncommon both after natural infection or vaccination especially against highly divergent subtypes. However, this kind of immunity has to be considered as the potential gold standard for really "broad-range" prophylactic or therapeutic approaches.

Until recently, heterosubtypic immunity was considered possible only after the elicitation of a T-cell immune response against highly conserved influenza inner proteins, such as M1, NP and NS1 proteins (Assarsson et al., 2008; Jameson et al., 1998). Indeed, an important role in the control of viral spread and in the viral clearance from infected tissues is played by cytotoxic T-lymphocytes (CTL) which recognize viral peptides presented via major histocompatibility type-I (MHC-I) on the surface of infected cells. It has been described that CTL depletion in animal models leads to higher viral titers and to a more severe clinical course of the infection (Liang et al., 1994). Other recent studies confirm the beneficial role of CTL response in limiting the severity of influenza infection also in humans (Kreijtz et al., 2008; Tu et al., 2010). More importantly, there is increasing evidence in humans that memory T-cells specific to conserved epitopes on the above-mentioned influenza proteins may protect from viral strains belonging to different subtypes (Kreijtz et al., 2007,2008; Lee et al., 2008; Tan et al., 2010; Tu et al., 2010). On the basis of this data new vaccinal strategies focused on the elicitation of a heterosubtypic protective T-cell response are under study as a possible support to the "classical" approach focused on eliciting neutralizing antibodies (Epstein and Price, 2010).

At the same time, several studies in mouse models suggest that also humoral immunity, especially in its IgG component, when directed against conserved epitopes contributes to heterosubtypic protection (Nguyen et al., 2001; Quan et al., 2008a,b; Takada et al., 2003; Tumpey et al., 2001). As an example, a heterosubtypic immune response was successfully elicited in infected β2-microglobulin knockout mice or in mice lacking the secretory IgA J-chain, evidencing that neither MHC-I-restricted T-cells nor secretory IgA are absolutely required for cross-protection (Epstein et al., 1997). In another study, the role of cross-reactive mucosal IgA has also been described in subjects vaccinated with intranasal inactivated vaccines (Tamura, 2010). Overall, a better comprehension of the role of humoral response in conferring such heterosubtypic anti-influenza immunity and of its interplay with T-cell response is therefore crucial for the development of new anti-influenza strategies. In this perspective, the role of well characterized mAbs may be extremely important.

In this review we will overview all major anti-influenza mAbs described in the literature, with particular attention reserved to those of human origin especially among those directed against HA. In each of the four paragraphs, a brief molecular description of the targets will be followed by comments on the prophylactic and therapeutic potential of available mAbs.

2. Monoclonal antibodies directed against influenza A hemagglutinin (HA)

Hemagglutinin (HA) is the major influenza surface protein, with approximately 500 molecules per virion. Sixteen different types of HAs (H1–H16) have been recognized, but among them only three subtypes (H1, H2 and H3) have been recognized to establish pandemics in the human population (Fouchier et al., 2005). The 16 HA subtypes are further classified on the basis of phylogenetic sequence in two groups, group 1 including, among others, H1, H2 and H5 subtypes, and group 2 including, among others, H3 subtype (Lambert and Fauci, 2010; Nabel and Fauci, 2010).

HA is synthesized as a single polypeptide precursor (HA0) which is cleaved by cellular proteases into two subunits, HA1 or the binding subunit, and HA2 or the fusion subunit (Fig. 1) (Gamblin and Skehel, 2010). The two subunits remain covalently linked to each other through a disulfide bond. On the viral envelope each HA molecule is organized in trimeric spike structures, with the globular HA1 domain mostly exposed on the surface and the HA2 fusogenic domains mainly constituting the fibrous stem of each trimer. The N-terminus of the HA2 domain contains a sequence of about 20 mostly hydrophobic aminoacids, particularly hidden in the HA structure, which constitutes the so-called "fusion peptide" (Gamblin and Skehel, 2010). The pivotal role of HA in the viral cycle and its exposition on the viral envelope make it the primary viral antigen targeted by the host's antibody response and the only antigen inducing a neutralizing antibody response. This is the reason why HA is the most variable influenza protein and why its variations, especially on the highly exposed HA1 subunit, are the main responsible for the immune escape of influenza viruses.

Neutralizing mAbs act by blocking either of the two functions of HA: virus binding to sialic acid on the cell surface or virus fusion with the endosomal membrane (Skehel and Wiley, 2000). Binding–blocking mAbs are typically directed against antigenic sites surrounding the receptor binding pocket in the membrane distal HA1 subunit (Wiley et al., 1981). Antibodies with similar features are the most abundant neutralizing antibodies produced in the

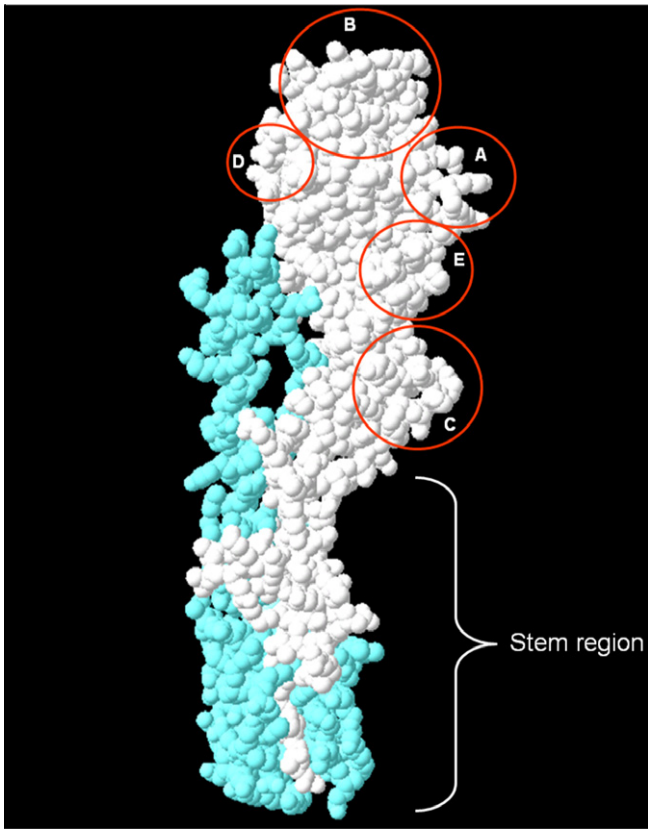

Fig. 1. Major antigenic sites on the globular head of HA. The five antigenic sites on the globular head of a HA monomer are schematically represented. Antigenic site D is not exposed in the intact trimer, and its role in eliciting neutralizing antibodies may be limited *in vivo*. The HA1 domain is represented in white, whereas the HA2 domain is depicted in turquoise

course of a natural infection, but are also those more easily escaped by viral hypervariability. On the other hand, relatively few examples of fusion-inhibiting mAbs are available. These extremely important antibodies are typically described to interact with the membrane proximal HA2 portion of the HA in the region of the fusion peptide, and are therefore directed against a functionally important region highly conserved even among different subtypes (Gamblin and Skehel, 2010; Vareckova et al., 2003).

2.1. Monoclonal antibodies directed against the HA globular domain

The globular domain of HA is exclusively constituted by residues belonging to the HA1 subunit, and its main role is to assure the binding between a well hidden binding pocket on it surface and sialic acid residues on cellular glycoproteins or glycolipids. This means that, on the globular head, a limited number of aminoacids are functionally conserved to allow the binding, but also that these residues are surrounded by a large number of residues easily prone to mutations without affecting the viral binding (Chutinimitkul et al., 2010; Gamblin and Skehel, 2010; Wang et al., 2009). This observation is extremely important to understand how influenza viruses escape neutralizing antibodies directed against this region. Indeed, the binding platform of an antibody is larger than the binding pocket on the globular head of HA; as a consequence, the binding of a neutralizing antibody may be easily escaped by mutating the aminoacids surrounding the viral binding pocket without affecting viral binding to the host cells (McDonald et al., 2007; Ping et al., 2008; Skehel et al., 1984). Interestingly, a recent paper evaluating viral mutants selected by neutralizing mAbs has shown that mutations around the binding pocket may also have a paradoxical positive effect on the viral binding determining an increased avidity to host cells (Hensley et al., 2009).

The role of monoclonal antibodies directed against the globular head has been crucial to understand its function and its antigenic organization. Classical studies using neutralizing mouse monoclonal antibodies identified five distinct antigenic sites (A-E) (Fig. 1) on the HA1 globular head region in the three-dimensional structure of the H3 HA molecule (A/Hong Kong/1/68) (Underwood, 1982; Wiley et al., 1981; Wilson et al., 1981). Similar antigenic sites of H1 (Caton et al., 1982) and H2 (Tsuchiya et al., 2001) subtypes were then identified on the basis of amino acid substitutions found in the HA sequences of variants that escaped from neutralization by the antibodies. Recently, it was suggested that the structures of antigenic sites of H5 (Kaverin et al., 2002; Kaverin et al., 2007) and H9 (Kaverin et al., 2004) subtypes may be different from those described for H1, H2, and H3 subtypes.

As a matter of fact, antibodies directed against these hypervariable antigenic sites are usually endowed with very potent, but limited in breadth, neutralizing activity, that is a classical example of homologous immunity. However, the recent 2009 pandemic has shown that antibodies directed against these antigenic sites may be important also in cross-protection. Monoclonal antibodies 2D1 and 2B12 cloned using circulating B cells from survivors of the 1918 pandemic and directed against the hypervariable Sa site on the globular head of H1 were shown to be effective *in vitro* (both) and *in vivo* (only 2D1) also against the 2009 pandemic virus (Krause et al., 2010; Yu et al., 2008). The partial cross-protection against temporarily distant strains was due to the nearly identity shown by the two viruses on the Sa site (Krause et al., 2010). This is one of the possible molecular mechanisms at the basis of the apparently paradoxical protection shown by older people against the 2009 pandemic virus (Wrammert et al., 2011). However, the same antibodies did not show any activity against recent H1N1 isolates antecedent to the pandemic strain (Yu et al., 2008). Other human monoclonal antibodies with similar characteristics have been selected against other influenza A subtype, as H5 (Lim et al., 2008; Simmons et al., 2007; Yang et al., 2010). An example is 3D1, that was cloned as a single-chain fragment from a phage-display library of human peripheral B cells, and that was shown to bind directly amino acid residues inside the sialic acid binding pocket (Lim et al., 2008; Yang et al., 2010). Although directly interacting with the binding pocket, also the binding of this antibody is certainly influenced by all surrounding residues not involved in the binding to sialic acid.

The effect of antigenic drift of the HA globular head on the binding of neutralizing mAbs has been clearly evidenced using a large panel (108 mAbs) of antibodies cloned in two different occasions from the same patient and directed against the 5 antigenic sites of the globular head of H3 subtype (Okada et al., 2010). These monoclonals were tested against 12 different H3N2 isolates ranging from 1968 to 2004, and showed the typical limited activity of mAbs directed against the globular head. Interestingly, the study of these mAbs showed a direct correlation between their binding activity and the viral evolution of the H3N2 subtype during the years showing that the neutralizing activity of the studied mAbs could be divided in three time groups with distinct strain specificity: 1968–1973, 1977–1993 and 1997–2003 (Okada et al., 2010).

Overall, the data presented in this paragraph evidence the importance as research tools of mAbs directed against the HA globular head, but at the same time clearly demonstrate that the therapeutic or prophylactic use of these mAbs may be problematic.

2.2. Monoclonal antibodies directed against the HA stem region

The stem region of HA is mainly formed by the HA2 domain, with a minimal but important contribution of several residues in

the N-terminus of HA1 (Gamblin and Skehel, 2010). The main function of the stem region is to mediate the fusion between the viral envelope and the endosomal membrane, following a low pH-induced conformational change of the stem (Gamblin and Skehel, 2010). This region is less exposed to the immune system and it is consequently less prone to mutate (Sui et al., 2009). As a consequence, the high sequence conservation observed even among different subtypes, especially in the fusion peptide, makes this region the ideal target for broadly neutralizing heterosubtypic mAbs (Karlsson Hedestam et al., 2008; Nabel and Fauci, 2010). Indeed, broadly neutralizing antibodies directed against this region may be elicited after natural infection or immunization, but they represent only a minimal part of the elicited repertoire (Corti et al., 2010). Several factors may contribute to the scarce immunogenicity of the stem region of HA, such as its inaccessibility on the virion surface, its close proximity to the envelope membrane and the outstanding immunodominance of the bulky globular head (Wang and Palese, 2009). The position on the viral particle and the presence of the highly immunogenic globular head probably limit the number of B-cells clones capable of physically interacting through their B-cell receptors with the stem region. This is shown by a recent paper evidencing the potential broad-range effectiveness of a novel vaccine construct based on a recombinant HA lacking the globular head (*headless HA*), in order to present the conserved HA stem to immune cells (Steel et al., 2010). More importantly, the close proximity to the membrane may also induce tolerance mechanisms since B-cell clones recognizing the stem may also auto-react with membrane components, as already described for antibodies directed against membrane-proximal residues in HIV-1 gp41 (Haynes et al., 2005; Verkoczy et al., 2010).

A help for the definitive comprehension of the factors limiting the elicitation of a broadly protective humoral response against the HA stem region may therefore come from the characterization of mAbs recognizing it.

The first mAb identified as targeting this region was a mouse monoclonal, named C179, that was shown to react against different HA subtypes belonging to phylogenetic group 1 (H1; H2; H5 and H6) and to neutralize infection by blocking the fusion step (Okuno et al., 1993; Okuno et al., 1994; Smirnov et al., 1999). This mAb has been mainly used as a control in research lab, and its potential use in passive immunoprophylaxis has never been deeply evaluated due to its murine origin and to its relative efficacy *in vivo* (Sakabe et al., 2009). Mouse mAbs with similar characteristics, but directed against group 2 subtypes, have also been reported (Wang et al., 2010b).

Recently, several groups have described human mAbs endowed with heterosubtypic activity isolated from synthetic (Sui et al., 2009) or immune donor-derived (Kashyap et al., 2008; Throsby et al., 2008) phage-display libraries, or from immortalized peripheral B-cells of a vaccinated patient (Corti et al., 2010). The main features of the most representatives of these mAbs are reported in Table 1. Intriguingly, most of these mAbs share a common genetic origin derived from a single V-gene germline (VH1–69), suggesting the presence of a conserved motif in this antibody subfamily determining the observed peculiar features (Corti et al., 2010; Sui et al., 2009; Throsby et al., 2008). Indeed, crystallization studies performed with two of these heterosubtypic mAbs confirmed that they interact with a highly conserved helical region in the HA membrane proximal stem and that both mAbs interact with the antigen only with the VH1–69-derived heavy chain CDR1 and CDR2 regions, but not with the CDR3 that usually confers antigen specificity to an antibody (Ekiert et al., 2009; Sui et al., 2009).

The reactivity and the *in vitro* and *in vivo* neutralizing activity of the above VH1–69-derived mAbs have been evaluated against a large panel of influenza strains belonging to different subtypes, indeed showing a heterosubtypic neutralizing activity (Friesen et al., 2010; Kashyap et al., 2010; Koudstaal et al., 2009; Sui et al., 2009; Throsby et al., 2008). The selection of antibodies with similar characteristics, especially from immunized patients, is of outstanding importance since it is the molecular proof that such antibodies may be stimulated by the natural infection or by vaccination. These antibodies may therefore represent a useful tool not only for possible new passive immunoprophylaxis strategies, but also for the design of new vaccinal approaches based on immunogens mimicking their epitope (Burton and Parren, 2000; Karlsson Hedestam et al., 2008; Nabel and Fauci, 2010). However, it is important to note that VH1–69 germline is usually reported in less than 2% of normal human B-cells (Brezinschek et al., 1995), and that its presence has been frequently described in autoimmune conditions (Manheimer-Lory et al., 1991; Perotti et al., 2008; Pos et al., 2009; Van Es et al., 1992), thus raising some doubts about the real efficacy and the safety of a possible HA stem region-based vaccinal approach.

The neutralizing activity of the above mAbs was shown to be limited to subtypes belonging to group 1, with no activity observed against group 2 viruses and in particular against H3N2 subtype. Under this perspective, an important finding was recently presented by our group at the Antivirals Congress celebrating the 30th anniversary of *Antiviral Research* covered by this issue of the journal (Mancini et al., 2010). We had already described two human monoclonals (PN-SIA28 and PN-SIA49) that as Fab fragments were able to neutralize H1N1 viruses encompassing the whole H1N1 pandemic of the last century and including the recent 2009 pandemic strain. This demonstrates the conservation of their epitope in all tested H1N1 isolates (Burioni et al., 2009a,b, 2010). Neither of the two mAbs derived from the VH1–69 germline (VH3–30 and VH3–23, respectively) evidencing that other germline genes may contribute to heterosubtypic anti-influenza humoral response. Intriguingly, when tested as whole IgG1 molecule, one of the two monoclonals (PN-SIA28) featured an unprecedented heterosubtypic activity extended to group 2 viruses, and in particular to H3N2 isolates. In all cases PN-SIA28 showed half maximal inhibitory concentration (IC_{50}) in the nanomolar range. The neutralizing activity against other subtypes belonging to group 1 has been evaluated confirming the broad heterosubtypic activity of this mAb (*unpublished data*).

The correct localization of the epitope recognized by PN-SIA28 on the HA molecule has not been obtained with crystallization studies yet, but several data confirm that it is located on the HA

Table 1
Major heterosubtypic neutralizing human monoclonal antibodies directed against influenza A hemagglutinin (HA) stem region.

mAb	Host	Specificity	Mechanism of action	Reference
F10	Human	HA subtypes belonging to group 1	Fusion inhibition	Sui et al. (2009)
CR6261	Human	HA subtypes belonging to group 1	Fusion inhibition	Ekiert et al. (2009), Throsby et al. (2008)
FC41	Human	HA subtypes belonging to group 1	Fusion inhibition	Corti et al. (2010)
A06	Human	HA subtypes belonging to group 1	Fusion inhibition	Kashyap et al. (2008, 2010)
PN-SIA 49	Human	HA subtypes belonging to group 1	Fusion inhibition	Mancini et al. (2010)
PN-SIA 28	Human	HA subtypes belonging to group 1 and group 2	Fusion inhibition	Mancini et al. (2010)

stem, such as the lack of hemagglutination inhibiting activity, the competition for binding observed with C179 (Burioni et al., 2009a,b, 2010) and the impaired binding against recombinant HA carrying alanine mutants in several positions of the stem region (*unpublished data*). The breadth of neutralizing activity of this mAb and its extension to H3N2 viruses confirm that its epitope is conserved among phylogenetically highly divergent subtypes, and that it is different from the epitopes described for the other mAbs with a group 1 restricted neutralizing activity. The better definition of this epitope will certainly provide the scientific community with an unprecedented tool for the design of new "universal" anti-influenza vaccinal strategies.

3. Monoclonal antibodies against influenza A neuraminidase (NA)

Like hemagglutinin, neuraminidase (NA) is an integral membrane glycoprotein sited on the viral envelope surface. The ratio of HA to NA is usually 4:1 to 5:1 and NA represents, as well as HA, a subtype-specific glycoprotein of influenza A viruses. NA is organized on the membrane as a homotetramer containing an enzymatically active head domain with sialidasic activity which allows the release of viral progeny from host cells (Gamblin and Skehel, 2010). NA is subjected, as HA, to antigenic shift and antigenic drift. While antigenic shift can lead to the emergence of new pandemic strains, the antigenic drift generates new seasonal strains no longer recognized by the specific NA humoral immune response or no longer inhibited by neuraminidase inhibitors. Most mutations of known effect on NA have been mapped on neuraminidase inhibitor-resistant isolates belonging to different subtypes (Abed et al., 2006; Besselaar et al., 2008; de Jong et al., 2005; Fiore et al., 2011; Fleming et al., 2010; Ramirez-Gonzalez et al., 2011; Sheu et al., 2008; Steinhoff, 2011; Wang et al., 2010a).

Viral NA plays a key role in host immune response as a major antigenic determinant in the course of natural infection or after vaccination. Indeed, studies in mice (Brett and Johansson, 2005; Johansson et al., 1998) and clinical trials demonstrated a positive correlation between serum neuraminidase-inhibiting (NI) antibody titers and vaccine effectiveness (Clements et al., 1986; Monto and Kendal, 1973). Moreover, it was observed that the protection conferred by anti-neuraminidase antibodies does not necessarily correlate with the titer of neutralizing anti-HA antibodies (Naikhin et al., 1983), the major determinants of protection. Indeed, the protective role of anti-NA antibodies has been thoroughly investigated, demonstrating that, in contrast to anti-HA antibodies, anti-NA antibodies do not neutralize virus infectivity, but limit the infection by impairing the efficient release of viruses from infected cells (Sylte and Suarez, 2009) thus determining decreased virus shedding and severity of illness. It has also been demonstrated that anti-NA antibodies may mediate antibody-dependent cell cytotoxicity (ADCC) mechanisms thereby contributing to the protective role of HA-specific neutralizing mAbs (Mozdzanowska et al., 1999).

During the past years several monoclonal antibodies directed against NA have been isolated (Table 2) and used in structural and functional studies on NA. Using an analogous method to those used for HA, antigenic sites were mapped on NA by analysis of changes in naturally existing strains and in variants selected using monoclonal antibodies. With this approach four antigenic sites were identified on influenza A virus NA, evidencing the presence of a quite conserved region (region 1) surrounded by other regions more subjected to aminoacidic changes. Antigenic region 2 can be further divided into four overlapping areas (2a–2d) based on the reactivity patterns of monoclonal antibodies with antigenic variants, chemically modified neuraminidase and the ability of the antibodies to inhibit enzyme activity of different molecular weight substrates (Jackson and Webster, 1982; Webster et al., 1984). Studies of the specificity of monoclonal antibodies inhibiting NA activity identified neutralizing conformational epitopes close to the enzyme active site. Moreover, these studies demonstrated that the native conformation of NA is necessary for the induction of NA inhibition (NI) antibodies, suggesting that the correlation between NA enzymatic activity and the correct NA protein-folding could be a good tool to asses the potency of influenza vaccines with respect to NA content (Sultana et al., 2011).

Overall, the potential prophylactic or therapeutic role of anti-NA mAbs is probably limited by two factors, that is the NA hypervariability, comparable to that observed for HA, and, more importantly, the lack of direct neutralizing activity, differently from that observed for anti-HA antibodies. However, the isolation of mAbs directed against conserved epitopes on the catalytic site of NA may play a role, in association with available neuraminidase inhibitors, in controlling the emergence of viral isolates resistant to drugs belonging to this class.

4. Monoclonal antibodies against M2 protein

The M2 protein of influenza A viruses is encoded on the seventh viral RNA segment together with the matrix protein 1 (M1) and is a transmembrane protein that forms a small proton-selective homotetrameric channel in the viral envelope, active at acidic pH, with roles in virus entry, assembly and budding (Iwatsuki-Horimoto et al., 2006; McCown and Pekosz, 2006; Mould et al., 2000; Pinto et al., 1992). This protein is vital for viral replication and is the target for the adamantanes, one of the two classes of available anti-influenza drugs.

M2 is composed of 97 amino acidic residues and its N terminus is directed toward the outside of the virus (Lamb et al., 1985). It has three structural domains: an amino-terminal extracellular (M2e) domain (residues 1–23), a transmembrane (TM) domain (residues 24–46) essential for its ion activity (Chizhmakov et al., 1996; Chizhmakov et al., 2003; Mould et al., 2000), and a cytoplasmic domain (residues 47–97). M2 is abundantly expressed on the surface of virus-infected cells but only a small number of M2 molecules is estimated to be incorporated into each virion (Lamb et al., 1985; Zebedee and Lamb, 1988; Zebedee et al., 1985).

Table 2
Major monoclonal antibodies directed against influenza A neuraminidase (NA).

mAb	Host	Specificity	Mechanism of action	Reference
HCA-3 HCA-2	Rabbit	NA (all major subtypes)	Not described	Gravel et al. (2010)
5B2 4C11 1E11	Mouse	NA (subtype-specific)	Inhibition of sialidase activity	Moreno et al. (2009)
Mem5	Mouse	NA (N2-specific)	Inhibition of sialidase activity	Gulati et al. (2002), Lee and Air (2006)
8H12	Mouse	NA (N1-specific)	Not described	Ho et al. (2009)
NC41	Mouse	NA (N9-specific)	Inhibition of sialidase activity	Colman et al. (1987), Tulip et al. (1992)
NC10	Mouse	NA (N9-specific)	Inhibition of sialidase activity	Colman et al. (1989), Malby et al. (1994)

The M2e domain is nearly invariant in all influenza A strains since the 1918 Spanish pandemic (Fiers et al., 2004) and for this reason M2e has been considered a promising candidate for the development of novel broad-spectrum, prophylactic and therapeutic anti-influenza strategies. In fact, several groups have reported that the immune response induced by M2-based vaccines can protect against influenza virus challenge, with particular attention reserved to M2e (De Filette et al., 2006; Denis et al., 2008; Ernst et al., 2006; Fan et al., 2004; Frace et al., 1999; Huleatt et al., 2008; Lalor et al., 2008; Liu et al., 2004a; Mozdzanowska et al., 2003; Neirynck et al., 1999; Slepushkin et al., 1995; Tompkins et al., 2007; Wu et al., 2007).

During a natural infection, antibodies against M2, and against M2e in particular, are absent or barely detectable in human sera (Feng et al., 2006). Due to its low representation on the virus surface and to the shielding by the larger and more abundant HA and NA proteins (Lamb et al.,1985; Zebedee and Lamb, 1988; Zebedee et al., 1985), M2e is in fact poorly immunogenic. It was shown that anti-M2 antibodies apparently neither bind efficiently to the virus nor neutralize it (Jegerlehner et al., 2004). On the contrary, anti-M2 antibodies apparently bind strongly to virus-infected cells (Jegerlehner et al., 2004) and through antibody-dependent cell-mediated cytotoxicity (ADCC) or antibody-mediated complement dependent cytotoxicity (CDC) can limit virus replication (Treanor et al., 1990).

The potential protective activity of anti-M2 antibodies was confirmed by studies with several anti-M2 mAb. The first anti-M2e mAb (14C2) providing evidence that anti-M2 immunity has antiviral activity was isolated in 1988 from M2-immunized mice (Zebedee and Lamb, 1988). When tested in plaque assay overlay, 14C2 could reduce the plaque size of many influenza A virus strains, thus indicating that this antibody was able to directly limit the viral replication. Moreover, passive administration of 14C2 could also reduce the level of replication of influenza A virus in the lungs of mice (Treanor et al., 1990).

Several subsequent studies with other murine mAbs confirmed that, among anti M2-antibodies, only anti-M2e mAbs featured *in vitro* and *in vivo* inhibitory activity against influenza viruses (Fu et al., 2009; Liu et al., 2003, 2004a; Zou et al., 2005). Interestingly, the inhibitory activity of some mAbs was reported to be extended also against influenza B viruses (Liu et al., 2003). Indeed, sequence analysis revealed that the first nine N terminal amino acid residues of M2e are extremely conserved even between the two main influenza types.

All the studies reported so far were based on the characterization of murine mAbs, but recently the development of transchromosomic mice and mammalian cell display techniques allowed the production of fully anti-M2e human antibodies. An anti-M2e mAb (Z3G1) was generated from transchromosomic mice engineered to produce fully human monoclonal antibodies (Wang et al., 2008). Z3G1 showed the ability to bind the majority of M2e variants from several viral isolates, including highly pathogenic avian strains. Moreover, passive immunotherapy with Z3G1 in mice demonstrated its significant prophylactic and therapeutic activity (Wang et al., 2008).

Other fully human anti-M2e monoclonal antibodies (D005, E040 and F052) were isolated from the peripheral blood mononuclear cells of an individual with high anti-M2e titers by mammalian cell display (Beerli et al., 2008, 2009). It was demonstrated that these antibodies bind M2 with high affinity and efficiently recognize M2 from recent H5N1 influenza A strains. All mAbs showed a potent prophylactic activity and therapeutic activity in a mouse model of influenza A infection against a single H1N1 strain (A/PR/8/34) (Beerli et al., 2009). In particular, D005 was able to fully protect mice with a single injection one day after infection and still showed some protection when administrated 3 days after challenge (Beerli et al., 2009).

All these studies emphasize that the anti-M2e immunity, directed against a remarkably conserved viral target, is capable of providing a significant level of protection against influenza A infections. The high degree of conservation of M2e is in part attributable to constraints resulting from its genetic relation to M1, the most conserved protein of the virus (Ito et al., 1991). In fact, the first 26 nucleotides of influenza A genome segment seven are employed to code both M1 and M2 proteins. An additional factor that contributes to the low degree of change seen in M2e is the absence of natural M2e-specific antibodies and thus pressure for change. Hence, there may be a concern that introducing immune pressure on M2e will promote the evolution of antigenic escape viruses. Indeed, the selection of such escape mutants was reported in an immunodeficient mouse model in which a panel of anti-M2e monoclonal antibodies was given to limit virus replication (Zharikova et al., 2005). However, the diversity of the escape mutants was restricted to a single residue, a proline in position 10 that mutated to leucine or histidine. On the other hand, no mutations were detected following multiple passages of virus under anti-M2e immune pressure in immunocompetent mice (Gerhard et al., 2006).

Overall, the possibility of the emergence of M2e-escape mutants following treatment with antibodies cannot be excluded. However, the fact that their diversity was demonstrated to be highly contained and considering that the escape variants would have to acquire a selective advantage compared to the wild-type, anti-M2e antibodies (Table 3) could be considered a feasible option for protection against influenza A infection.

Table 3
Monoclonal antibodies directed against M2 protein.

mAb	Host	Specificity	Mechanism of action	Reference
14C2	Mouse	M2e	Direct interference with M2 functions (?);ADCC CDC	Zebedee and Lamb (1988) Treanor et al. (1990)
8c6 1B12	Mouse	M2e EVETPIRN (aa 6–13)	ADCC CDC	Liu et al. (2004b)
L18 O19 P6 S1	Mouse	M2e	ADCC CDC	Fu et al. (2009)
Z3G1	Human (transchromosomic mice)	M2e LLTEVETPIR (aa 3–12)	ADCC CDC	Wang et al. (2008)
D005 E040 F052	Human	M2e	ADCC CDC	Beerli et al. (2009)

M2e: M2 extracellular domain. Specific linear epitopes are reported.
ADCC: antibody-dependent cell cytotoxicity
CDC: complement-dependent cytotoxicity

5. Monoclonal antibodies against internal proteins

The protective potential of antibodies direct against the internal proteins of influenza A has often been dismissed because of the assumption that an effective antibody response neutralizes the virus by preventing its attachment to host cells or its fusion with the endosomal membrane. However, the Fc region of an antibody can activate a variety of antiviral effector functions (Huber et al., 2001; Huber et al., 2006; Leopold et al., 2006; Muckelbauer et al., 1995; Muckelbauer and Rossmann, 1997; Palmer et al., 2000). In fact, non-neutralizing anti-influenza antibodies, such as anti-NP, have been shown to induce complement-mediated cytolysis (Yewdell et al., 1981), increase T cell responses associated with enhanced dendritic cell function (Zheng et al., 2007), and reduce viral replication in culture (Sambhara et al., 2001). Moreover, studies with anti-hepatitis C virus antibodies has also demonstrated the potential of the intracellular expression of monoclonal antibodies directed against highly conserved inner viral proteins (Chandra et al., 2010; Prabhu et al., 2004). Therefore, antibodies to conserved internal viral proteins may promote early viral clearance through some combination of these mechanisms.

5.1. Anti-nucleoprotein (NP) monoclonal antibodies

Influenza A virus has a segmented negative-sense single-stranded RNA genome. Each RNA segment is associated with nucleoprotein (NP) and with the polymerase complex consisting of PB1, PB2 (basic polymerases 1 and 2) and PA (acid polymerase). These ribonucleoparticle (RNP) complexes are surrounded by a layer of the matrix protein, M1 (Noda et al., 2006).

NP is a conserved highly basic protein that binds to single-strand RNA in a non-sequence specific manner (Baudin et al., 1994). Both natural infection with influenza virus and vaccination with recombinant NP elicit NP-specific antibodies (Sukeno et al., 1979). However, anti-NP antibodies were considered to be ineffective because they do not neutralize the virus and because passive transfer of such antibodies do not protect naive immunodeficient SCID recipient mice (Ulmer et al., 1993). However, it has recently been shown that anti-NP monoclonal antibodies form immune complexes which can promote dendritic cell maturation, Th1 cytokine production and anti-influenza $CD8^+$ CTL responses in naive immunocompetent mice (Carragher et al., 2008; Zheng et al., 2007). Immunization with NP was effective in reducing morbidity and viral titers after challenge with influenza virus in a mouse model (Carragher et al., 2008). Importantly, antibody-deficient mice were not protected by this vaccination strategy and immune serum could transfer these protective effects to naive hosts in an antibody-dependent manner (Carragher et al., 2008).

Single-chain intracellular antibodies specifically interacting with influenza NP were recently described (Mukhtar et al., 2009). These anti-NP intrabodies significantly inhibited influenza A virus transcription and replication through blocking the interaction of NP with PB1 and PB2. The broad inhibitory effects of anti-NP intrabodies on viral transcription and replication provides proof-of-concept that the intracellular inhibition of NP may be considered as a possible future broad-range anti-influenza strategy (Mukhtar et al., 2009).

5.2. Anti-polymerase complex monoclonal antibodies

The influenza virus RNA-dependent RNA polymerase complex consists of PB1, PB2 and PA (Fodor et al., 2002). Because of the essential role of the polymerase proteins in the influenza virus life cycle, targeting the polymerase complex could provide a novel strategy for the development of antiviral compounds against the influenza A virus. The only described human monoclonal antibodies against the polymerase proteins are several single chain variable antibody fragments (HuScFv) which, according to the authors, should be engineered to develop cell-penetrating antibodies that could interfere with the functions of the intracellular polymerase subunits (Thathaisong et al., 2008).

5.3. Anti-M1 protein monoclonal antibodies

The influenza A virus matrix protein (M1) is the most abundant structural protein of influenza virus and is highly conserved among type A viruses (McCauley and Mahy, 1983). This viral protein has an important role in virus replication (Bui et al., 2000; Nayak et al., 2004; Whittaker et al., 1996) making both M1 and the M1-coding RNA segment attractive targets for drug inhibitors, siRNA and therapeutic antibodies (Hui et al., 2004; Kawaoka et al., 1990).

Recently, a HuScFv (PEN-HuScFv) specifically binding the M1 of different influenza A subtypes was produced using phage display technology (Poungpair et al., 2009). PEN-HuScFv was demonstrated to inhibit the binding of recombinant M1 to viral genomic RNA in vitro (Poungpair et al., 2009). To interfere with the multiple biological functions of the M1, it is necessary that the HuScFv is able to access its intracellular target. Therefore, PEN-HuScFv was engineered into a cell-penetrable format (transbody) by molecular linking of the gene encoding the HuScFv to the penetratin (PEN) gene, the protein transduction domain of the *Drosophila* homeodomain (Derossi et al., 1994). The PEN-HuScFv, when used to treat influenza-infected cells, reduced the number of viruses released from the cells indicating that the M1-specific transbody affected the influenza A virus life cycle in living mammalian cells (Poungpair et al., 2010).

The role of mAbs directed against influenza inner proteins (Table 4) deserves further investigation, but the non-neutralizing nature of these mAbs and the need of a functionally intact cellular branch of the immune system make their potential prophylactic or therapeutic role highly uncertain, especially in high-risk patients such as the children and the elderly. Other possible strategies, such as their intracellular expression to interfere with the different functions of the intracellular viral proteins seem still far from a real prophylactic or therapeutic application.

6. Concluding remarks

Monoclonal antibodies have been used in many occasions as useful tools in the research on influenza viruses. Their use has allowed shedding some light on the protective mechanisms of the humoral response against these highly variable viruses. It is now time to look at monoclonal antibodies also as potential prophylactic and therapeutic tools against these viruses. The potential therapeutic role of passively administered antibodies has somehow already been evaluated in humans, as demonstrated during the 1918 pandemic (Beigel and Bray, 2008; Luke et al., 2006), or even more recently during the H5N1 outbreaks (Beigel and Bray, 2008; Zhou et al., 2007), by the survival rates reported for severely ill patients treated with blood products from convalescent subjects. No data are available about the possible prophylactic role of passively administered antibodies in humans, but several studies in animal models give hope for their possible use in at-risk categories (Corti et al., 2010; Friesen et al., 2010; Kashyap et al., 2010; Simmons et al., 2007; Throsby et al., 2008) or in combination with the available antivirals (Koudstaal et al., 2009). The identification of human monoclonal antibodies against broadly conserved epitopes on hemagglutinin, the major protective antigenic determinant of influenza, and their efficacy against different subtypes in *in vitro* and *in vivo* studies may be a great step forward in this direction.

Table 4
Monoclonal antibodies directed against influenza virus inner proteins.

mAb	Host	Specificity	Mechanism of action	Reference
aN02 aN77 aN110 aN122 aN130	Human	NP	Inhibition of viral transcription and replication through blocking the interaction of NP with PB1 and PB2	Mukhtar et al. (2009)
b1-1 b1-5, b1-6 b1-7, b1-8 b1-10	Human	PB1	Not described in detail	Thathaisong et al. (2008)
b2-1, b2-4 b2-9, b2-10		PB2		
a1, a3, a4, a5, a6, a7, a8, a9		PA		
PEN-HuScFv	Human	M1	Inhibition of binding of M1 to vRNA. Reduction of the viruses released from the cell.	Poungpair et al. (2010)

Further tests and trials are certainly needed and will certainly follow in the next few years to determine the safety and the effectiveness of this approach.

The availability of different broadly protecting antibodies directed against different crucial epitopes (and possibly against different influenza proteins) may allow the development of antibody cocktails with broad protective activity against phylogenetically distant influenza viruses. The parallel targeting of distinct crucial viral epitopes on different proteins may also minimize the risk of the rapid emergence of viral escape mutants with unaltered viral fitness (Clementi, 2008), which is now the case for drug-resistant escape mutants.

Finally, the availability of monoclonal antibodies with such characteristics could also allow the design of a new generation of influenza immunogens capable of mimicking there epitopes and, therefore, eliciting a vaccine-induced broadly protective immune response.

References

Abed, Y., Baz, M., Boivin, G., 2006. Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. Antivir. Ther. 11, 971–976.

Ansaldi, F., Canepa, P., Parodi, V., Bacilieri, S., Orsi, A., Compagnino, F., Icardi, G., Durando, P., 2009. Adjuvanted seasonal influenza vaccines and perpetual viral metamorphosis: the importance of cross-protection. Vaccine 27, 3345–3348.

Assarsson, E., Bui, H.H., Sidney, J., Zhang, Q., Glenn, J., Oseroff, C., Mbawuike, I.N., Alexander, J., Newman, M.J., Grey, H., Sette, A., 2008. Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. J. Virol. 82, 12241–12251.

Baudin, F., Bach, C., Cusack, S., Ruigrok, R.W., 1994. Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. EMBO J. 13, 3158–3165.

Beerli, R.R., Bauer, M., Buser, R.B., Gwerder, M., Muntwiler, S., Maurer, P., Saudan, P., Bachmann, M.F., 2008. Isolation of human monoclonal antibodies by mammalian cell display. Proc. Natl. Acad. Sci. USA 105, 14336–14341.

Beerli, R.R., Bauer, M., Schmitz, N., Buser, R.B., Gwerder, M., Muntwiler, S., Renner, W.A., Saudan, P., Bachmann, M.F., 2009. Prophylactic and therapeutic activity of fully human monoclonal antibodies directed against influenza A M2 protein. Virol J. 6, 224.

Beigel, J., Bray, M., 2008. Current and future antiviral therapy of severe seasonal and avian influenza. Antiviral Res. 78, 91–102.

Besselaar, T.G., Naidoo, D., Buys, A., Gregory, V., McAnerney, J., Manamela, J.M., Blumberg, L., Schoub, B.D., 2008. Widespread oseltamivir resistance in influenza A viruses (H1N1), South Africa. Emerg. Infect. Dis. 14, 1809–1810.

Brett, I.C., Johansson, B.E., 2005. Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology 339, 273–280.

Brezinschek, H.P., Brezinschek, R.I., Lipsky, P.E., 1995. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J. Immunol. 155, 190–202.

Bui, M., Wills, E.G., Helenius, A., Whittaker, G.R., 2000. Role of the influenza virus M1 protein in nuclear export of viral ribonucleoproteins. J. Virol. 74, 1781–1786.

Burch, J., Corbett, M., Stock, C., Nicholson, K., Elliot, A.J., Duffy, S., Westwood, M., Palmer, S., Stewart, L., 2009a. Prescription of anti-influenza drugs for healthy adults: a systematic review and meta-analysis. Lancet Infect. Dis. 9, 537–545.

Burch, J., Paulden, M., Conti, S., Stock, C., Corbett, M., Welton, N.J., Ades, A.E., Sutton, A., Cooper, N., Elliot, A.J., Nicholson, K., Duffy, S., McKenna, C., Stewart, L., Westwood, M., Palmer, S., 2009b. Antiviral drugs for the treatment of influenza: a systematic review and economic evaluation. Health Technol. Assess. 13, 1–265 (iii-iv).

Burioni, R., Canducci, F., Clementi, M., 2009a. Pregnancy and H1N1 infection. Lancet 374, 1417 (author reply 1417–1418).

Burioni, R., Canducci, F., Mancini, N., Clementi, N., Sassi, M., De Marco, D., Saita, D., Diotti, R.A., Sautto, G., Sampaolo, M., Clementi, M., 2009b. Molecular cloning of the first human monoclonal antibodies neutralizing with high potency swine-origin influenza A pandemic virus (S-OIV). New Microbiol. 32, 319–324.

Burioni, R., Canducci, F., Mancini, N., Clementi, N., Sassi, M., De Marco, D., Diotti, R.A., Saita, D., Sampaolo, M., Sautto, G., Pianezze, M., Clementi, M., 2010. Monoclonal antibodies isolated from human B cells neutralize a broad range of H1 subtype influenza A viruses including swine-origin Influenza virus (S-OIV). Virology 399, 144–152.

Burton, D.R., Parren, P.W., 2000. Vaccines and the induction of functional antibodies: time to look beyond the molecules of natural infection? Nat. Med. 6, 123–125.

Carragher, D.M., Kaminski, D.A., Moquin, A., Hartson, L., Randall, T.D., 2008. A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. J. Immunol. 181, 4168–4176.

Carrat, F., Flahault, A., 2007. Influenza vaccine: the challenge of antigenic drift. Vaccine 25, 6852–6862.

Caton, A.J., Brownlee, G.G., Yewdell, J.W., Gerhard, W., 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell 31, 417–427.

Chandra, P.K., Hazari, S., Poat, B., Gunduz, F., Prabhu, R., Liu, G., Burioni, R., Clementi, M., Garry, R.F., Dash, S., 2010. Intracytoplasmic stable expression of IgG1 antibody targeting NS3 helicase inhibits replication of highly efficient hepatitis C Virus 2a clone. Virol. J. 7, 118–129.

Cheng, P.K., Leung, T.W., Ho, E.C., Leung, P.C., Ng, A.Y., Lai, M.Y., Lim, W.W., 2009. Oseltamivir- and amantadine-resistant influenza viruses A (H1N1). Emerg. Infect. Dis. 15, 966–968.

Chizhmakov, I.V., Geraghty, F.M., Ogden, D.C., Hayhurst, A., Antoniou, M., Hay, A.J., 1996. Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells. J. Physiol. 494, 329–336.

Chizhmakov, I.V., Ogden, D.C., Geraghty, F.M., Hayhurst, A., Skinner, A., Betakova, T., Hay, A.J., 2003. Differences in conductance of M2 proton channels of two influenza viruses at low and high pH. J. Physiol. 546, 427–438.

Chutinimitkul, S., Herfst, S., Steel, J., Lowen, A.C., Ye, J., van Riel, D., Schrauwen, E.J., Bestebroer, T.M., Koel, B., Burke, D.F., Sutherland-Cash, K.H., Whittleston, C.S., Russell, C.A., Wales, D.J., Smith, D.J., Jonges, M., Meijer, A., Koopmans, M., Rimmelzwaan, G.F., Kuiken, T., Osterhaus, A.D., Garcia-Sastre, A., Perez, D.R., Fouchier, R.A., 2010. Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. J. Virol. 84, 11802–11813.

Clementi, M., 2008. Perspectives and opportunities for novel antiviral treatments targeting virus fitness. Clin. Microbiol. Infect. 14, 629–631.

Clements, M.L., Betts, R.F., Tierney, E.L., Murphy, B.R., 1986. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J. Clin. Microbiol. 24, 157–160.

Colman, P.M., Laver, W.G., Varghese, J.N., Baker, A.T., Tulloch, P.A., Air, G.M., Webster, R.G., 1987. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. Nature 326, 358–363.

Colman, P.M., Tulip, W.R., Varghese, J.N., Tulloch, P.A., Baker, A.T., Laver, W.G., Air, G.M., Webster, R.G., 1989. Three-dimensional structures of influenza virus

neuraminidase-antibody complexes. Philos. Trans. R. Soc. Lond. B Biol. Sci. 323, 511–518.
Corti, D., Suguitan Jr., A.L., Pinna, D., Silacci, C., Fernandez-Rodriguez, B.M., Vanzetta, F., Santos, C., Luke, C.J., Torres-Velez, F.J., Temperton, N.J., Weiss, R.A., Sallusto, F., Subbarao, K., Lanzavecchia, A., 2010. Heterosubtypic neutralizing antibodies are produced by individuals immunized with a seasonal influenza vaccine. J. Clin. Invest. 120, 1663–1673.
De Filette, M., Fiers, W., Martens, W., Birkett, A., Ramne, A., Lowenadler, B., Lycke, N., Jou, W.M., Saelens, X., 2006. Improved design and intranasal delivery of an M2e-based human influenza A vaccine. Vaccine 24, 6597–6601.
de Jong, M.D., Tran, T.T., Truong, H.K., Vo, M.H., Smith, G.J., Nguyen, V.C., Bach, V.C., Phan, T.Q., Do, Q.H., Guan, Y., Peiris, J.S., Tran, T.H., Farrar, J., 2005. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N. Engl. J. Med. 353, 2667–2672.
Denis, J., Acosta-Ramirez, E., Zhao, Y., Hamelin, M.E., Koukavica, I., Baz, M., Abed, Y., Savard, C., Pare, C., Lopez Macias, C., Boivin, G., Leclerc, D., 2008. Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. Vaccine 26, 3395–3403.
Derossi, D., Joliot, A.H., Chassaing, G., Prochiantz, A., 1994. The third helix of the Antennapedia homeodomain translocates through biological membranes. J. Biol. Chem. 269, 10444–10450.
Dormitzer, P.R., Galli, G., Castellino, F., Golding, H., Khurana, S., Del Giudice, G., Rappuoli, R., 2011. Influenza vaccine immunology. Immunol. Rev. 239, 167–177.
Ekiert, D.C., Bhabha, G., Elsliger, M.A., Friesen, R.H., Jongeneelen, M., Throsby, M., Goudsmit, J., Wilson, I.A., 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324, 246–251.
Epstein, S.L., Price, G.E., 2010. Cross-protective immunity to influenza A viruses. Expert Rev. Vaccines 9, 1325–1341.
Epstein, S.L., Misplon, J.A., Lawson, C.M., Subbarao, E.K., Connors, M., Murphy, B.R., 1993. Beta 2-microglobulin-deficient mice can be protected against influenza A infection by vaccination with vaccinia-influenza recombinants expressing hemagglutinin and neuraminidase. J. Immunol. 150, 5484–5493.
Epstein, S.L., Lo, C.Y., Misplon, J.A., Lawson, C.M., Hendrickson, B.A., Max, E.E., Subbarao, K., 1997. Mechanisms of heterosubtypic immunity to lethal influenza A virus infection in fully immunocompetent, T cell-depleted, beta2-microglobulin-deficient, and J chain-deficient mice. J. Immunol. 158, 1222–1230.
Ernst, W.A., Kim, H.J., Tumpey, T.M., Jansen, A.D., Tai, W., Cramer, D.V., Adler-Moore, J.P., Fujii, G., 2006. Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. Vaccine 24, 5158–5168.
Fan, J., Liang, X., Horton, M.S., Perry, H.C., Citron, M.P., Heidecker, G.J., Fu, T.M., Joyce, J., Przysiecki, C.T., Keller, P.M., Garsky, V.M., Ionescu, R., Rippeon, Y., Shi, L., Chastain, M.A., Condra, J.H., Davies, M.E., Liao, J., Emini, E.A., Shiver, J.W., 2004. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine 22, 2993–3003.
Feng, J., Zhang, M., Mozdzanowska, K., Zharikova, D., Hoff, H., Wunner, W., Couch, R.B., Gerhard, W., 2006. Influenza A virus infection engenders a poor antibody response against the ectodomain of matrix protein 2. Virol. J. 3, 102.
Fiers, W., De Filette, M., Birkett, A., Neirynck, S., Min Jou, W., 2004. A "universal" human influenza A vaccine. Virus Res. 103, 173–176.
Fiore, A.E., Fry, A., Shay, D., Gubareva, L., Bresee, J.S., Uyeki, T.M., 2011. Antiviral agents for the treatment and chemoprophylaxis of influenza–-recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Surveill. Summ. 60, 1–24.
Fleming, D.M., Andrews, N.J., Ellis, J.S., Bermingham, A., Sebastianpillai, P., Elliot, A.J., Miller, E., Zambon, M., 2010. Estimating influenza vaccine effectiveness using routinely collected laboratory data. J. Epidemiol. Commun. Health 64, 1062–1067.
Fodor, E., Crow, M., Mingay, L.J., Deng, T., Sharps, J., Fechter, P., Brownlee, G.G., 2002. A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. J. Virol. 76, 8989–9001.
Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D., 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J. Virol. 79, 2814–2822.
Frace, A.M., Klimov, A.I., Rowe, T., Black, R.A., Katz, J.M., 1999. Modified M2 proteins produce heterotypic immunity against influenza A virus. Vaccine 17, 2237–2244.
Friesen, R.H., Koudstaal, W., Koldijk, M.H., Weverling, G.J., Brakenhoff, J.P., Lenting, P.J., Stittelaar, K.J., Osterhaus, A.D., Kompier, R., Goudsmit, J., 2010. New class of monoclonal antibodies against severe influenza: prophylactic and therapeutic efficacy in ferrets. PLoS One 5, e9106.
Fu, T.M., Freed, D.C., Horton, M.S., Fan, J., Citron, M.P., Joyce, J.G., Garsky, V.M., Casimiro, D.R., Zhao, Q., Shiver, J.W., Liang, X., 2009. Characterizations of four monoclonal antibodies against M2 protein ectodomain of influenza A virus. Virology 385, 218–226.
Gamblin, S.J., Skehel, J.J., 2010. Influenza hemagglutinin and neuraminidase membrane glycoproteins. J. Biol. Chem. 285, 28403–28409.
Gerhard, W., Mozdzanowska, K., Furchner, M., Washko, G., Maiese, K., 1997. Role of the B-cell response in recovery of mice from primary influenza virus infection. Immunol. Rev. 159, 95–103.
Gerhard, W., Mozdzanowska, K., Zharikova, D., 2006. Prospects for universal influenza virus vaccine. Emerg. Infect. Dis. 12, 569–574.
Girard, M.P., Tam, J.S., Assossou, O.M., Kieny, M.P., 2010. The 2009 A (H1N1) influenza virus pandemic: A review. Vaccine 28, 4895–4902.
Gravel, C., Li, C., Wang, J., Hashem, A.M., Jaentschke, B., Xu, K.W., Lorbetskie, B., Gingras, G., Aubin, Y., Van Domselaar, G., Girard, M., He, R., Li, X., 2010. Qualitative and quantitative analyses of virtually all subtypes of influenza A and B viral neuraminidases using antibodies targeting the universally conserved sequences. Vaccine 28, 5774–5784.
Gulati, U., Hwang, C.C., Venkatramani, L., Gulati, S., Stray, S.J., Lee, J.T., Laver, W.G., Bochkarev, A., Zlotnick, A., Air, G.M., 2002. Antibody epitopes on the neuraminidase of a recent H3N2 influenza virus (A/Memphis/31/98). J. Virol. 76, 12274–12280.
Haynes, B.F., Fleming, J., St Clair, E.W., Katinger, H., Stiegler, G., Kunert, R., Robinson, J., Scearce, R.M., Plonk, K., Staats, H.F., Ortel, T.L., Liao, H.X., Alam, S.M., 2005. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science 308, 1906–1908.
Hensley, S.E., Das, S.R., Bailey, A.L., Schmidt, L.M., Hickman, H.D., Jayaraman, A., Viswanathan, K., Raman, R., Sasisekharan, R., Bennink, J.R., Yewdell, J.W., 2009. Hemagglutinin receptor binding avidity drives influenza A virus antigenic drift. Science 326, 734–736.
Ho, H.T., Qian, H.L., He, F., Meng, T., Szyporta, M., Prabhu, N., Prabakaran, M., Chan, K.P., Kwang, J., 2009. Rapid detection of H5N1 subtype influenza viruses by antigen capture enzyme-linked immunosorbent assay using H5- and N1-specific monoclonal antibodies. Clin. Vaccine Immunol. 16, 726–732.
Huber, V.C., Lynch, J.M., Bucher, D.J., Le, J., Metzger, D.W., 2001. Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. J. Immunol. 166, 7381–7388.
Huber, V.C., McKeon, R.M., Brackin, M.N., Miller, L.A., Keating, R., Brown, S.A., Makarova, N., Perez, D.R., Macdonald, G.H., McCullers, J.A., 2006. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol. 13, 981–990.
Hui, E.K., Yap, E.M., An, D.S., Chen, I.S., Nayak, D.P., 2004. Inhibition of influenza virus matrix (M1) protein expression and virus replication by U6 promoter-driven and lentivirus-mediated delivery of siRNA. J. Gen. Virol. 85, 1877–1884.
Huleatt, J.W., Nakaar, V., Desai, P., Huang, Y., Hewitt, D., Jacobs, A., Tang, J., McDonald, W., Song, L., Evans, R.K., Umlauf, S., Tussey, L., Powell, T.J., 2008. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine 26, 201–214.
Ito, T., Gorman, O.T., Kawaoka, Y., Bean, W.J., Webster, R.G., 1991. Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J. Virol. 65, 5491–5498.
Iwatsuki-Horimoto, K., Horimoto, T., Noda, T., Kiso, M., Maeda, J., Watanabe, S., Muramoto, Y., Fujii, K., Kawaoka, Y., 2006. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. J. Virol. 80, 5233–5240.
Jackson, D.C., Webster, R.G., 1982. A topographic map of the enzyme active center and antigenic sites on the neuraminidase of influenza virus A/Tokyo/3/67 (H2N2). Virology 123, 69–77.
Jameson, J., Cruz, J., Ennis, F.A., 1998. Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J. Virol. 72, 8682–8689.
Jegerlehner, A., Schmitz, N., Storni, T., Bachmann, M.F., 2004. Influenza A vaccine based on the extracellular domain of M2: weak protection mediated via antibody-dependent NK cell activity. J. Immunol. 172, 5598–5605.
Johansson, B.E., Matthews, J.T., Kilbourne, E.D., 1998. Supplementation of conventional influenza A vaccine with purified viral neuraminidase results in a balanced and broadened immune response. Vaccine 16, 1009–1015.
Karlsson Hedestam, G.B., Fouchier, R.A., Phogat, S., Burton, D.R., Sodroski, J., Wyatt, R.T., 2008. The challenges of eliciting neutralizing antibodies to HIV-1 and to influenza virus. Nat. Rev. Microbiol. 6, 143–155.
Kashyap, A.K., Steel, J., Oner, A.F., Dillon, M.A., Swale, R.E., Wall, K.M., Perry, K.J., Faynboym, A., Ilhan, M., Horowitz, M., Horowitz, L., Palese, P., Bhatt, R.R., Lerner, R.A., 2008. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc. Natl. Acad. Sci. USA 105, 5986–5991.
Kashyap, A.K., Steel, J., Rubrum, A., Estelles, A., Briante, R., Ilyushina, N.A., Xu, L., Swale, R.E., Faynboym, A.M., Foreman, P.K., Horowitz, M., Horowitz, L., Webby, R., Palese, P., Lerner, R.A., Bhatt, R.R., 2010. Protection from the 2009 H1N1 pandemic influenza by an antibody from combinatorial survivor-based libraries. PLoS Pathog. 6, e1000990.
Kaverin, N.V., Rudneva, I.A., Ilyushina, N.A., Varich, N.L., Lipatov, A.S., Smirnov, Y.A., Govorkova, E.A., Gitelman, A.K., Lvov, D.K., Webster, R.G., 2002. Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. J. Gen. Virol. 83, 2497–2505.
Kaverin, N.V., Rudneva, I.A., Ilyushina, N.A., Lipatov, A.S., Krauss, S., Webster, R.G., 2004. Structural differences among hemagglutinins of influenza A virus subtypes are reflected in their antigenic architecture: analysis of H9 escape mutants. J. Virol. 78, 240–249.
Kaverin, N.V., Rudneva, I.A., Govorkova, E.A., Timofeeva, T.A., Shilov, A.A., Kochergin-Nikitsky, K.S., Krylov, P.S., Webster, R.G., 2007. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J. Virol. 81, 12911–12917.
Kawaoka, Y., Yamnikova, S., Chambers, T.M., Lvov, D.K., Webster, R.G., 1990. Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology 179, 759–767.

Kitching, A., Roche, A., Balasegaram, S., Heathcock, R., Maguire, H., 2009. Oseltamivir adherence and side effects among children in three London schools affected by influenza A(H1N1)v, May 2009 - an internet-based cross-sectional survey. Eur. Surveill. 14, 19287.

Koudstaal, W., Koldijk, M.H., Brakenhoff, J.P., Cornelissen, L.A., Weverling, G.J., Friesen, R.H., Goudsmit, J., 2009. Pre- and postexposure use of human monoclonal antibody against H5N1 and H1N1 influenza virus in mice. viable alternative to oseltamivir. J. Infect. Dis. 200, 1870–1873.

Krause, J.C., Tumpey, T.M., Huffman, C.J., McGraw, P.A., Pearce, M.B., Tsibane, T., Hai, R., Basler, C.F., Crowe Jr., J.E., 2010. Naturally occurring human monoclonal antibodies neutralize both 1918 and 2009 pandemic influenza A (H1N1) viruses. J. Virol. 84, 3127–3130.

Kreijtz, J.H., Bodewes, R., van Amerongen, G., Kuiken, T., Fouchier, R.A., Osterhaus, A.D., Rimmelzwaan, G.F., 2007. Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice. Vaccine 25, 612–620.

Kreijtz, J.H., de Mutsert, G., van Baalen, C.A., Fouchier, R.A., Osterhaus, A.D., Rimmelzwaan, G.F., 2008. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J. Virol. 82, 5161–5166.

Lalor, P.A., Webby, R.J., Morrow, J., Rusalov, D., Kaslow, D.C., Rolland, A., Smith, L.R., 2008. Plasmid DNA-based vaccines protect mice and ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus. J. Infect. Dis. 197, 1643–1652.

Lamb, R.A., Zebedee, S.L., Richardson, C.D., 1985. Influenza virus M2 protein is an integral membrane protein expressed on the infected-cell surface. Cell 40, 627–633.

Lambert, L.C., Fauci, A.S., 2010. Influenza vaccines for the future. N. Engl. J. Med. 363, 2036–2044.

Lee, J.T., Air, G.M., 2006. Interaction between a 1998 human influenza virus N2 neuraminidase and monoclonal antibody Mem5. Virology 345, 424–433.

Lee, L.Y., Ha do, L.A., Simmons, C., de Jong, M.D., Chau, N.V., Schumacher, R., Peng, Y.C., McMichael, A.J., Farrar, J.J., Smith, G.L., Townsend, A.R., Askonas, B.A., Rowland-Jones, S., Dong, T., 2008. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J. Clin. Invest. 118, 3478–3490.

Leopold, P.L., Wendland, R.L., Vincent, T., Crystal, R.G., 2006. Neutralized adenovirus-immune complexes can mediate effective gene transfer via an Fc receptor-dependent infection pathway. J. Virol. 80, 10237–10247.

Liang, S., Mozdzanowska, K., Palladino, G., Gerhard, W., 1994. Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. J. Immunol. 152, 1653–1661.

Lim, A.P., Wong, S.K., Chan, A.H., Chan, C.E., Ooi, E.E., Hanson, B.J., 2008. Epitope characterization of the protective monoclonal antibody VN04–2 shows broadly neutralizing activity against highly pathogenic H5N1. Virol. J. 5, 80.

Liu, W., Li, H., Chen, Y.H., 2003. N-terminus of M2 protein could induce antibodies with inhibitory activity against influenza virus replication. FEMS Immunol. Med. Microbiol. 35, 141–146.

Liu, W., Peng, Z., Liu, Z., Lu, Y., Ding, J., Chen, Y.H., 2004a. High epitope density in a single recombinant protein molecule of the extracellular domain of influenza A virus M2 protein significantly enhances protective immunity. Vaccine 23, 366–371.

Liu, W., Zou, P., Chen, Y.H., 2004b. Monoclonal antibodies recognizing EVETPIRN epitope of influenza A virus M2 protein could protect mice from lethal influenza A virus challenge. Immunol. Lett. 93, 131–136.

Luke, T.C., Kilbane, E.M., Jackson, J.L., Hoffman, S.L., 2006. Meta-analysis: convalescent blood products for Spanish influenza pneumonia: a future H5N1 treatment? Ann. Intern. Med. 145, 599–609.

Malby, R.L., Tulip, W.R., Harley, V.R., McKimm-Breschkin, J.L., Laver, W.G., Webster, R.G., Colman, P.M., 1994. The structure of a complex between the NC10 antibody and influenza virus neuraminidase and comparison with the overlapping binding site of the NC41 antibody. Structure 2, 733–746.

Mancini, N., Solforosi, L., De Marco, D., and Clementi, N. (2010). Heterosubtypic neutralizing activity of two human monoclonal antibodies recognizing epitopes broadly shared among different influenza subtypes. Antiviral Congress, Amsterdam, The Netherlands, 7-9 November 2010. Abstract P2.54.

Manheimer-Lory, A., Katz, J.B., Pillinger, M., Ghossein, C., Smith, A., Diamond, B., 1991. Molecular characteristics of antibodies bearing an anti-DNA-associated idiotype. J. Exp. Med. 174, 1639–1652.

McCauley, J.W., Mahy, B.W., 1983. Structure and function of the influenza virus genome. Biochem. J. 211, 281–294.

McCown, M.F., Pekosz, A., 2006. Distinct domains of the influenza a virus M2 protein cytoplasmic tail mediate binding to the M1 protein and facilitate infectious virus production. J. Virol. 80, 8178–8189.

McDonald, N.J., Smith, C.B., Cox, N.J., 2007. Antigenic drift in the evolution of H1N1 influenza A viruses resulting from deletion of a single amino acid in the haemagglutinin gene. J. Gen. Virol. 88, 3209–3213.

Monto, A.S., Kendal, A.P., 1973. Effect of neuraminidase antibody on Hong Kong influenza. Lancet 1, 623–625.

Monto, A.S., Ansaldi, F., Aspinall, R., McElhaney, J.E., Montano, L.F., Nichol, K.L., Puig-Barbera, J., Schmitt, J., Stephenson, I., 2009. Influenza control in the 21st century: optimizing protection of older adults. Vaccine 27, 5043–5053.

Moreno, A., Brocchi, E., Lelli, D., Gamba, D., Tranquillo, M., Cordioli, P., 2009. Monoclonal antibody based ELISA tests to detect antibodies against neuraminidase subtypes 1, 2 and 3 of avian influenza viruses in avian sera. Vaccine 27, 4967–4974.

Mould, J.A., Drury, J.E., Frings, S.M., Kaupp, U.B., Pekosz, A., Lamb, R.A., Pinto, L.H., 2000. Permeation and activation of the M2 ion channel of influenza A virus. J. Biol. Chem. 275, 31038–31050.

Mozdzanowska, K., Maiese, K., Furchner, M., Gerhard, W., 1999. Treatment of influenza virus-infected SCID mice with nonneutralizing antibodies specific for the transmembrane proteins matrix 2 and neuraminidase reduces the pulmonary virus titer but fails to clear the infection. Virology 254, 138–146.

Mozdzanowska, K., Feng, J., Eid, M., Kragol, G., Cudic, M., Otvos Jr., L., Gerhard, W., 2003. Induction of influenza type A virus-specific resistance by immunization of mice with a synthetic multiple antigenic peptide vaccine that contains ectodomains of matrix protein 2. Vaccine 21, 2616–2626.

Muckelbauer, J.K., Rossmann, M.G., 1997. The structure of coxsackievirus B3. Curr. Top. Microbiol. Immunol. 223, 191–208.

Muckelbauer, J.K., Kremer, M., Minor, I., Tong, L., Zlotnick, A., Johnson, J.E., Rossmann, M.G., 1995. Structure determination of coxsackievirus B3 to 3.5 A resolution. Acta Crystallogr. D Biol. Crystallogr 51, 871–887.

Mukhtar, M.M., Li, S., Li, W., Wan, T., Mu, Y., Wei, W., Kang, L., Rasool, S.T., Xiao, Y., Zhu, Y., Wu, J., 2009. Single-chain intracellular antibodies inhibit influenza virus replication by disrupting interaction of proteins involved in viral replication and transcription. Int. J. Biochem. Cell. Biol. 41, 554–560.

Nabel, G.J., Fauci, A.S., 2010. Induction of unnatural immunity: prospects for a broadly protective universal influenza vaccine. Nat. Med. 16, 1389–1391.

Naikhin, A.N., Tsaritsina, I.M., Oleinikova, E.V., Syrodoeva, L.G., Korchanova, N.L., Denisov, G.M., Ya Shvartsman, S., 1983. The importance of antineuraminidase antibodies in resistance to influenza A and immunologic memory for their synthesis. J. Hyg. (Lond.) 91, 131–138.

Nayak, D.P., Hui, E.K., Barman, S., 2004. Assembly and budding of influenza virus. Virus Res. 106, 147–165.

Neirynck, S., Deroo, T., Saelens, X., Vanlandschoot, P., Jou, W.M., Fiers, W., 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat. Med. 5, 1157–1163.

Nguyen, H.H., van Ginkel, F.W., Vu, H.L., McGhee, J.R., Mestecky, J., 2001. Heterosubtypic immunity to influenza A virus infection requires B cells but not CD8+ cytotoxic T lymphocytes. J. Infect. Dis. 183, 368–376.

Noda, T., Sagara, H., Yen, A., Takada, A., Kida, H., Cheng, R.H., Kawaoka, Y., 2006. Architecture of ribonucleoprotein complexes in influenza A virus particles. Nature 439, 490–492.

Okada, J., Ohshima, N., Kubota-Koketsu, R., Ota, S., Takase, W., Azuma, M., Iba, Y., Nakagawa, N., Yoshikawa, T., Nakajima, Y., Ishikawa, T., Asano, Y., Okuno, Y., Kurosawa, Y., 2010. Monoclonal antibodies in man that neutralized H3N2 influenza viruses were classified into three groups with distinct strain specificity: 1968–1973, 1977–1993 and 1997–2003. Virology 397, 322–330.

Okuno, Y., Isegawa, Y., Sasao, F., Ueda, S., 1993. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J. Virol. 67, 2552–2558.

Okuno, Y., Matsumoto, K., Isegawa, Y., Ueda, S., 1994. Protection against the mouse-adapted A/FM/1/47 strain of influenza A virus in mice by a monoclonal antibody with cross-neutralizing activity among H1 and H2 strains. J. Virol. 68, 517–520.

Palese, P. (2004). Influenza: old and new threats. *Nat. Med.* **10** (Suppl.), S82-S87.

Palladino, G., Mozdzanowska, K., Washko, G., Gerhard, W., 1995. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J. Virol. 69, 2075–2081.

Palmer, P., Charley, B., Rombaut, B., Daeron, M., Lebon, P., 2000. Antibody-dependent induction of type I interferons by poliovirus in human mononuclear blood cells requires the type II fcgamma receptor (CD32). Virology 278, 86–94.

Perotti, M., Ghidoli, N., Altara, R., Diotti, R.A., Clementi, N., De Marco, D., Sassi, M., Clementi, M., Burioni, R., Mancini, N., 2008. Hepatitis C virus (HCV)-driven stimulation of subfamily-restricted natural IgM antibodies in mixed cryoglobulinemia. Autoimmun. Rev. 7, 468–472.

Ping, J., Li, C., Deng, G., Jiang, Y., Tian, G., Zhang, S., Bu, Z., Chen, H., 2008. Single-amino-acid mutation in the HA alters the recognition of H9N2 influenza virus by a monoclonal antibody. Biochem. Biophys. Res. Commun. 371, 168–171.

Pinto, L.H., Holsinger, L.J., Lamb, R.A., 1992. Influenza virus M2 protein has ion channel activity. Cell 69, 517–528.

Pos, W., Luken, B.M., Hovinga, J.A., Turenhout, E.A., Scheiflinger, F., Dong, J.F., Fijnheer, R., Voorberg, J., 2009. VH1–69 germline encoded antibodies directed towards ADAMTS13 in patients with acquired thrombotic thrombocytopenic purpura. J. Thromb. Haemost. 7, 421–428.

Poungpair, O., Chaicumpa, W., Kulkeaw, K., Maneewatch, S., Thueng-in, K., Srimanote, P., Tongtawe, P., Songserm, T., Lekcharoensuk, P., Tapchaisri, P., 2009. Human single chain monoclonal antibody that recognizes matrix protein of heterologous influenza A virus subtypes. J. Virol. Methods 159, 105–111.

Poungpair, O., Pootong, A., Maneewatch, S., Srimanote, P., Tongtawe, P., Songserm, T., Tapchaisri, P., Chaicumpa, W., 2010. A human single chain transbody specific to matrix protein (M1) interferes with the replication of influenza A virus. Bioconjug. Chem. 21, 1134–1141.

Prabhu, R., Khalap, N., Burioni, R., Clementi, M., Garry, R.F., Dash, S., 2004. Inhibition of hepatitis C virus nonstructural protein, helicase activity, and viral replication by a recombinant human antibody clone. Am. J. Pathol. 165, 1163–1173.

Quan, F.S., Compans, R.W., Nguyen, H.H., Kang, S.M., 2008a. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. J. Virol. 82, 1350–1359.

Quan, F.S., Steinhauer, D., Huang, C., Ross, T.M., Compans, R.W., Kang, S.M., 2008b. A bivalent influenza VLP vaccine confers complete inhibition of virus replication in lungs. Vaccine 26, 3352–3361.

Ramirez-Gonzalez, J.E., Gonzalez-Duran, E., Alcantara-Perez, P., Wong-Arambula, C., Olivera-Diaz, H., Cortez-Ortiz, I., Barrera-Badillo, G., Nguyen, H., Gubareva, L., Lopez-Martinez, I., Diaz-Quinonez, J.A., Lezana-Fernandez, M.A., Gatell-Ramirez, H.L., Cordova Villalobos, J.A., Hernandez-Avila, M., Alpuche-Aranda, C., 2011. Oseltamivir-Resistant Pandemic (H1N1) 2009 Virus, Mexico. Emerg. Infect. Dis. 17, 283–286.

Renegar, K.B., Small Jr., P.A., Boykins, L.G., Wright, P.F., 2004. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. J. Immunol. 173, 1978–1986.

Sakabe, S., Iwatsuki-Horimoto, K., Horimoto, T., Nidom, C.A., Le, M.Q., Takano, R., Kubota-Koketsu, R., Okuno, Y., Ozawa, M., Kawaoka, Y.A., 2009. A cross-reactive neutralizing monoclonal antibody protects mice from H5N1 and pandemic (H1N1) 2009 virus infection. Antiviral Res. 88, 249–255.

Sambhara, S., Kurichh, A., Miranda, R., Tumpey, T., Rowe, T., Renshaw, M., Arpino, R., Tamane, A., Kandil, A., James, O., Underdown, B., Klein, M., Katz, J., Burt, D., 2001. Heterosubtypic immunity against human influenza A viruses, including recently emerged avian H5 and H9 viruses, induced by FLU-ISCOM vaccine in mice requires both cytotoxic T-lymphocyte and macrophage function. Cell. Immunol. 211, 143–153.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V., 2008. Surveillance for neuraminidase inhibitor resistance among human influenza A and B viruses circulating worldwide from 2004 to 2008. Antimicrob. Agents Chemother. 52, 3284–3292.

Simmons, C.P., Bernasconi, N.L., Suguitan, A.L., Mills, K., Ward, J.M., Chau, N.V., Hien, T.T., Sallusto, F., Hado, Q., Farrar, J., de Jong, M.D., Lanzavecchia, A., Subbarao, K., 2007. Prophylactic and therapeutic efficacy of human monoclonal antibodies against H5N1 influenza. PLoS Med. 4, e178.

Skehel, J.J., Wiley, D.C., 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. 69, 531–569.

Skehel, J.J., Stevens, D.J., Daniels, R.S., Douglas, A.R., Knossow, M., Wilson, I.A., Wiley, D.C., 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. USA 81, 1779–1783.

Slepushkin, V.A., Katz, J.M., Black, R.A., Gamble, W.C., Rota, P.A., Cox, N.J., 1995. Protection of mice against influenza A virus challenge by vaccination with baculovirus-expressed M2 protein. Vaccine 13, 1399–1402.

Smirnov, Y.A., Lipatov, A.S., Gitelman, A.K., Okuno, Y., Van Beek, R., Osterhaus, A.D., Claas, E.C., 1999. An epitope shared by the hemagglutinins of H1, H2, H5, and H6 subtypes of influenza A virus. Acta Virol. 43, 237–244.

Stanekova, Z., Vareckova, E., 2010. Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. Virol. J. 7, 351.

Steel, J., Lowen, A.C., Wang, T.T., Yondola, M., Gao, Q., Haye, K., Garcia-Sastre, A., Palese, P., 2010. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio 1 (1).

Steinhoff, M.C., 2011. Influenza in young children: burden, immunisation, and policy. Lancet Infect. Dis. 11, 2–3.

Sui, J., Hwang, W.C., Perez, S., Wei, G., Aird, D., Chen, L.M., Santelli, E., Stec, B., Cadwell, G., Ali, M., Wan, H., Murakami, A., Yammanuru, A., Han, T., Cox, N.J., Bankston, L.A., Donis, R.O., Liddington, R.C., Marasco, W.A., 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat. Struct. Mol. Biol. 16, 265–273.

Sukeno, N., Otsuki, Y., Konno, J., Yamane, N., Odagiri, T., Arikawa, J., Ishida, N., 1979. Anti-nucleoprotein antibody response in influenza A infection. Tohoku J. Exp. Med. 128, 241–249.

Sultana, I., Gao, J., Markoff, L., Eichelberger, M.C., 2011. Influenza neuraminidase-inhibiting antibodies are induced in the presence of zanamivir. Vaccine 29, 2601–2606.

Sylte, M.J., Suarez, D.L., 2009. Influenza neuraminidase as a vaccine antigen. Curr. Top. Microbiol. Immunol. 333, 227–241.

Takada, A., Matsushita, S., Ninomiya, A., Kawaoka, Y., Kida, H., 2003. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. Vaccine 21, 3212–3218.

Tamura, S., 2010. Studies on the usefulness of intranasal inactivated influenza vaccines. Vaccine 28, 6393–6397.

Tan, P.T., Heiny, A.T., Miotto, O., Salmon, J., Marques, E.T., Lemonnier, F., August, J.T., 2010. Conservation and diversity of influenza A H1N1 HLA-restricted T cell epitope candidates for epitope-based vaccines. PLoS One 5, e8754.

Thathaisong, U., Maneewatch, S., Kulkeaw, K., Thueng-In, K., Poungpair, O., Srimanote, P., Songserm, T., Tongtawe, P., Tapchaisri, P., Chaicumpa, W., 2008. Human monoclonal single chain antibodies (HuScFv) that bind to the polymerase proteins of influenza A virus. Asian Pac. J. Allergy Immunol. 26, 23–35.

Throsby, M., van den Brink, E., Jongeneelen, M., Poon, L.L., Alard, P., Cornelissen, L., Bakker, A., Cox, F., van Deventer, E., Guan, Y., Cinatl, J., ter Meulen, J., Lasters, I., Carsetti, R., Peiris, M., de Kruif, J., Goudsmit, J., 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 3, e3942.

Tompkins, S.M., Zhao, Z.S., Lo, C.Y., Misplon, J.A., Liu, T., Ye, Z., Hogan, R.J., Wu, Z., Benton, K.A., Tumpey, T.M., Epstein, S.L., 2007. Matrix protein 2 vaccination and protection against influenza viruses, including subtype H5N1. Emerg. Infect. Dis. 13, 426–435.

Treanor, J.J., Tierney, E.L., Zebedee, S.L., Lamb, R.A., Murphy, B.R., 1990. Passively transferred monoclonal antibody to the M2 protein inhibits influenza A virus replication in mice. J. Virol. 64, 1375–1377.

Tsuchiya, E., Sugawara, K., Hongo, S., Matsuzaki, Y., Muraki, Y., Li, Z.N., Nakamura, K., 2001. Antigenic structure of the haemagglutinin of human influenza A/H2N2 virus. J. Gen. Virol. 82, 2475–2484.

Tu, W., Mao, H., Zheng, J., Liu, Y., Chiu, S.S., Qin, G., Chan, P.L., Lam, K.T., Guan, J., Zhang, L., Guan, Y., Yuen, K.Y., Peiris, J.S., Lau, Y.L., 2010. Cytotoxic T lymphocytes established by seasonal human influenza cross-react against 2009 pandemic H1N1 influenza virus. J. Virol. 84, 6527–6535.

Tulip, W.R., Varghese, J.N., Laver, W.G., Webster, R.G., Colman, P.M., 1992. Refined crystal structure of the influenza virus N9 neuraminidase-NC41 Fab complex. J. Mol. Biol. 227, 122–148.

Tumpey, T.M., Renshaw, M., Clements, J.D., Katz, J.M., 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J. Virol. 75, 5141–5150.

Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., Hawe, L.A., Leander, K.R., Martinez, D., Perry, H.C., Shiver, J.W., Montgomery, D.L., Liu, M.A., 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259, 1745–1749.

Underwood, P.A., 1982. Mapping of antigenic changes in the haemagglutinin of Hong Kong influenza (H3N2) strains using a large panel of monoclonal antibodies. J. Gen. Virol. 62, 153–169.

VanEs, J.H., Aanstoot, H., Gmelig-Meyling, F.H., Derksen, R.H., Logtenberg, T., 1992. A human systemic lupus erythematosus-related anti-cardiolipin/single-stranded DNA autoantibody is encoded by a somatically mutated variant of the developmentally restricted 51P1 VH gene. J. Immunol. 149, 2234–2240.

Vareckova, E., Mucha, V., Wharton, S.A., Kostolansky, F., 2003. Inhibition of fusion activity of influenza A haemagglutinin mediated by HA2-specific monoclonal antibodies. Arch. Virol. 148, 469–486.

Verkoczy, L., Diaz, M., Holl, T.M., Ouyang, Y.B., Bouton-Verville, H., Alam, S.M., Liao, H.X., Kelsoe, G., Haynes, B.F., 2010. Autoreactivity in an HIV-1 broadly reactive neutralizing antibody variable region heavy chain induces immunologic tolerance. Proc. Natl. Acad. Sci. USA 107, 181–186.

Wang, T.T., Palese, P., 2009. Universal epitopes of influenza virus hemagglutinins? Nat. Struct. Mol. Biol. 16, 233–234.

Wang, R., Song, A., Levin, J., Dennis, D., Zhang, N.J., Yoshida, H., Koriazova, L., Madura, L., Shapiro, L., Matsumoto, A., Yoshida, H., Mikayama, T., Kubo, R.T., Sarawar, S., Cheroutre, H., Kato, S., 2008. Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. Antiviral Res. 80, 168–177.

Wang, C.C., Chen, J.R., Tseng, Y.C., Hsu, C.H., Hung, Y.F., Chen, S.W., Chen, C.M., Khoo, K.H., Cheng, T.J., Cheng, Y.S., Jan, J.T., Wu, C.Y., Ma, C., Wong, C.H., 2009. Glycans on influenza hemagglutinin affect receptor binding and immune response. Proc. Natl. Acad. Sci. USA 106, 18137–18142.

Wang, B., Dwyer, D.E., Blyth, C.C., Soedjono, M., Shi, H., Kesson, A., Ratnamohan, M., McPhie, K., Cunningham, A.L., Saksena, N.K., 2010a. Detection of the rapid emergence of the H275Y mutation associated with oseltamivir resistance in severe pandemic influenza virus A/H1N1 09 infections. Antiviral Res. 87, 16–21.

Wang, T.T., Tan, G.S., Hai, R., Pica, N., Petersen, E., Moran, T.M., Palese, P., 2010b. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. PLoS Pathog. 6, e1000796.

Webster, R.G., Brown, L.E., Laver, W.G., 1984. Antigenic and biological characterization of influenza virus neuraminidase (N2) with monoclonal antibodies. Virology 135, 30–42.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., Kawaoka, Y., 1992. Evolution and ecology of influenza A viruses. Microbiol Rev. 56, 152–179.

Whittaker, G., Bui, M., Helenius, A., 1996. The role of nuclear import and export in influenza virus infection. Trends Cell. Biol. 6, 67–71.

Wiley, D.C., Wilson, I.A., Skehel, J.J., 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 289, 373–378.

Wilson, I.A., Skehel, J.J., Wiley, D.C., 1981. Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature 289, 366–373.

Wrammert, J., Koutsonanos, D., Li, G.M., Edupuganti, S., Sui, J., Morrissey, M., McCausland, M., Skountzou, I., Hornig, M., Lipkin, W.I., Mehta, A., Razavi, B., Del Rio, C., Zheng, N.Y., Lee, J.H., Huang, M., Ali, Z., Kaur, K., Andrews, S., Amara, R.R., Wang, Y., Das, S.R., O'Donnell, C.D., Yewdell, J.W., Subbarao, K., Marasco, W.A., Mulligan, M.J., Compans, R., Ahmed, R., Wilson, P.C., 2011. Broadly cross-reactive antibodies dominate the human B cell response against 2009 pandemic H1N1 influenza virus infection. J. Exp. Med. 208, 181–193.

Wu, F., Huang, J.H., Yuan, X.Y., Huang, W.S., Chen, Y.H., 2007. Characterization of immunity induced by M2e of influenza virus. Vaccine 25, 8868–8873.

Yang, J., Yoshida, R., Kariya, Y., Zhang, X., Hashiguchi, S., Nakashima, T., Suda, Y., Takada, A., Ito, Y., Sugimura, K., 2010. Characterization of human single-chain antibodies against highly pathogenic avian influenza H5N1 viruses: mimotope and neutralizing activity. J. Biochem. 148, 507–515.

Yewdell, J.W., Frank, E., Gerhard, W., 1981. Expression of influenza A virus internal antigens on the surface of infected P815 cells. J. Immunol. 126, 1814–1819.

Yu, X., Tsibane, T., McGraw, P.A., House, F.S., Keefer, C.J., Hicar, M.D., Tumpey, T.M., Pappas, C., Perrone, L.A., Martinez, O., Stevens, J., Wilson, I.A., Aguilar, P.V., Altschuler, E.L., Basler, C.F., Crowe Jr., J.E., 2008. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature 455, 532–536.

Zebedee, S.L., Lamb, R.A., 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. J. Virol. 62, 2762–2772.

Zebedee, S.L., Richardson, C.D., Lamb, R.A., 1985. Characterization of the influenza virus M2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. J. Virol. 56, 502–511.

Zharikova, D., Mozdzanowska, K., Feng, J., Zhang, M., Gerhard, W., 2005. Influenza type A virus escape mutants emerge in vivo in the presence of antibodies to the ectodomain of matrix protein 2. J. Virol. 79, 6644–6654.

Zheng, B., Zhang, Y., He, H., Marinova, E., Switzer, K., Wansley, D., Mbawuike, I., Han, S., 2007. Rectification of age-associated deficiency in cytotoxic T cell response to influenza A virus by immunization with immune complexes. J. Immunol. 179, 6153–6159.

Zhou, B., Zhong, N., Guan, Y., 2007. Treatment with convalescent plasma for influenza A (H5N1) infection. N. Engl. J. Med. 357, 1450–1451.

Zou, P., Liu, W., Chen, Y.H., 2005. The epitope recognized by a monoclonal antibody in influenza A virus M2 protein is immunogenic and confers immune protection. Int. Immunopharmacol. 5, 631–635.