Emerging Microbes and Infections (2012) 1, e13; doi:10.1038/emi.2012.1

www.nature.com/emi

REVIEW

Receptor-binding domains of spike proteins of emerging or re-emerging viruses as targets for development of antiviral vaccines

Shibo Jiang[1,2], Lu Lu[1], Qi Liu[1,3], Wei Xu[1] and Lanying Du[2]

A number of emerging and re-emerging viruses have caused epidemics or pandemics of infectious diseases leading to major devastations throughout human history. Therefore, developing effective and safe vaccines against these viruses is clearly important for the protection of at-risk populations. Our previous studies have shown that the receptor-binding domain (RBD) in the spike protein of severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) is a key target for the development of SARS vaccines. In this review, we highlight some key advances in the development of antiviral vaccines targeting the RBDs of spike proteins of emerging and re-emerging viruses, using SARS-CoV, influenza virus, Hendra virus (HeV) and Nipah virus (NiV) as examples.
Emerging Microbes and Infections (2012) **1,** e13; doi:10.1038/emi.2012.1; published online 8 August 2012

Keywords: Hendra virus; influenza virus; Nipah virus; receptor-binding domain; SARS-CoV; spike protein; vaccine; virus

INTRODUCTION

Epidemics and pandemics of emerging and re-emerging viral infectious diseases, such as severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV),[1–3] influenza virus,[4] Hendra virus (HeV) and Nipah virus (NiV),[5,6] have posed a great threat to public health, human safety and economic stability worldwide. Therefore, development of effective and safe vaccines to prevent these diseases is urgently needed.

Safety concerns have arisen over the use of whole inactivated or live-attenuated viruses for the development of antiviral vaccines;[7] therefore, scientists have been attempting to design vaccines based on the spike proteins of emerging and re-emerging viruses. Indeed, spike protein-based vaccines are safer and more effective than vaccines based on inactivated or live-attenuated viruses.[8] However, new concerns have been raised over the use of full-length viral spike proteins for developing subunit vaccines because some of the non-neutralizing, or immunodominant, epitopes in the spike proteins may induce antibodies that enhance, rather than neutralize, viral infection, or otherwise cause harmful inflammatory responses in vaccinated hosts.[9,10] Therefore, selection of the key functional domains that contain neutralizing and/or T-cell epitopes as the immunogens appears to be an important strategy in developing antiviral vaccines.

Infection is initiated upon binding of the viral spike protein with the receptor or coreceptor on the target cell surface, as mediated by the spike protein's receptor-binding domain (RBD) (Figure 1). Therefore,

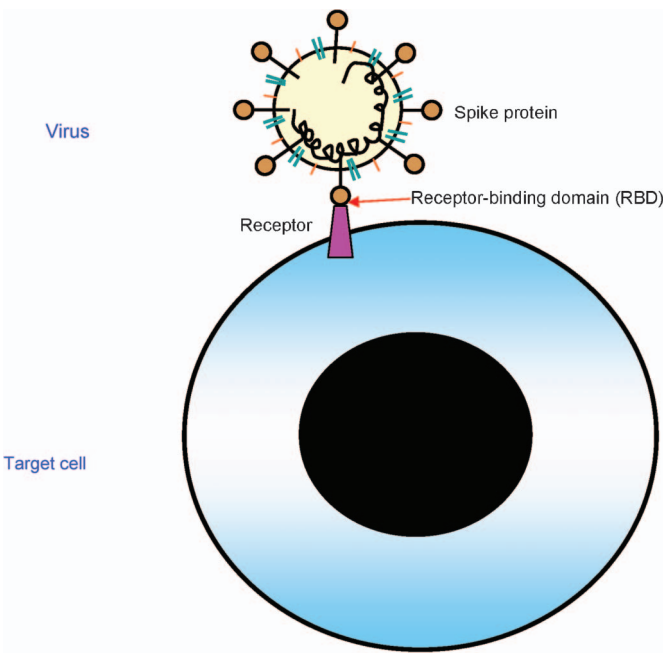

Figure 1 The interaction between the receptor-binding domain in the spike of an emerging virus and the corresponding receptor on the target cell.

[1]MOE/MOH Key Laboratory of Medical Molecular Virology, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai 200032, China; [2]Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY 10065, USA and [3]Department of Medical Microbiology and Immunology, School of Basic Medicine, Dali University, Dali 671000, China
Correspondence: SB Jiang
E-mail: shibojiang@fudan.edu.cn
Received 7 March 2012; revised 10 March 2012; accepted 12 March 2012

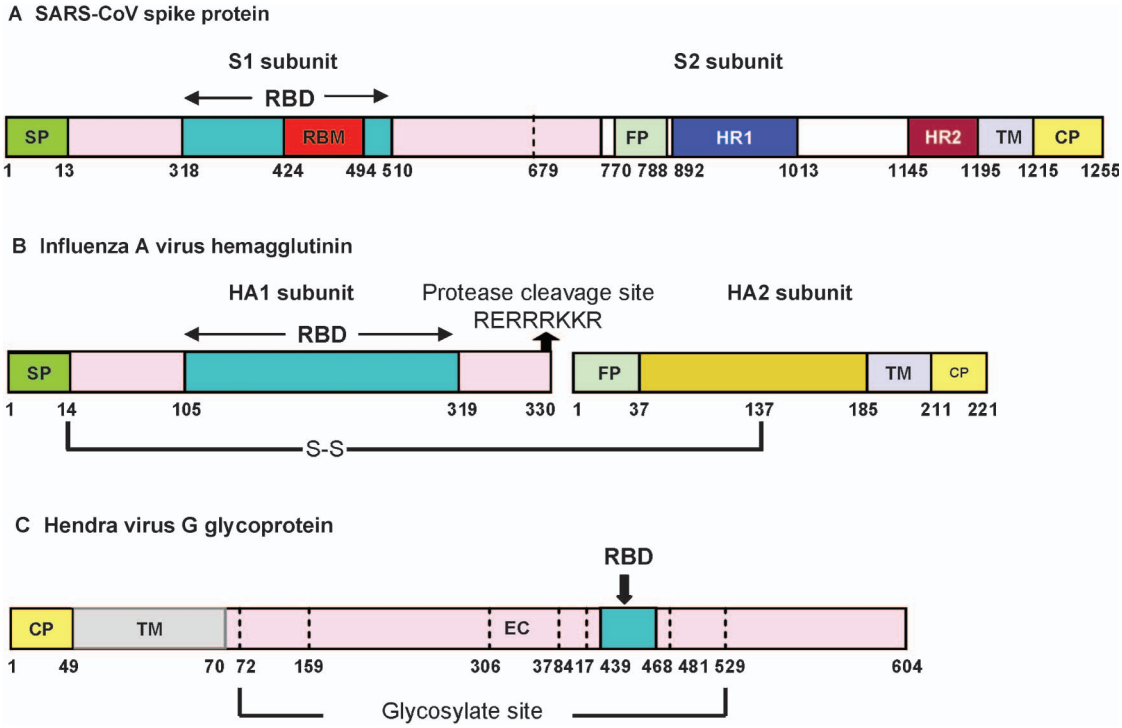

Figure 2 The RBDs in the envelope glycoproteins of SARS-CoV, influenza virus and Hendra virus. (**A**) RBD in the SARS-CoV spike protein.[14,17,79] The residue numbers of each region represent their positions in the S protein of SARS-CoV. (**B**) RBD in influenza virus HA. Taking H5N1 as an example, HA consists of a globular 'head' HA1 subunit containing the receptor-binding domain RBD and a membrane proximal HA2 subunit with α-helical stalk region (FP). The leading sequence (SP) is located at the N-terminus of HA1. There is a protease cleavage site between HA1 and HA2 subunits characterized by specific amino acid sequences, e.g., RERRRKKR. HA1 and HA2 subunits were connected by interchain disulfide bond from HA1-aa14 to HA2-aa137.[80] (**C**) RBD in the Hendra virus G glycoprotein. There are many glycosylate sites in the extracellular domain located in 72, 159, 306, 378, 417, 481 and 529, respectively. Residues from aa 439 to aa 468, which are located in RBD domain, are an important domain within G for receptor interaction. CP, cytoplasm domain; EC, extracellular domain; FP, fusion peptide; HA, hemagglutinin; HR, heptad repeat; RBM, receptor-binding motif; TM, transmembrane domain; SP, signal peptide.

the RBD plays an important role in viral attachment, fusion and entry and may serve as an attractive target for designing antiviral vaccines. Our previous studies have demonstrated that the vaccines based on the RBD in the spike protein of SARS-CoV (Figure 2A) induced the most potent neutralizing antibody responses and protective immunity in vaccinated animals,[11–17] suggesting that the RBDs in the spike proteins of other viruses that cause emerging and re-emerging infectious diseases, such as influenza virus (Figure 2B), HeV and NiV (Figure 2C), may also serve as targets for developing vaccines to prevent corresponding infectious diseases.[18]

Here, we reviewed important advances in the development of antiviral vaccines that mainly target the RBD of spike proteins of some viruses which have caused serious emerging and re-emerging infectious diseases during the past three decades, and assess the feasibility of applying similar strategies in the design and development of vaccines against other emerging and re-emerging viruses.

DEVELOPMENT OF SARS VACCINES TARGETING THE RBD OF SARS-COV SPIKE PROTEIN

SARS, as the first new infectious disease identified in the twenty-first century, is an acute and severe respiratory disease. After it originated in Guangdong Province of China in 2002,[3] SARS rapidly spread to 29 countries and areas around the world. SARS-CoV, the causative agent, was identified in April 2003.[19] A total of 8098 SARS cases with 774 deaths were reported during the 2003 outbreak. Although SARS-CoV was believed to be eliminated from human circulation in July 2003, its brief recurrence in laboratory workers in China between late 2003 and early 2004 and its ubiquity in nature both pose a threat for its re-emergence in human populations through zoonotic reintroduction, laboratory escape, or bioterrorism.[20] Thus, there is an urgent need for effective strategies against this devastating viral agent, especially in high-risk groups, including the elderly, healthcare workers and laboratory personnel.

SARS-CoV primarily transmits via droplets (respiratory secretions) and close person-to-person contact. It may exist in respiratory secretions, stools, urines and sweats in SARS patients. After entering the body, the virus binds to its receptor angiotensin-converting enzyme 2,[21,22] which expresses on the primary target cells, pneumocytes and enterocytes in the respiratory system, and other susceptible cells, such as intestinal mucosal cells, renal tubular epithelial cells, cerebral neurons and immune cells.[23] After permeating these cells, the virus replicates and is released to infect new target cells (Figure 3). The production and activation levels of proinflammatory chemokines and cytokines are remarkably elevated, causing significant lung tissue damage that results in atypical pneumonia with rapid respiratory deterioration and failure.[24]

The S protein and its RBD of SARS-CoV

SARS-CoV is an enveloped virus containing a single and positive-stranded RNA. The genome RNA encodes nonstructural replicase polyprotein and structural proteins, including spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins.[2,25] SARS-CoV

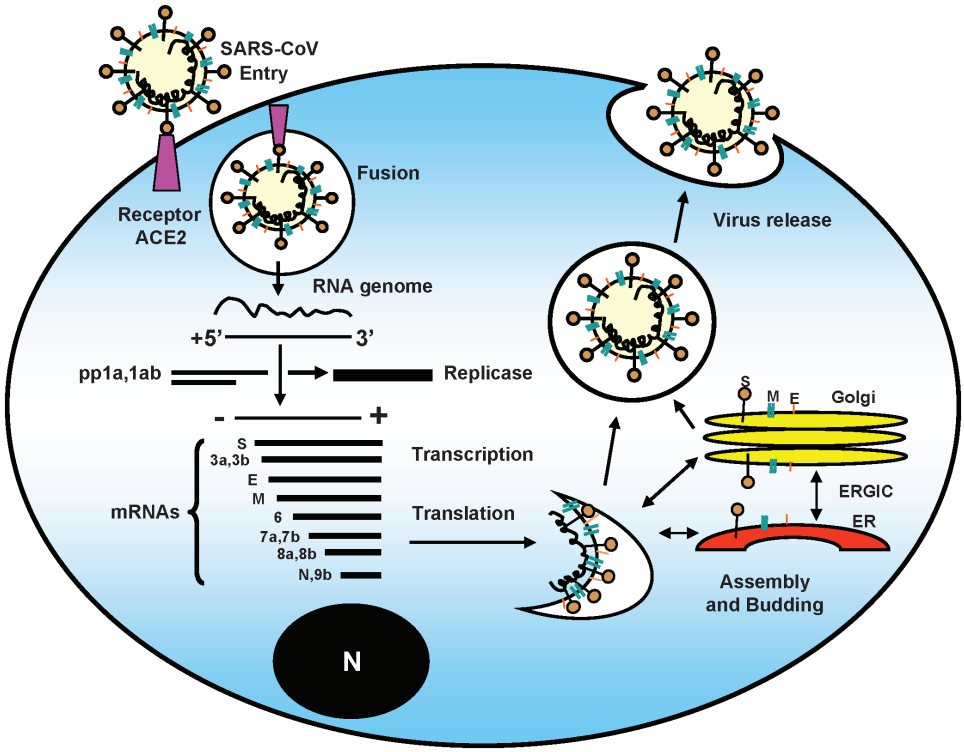

Figure 3 The SARS-CoV life cycle in host cells and its S protein structure. Life cycle of SARS-CoV. SARS-CoV begins its life cycle when its S protein binds to the cellular receptor ACE2. After receptor binding, the conformation change in the S protein facilitates viral envelope fusion with the cell membrane through the endosomal pathway. Then SARS-CoV releases RNA into the host cell. Genome RNA is translated into viral replicase polyproteins pp1a and 1ab, which are then cleaved into small products by viral proteinases. At the same time, polymerase, which produces a series of subgenomic mRNAs by discontinuous transcription, is finally translated into relevant viral proteins. Viral proteins and genome RNA are subsequently assembled into virions in the ER and Golgi, which are budding into the lumen of the ERGIC and then transported via vesicles and released out of the cell. ACE2, angiotensin-converting enzyme 2; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment.

infection can trigger a series of humoral and cellular immune responses, mainly targeting the S protein.[26,27]

The S protein of SARS-CoV is a type I transmembrane glycoprotein with its N terminus on the surface of the infected cell or the virus particle. Its precursor, consisting of 1255 amino acids (aa), can be cleaved by proteases into the surface subunit S1 (aa 17–680) and the transmembrane unit S2 (aa 681–1195)[28,29] (Figure 2A). S1 recognizes and binds to host cell receptors, leading to the conformational changes of S2 that mediate viral fusion between the viral envelope and the host cell membrane.[30]

The minimal RBD of SARS-CoV S protein is located in the S1 subunit (aa 318–510) and is responsible for viral binding to host cell receptors[31,32] (Figure 2A). Besides the main receptor, angiotensin-converting enzyme 2, there are several alternative receptors, such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin and/or liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin.[33] Two residues (aa 479 and aa 487) in RBD determine SARS progression and tropism, and their mutations may enhance animal-to-human or human-to-human transmission.[29] Some residues (aa 109, 118, 119, 158, 227, 589 and 699) in S protein are critical for dendritic cell/lymph node-specific intercellular adhesion molecule-3-grabbing integrin-mediated virus entry.[34]

Vaccines based on the RBD of SARS-CoV S protein

The roles of S protein in receptor binding and membrane fusion make it a perfect target for vaccine and antiviral development. Several vaccine candidates based on the full-length S protein of SARS-CoV have shown high immunogenicity in inducing neutralizing antibody responses and protection against SARS-CoV challenge.[8,9] However, these vaccines may also induce harmful immune responses that cause liver damage in the vaccinated animals[10] and/or enhance SARS-CoV infection through an antibody-dependent mechanism.[9] All of these have raised significant concerns about the safety and ultimate protective efficacy of vaccines based on the entire S protein.

Previous studies indicated that RBDs of S protein of coronaviruses (mouse hepatitis virus and human coronavirus 229E contain major antigenic determinants that can induce neutralizing antibody responses.[35,36] We therefore postulated that RBD of SARS-CoV may also be the target of neutralizing antibodies. Indeed, we found that a majority of the neutralizing antibodies in the sera of SARS patients and animals immunized with the inactivated SARS-CoV strongly reacted with the RBD of SARS-CoV S protein since depletion of the RBD-specific antibodies resulted in elimination of the neutralizing activity.[12,37] Chen *et al.*[38] also demonstrated that recombinant RBD could absorb and remove most neutralizing antibodies from the antisera of mice, rabbits and monkeys immunized with a live-attenuated modified vaccinia Ankara virus expressing the full-length S protein. We have constructed several subunit SARS vaccine candidates using recombinant RBD expressed in *Escherichia coli*, insect and mammalian cells and used them to immunize mice. We found that all of them could induce highly potent and broad cross-reactive neutralizing antibody responses[11,17] and protective immunity against challenge with live SARS-CoV. We also demonstrated that intramuscular and mucosal immunization of animals with the adeno-associated virus-based

RBD vaccine could elicit strong neutralizing antibody responses and protection against SARS-CoV challenge.[15–17] These findings suggest that this RBD-based vaccine has considerable potential for development into a safe and effective SARS vaccine to prevent future SARS epidemics.

Therefore, our previous studies on SARS vaccines have proven the concept of RBD-based vaccines and have shown that this strategy could also be applied to the development of vaccines against other infectious diseases.

RATIONAL DESIGN OF INFLUENZA VACCINES BASED ON NEUTRALIZING EPITOPES IN THE RBD IN THE HEMAGGLUTININ OF INFLUENZA A VIRUSES

Influenza pandemics occur frequently in the human population with substantial morbidity and mortality. Three major influenza pandemics have occurred in the last century, respectively caused by different subtypes of influenza A virus (IAV), including 1918 H1N1 ('Spanish Flu'), 1957 H2N2 ('Asian Flu') and 1968 H3N2 ('Hong Kong Flu').[39] In 2009, a swine-origin IAV (S-OIV), H1N1, which originated in Mexico and then spread globally, caused the first influenza pandemic of the twenty-first century.[40] Several other subtypes of IAVs were reported to cross the species barrier to infect humans. For example, in March 1999, avian IAV H9N2 was seen to infect two persons in Hong Kong, resulting in mild influenza.[41] In 2003, another avian IAV subtype H7N7 was reported to infect 89 humans with one death during a fowl plague outbreak in the Netherlands.[42] Furthermore, the highly pathogenic avian influenza A virus (HPAI) H5N1 has become a continuing threat to public health worldwide since its first report in 2003.[43] As of 20th January 2012, 582 cases of H5N1 have been reported to the World Health Organization with a mortality rate approaching 60% (http://www.who.int/influenza/human_animal_interface/EN_GIP_20120120Cumulative NumberH5N1cases.pdf).

IAV hemagglutinin and its RBD

IAVs belong to the family of Orthomyxoviridae. The genome of the virus consists of eight single-stranded, negative-sense RNA segments, encoding several important proteins, including surface proteins hemagglutinin (HA), neuraminidase (NA) and matrix 2 protein (M2), nucleoprotein (NP), matrix protein 1 (M1), non-structural proteins (NS1, NS2), as well as polymerase proteins PB1, PB2 and PA.[44] HA and NA are two important envelope glycoproteins of the virus, and all IAVs are classified into 16 antigenically distinct HAs (H1–H16) and 9 different NAs (N1–N9), according to the serotypes of HA and NA.

The HA of IAVs is a homotrimer and plays important roles in virus infection. Each of the single-chain monomers is initially synthesized as a precursor polypeptide, HA0, which is then cleaved by host proteases into two subunits, HA1 and HA2. The HA1 subunit is mainly responsible for receptor binding. It mediates viral attachment to the cellular receptors of the target cells known as sialic acid of glycoproteins and glycolipids.[45] Different HAs of IAVs recognize different sialic acid receptors. For example, the HAs from humans bind preferentially to sialic acids in the α (2,6) linkage, while those from avians and equines recognize α (2,3) linkages. It appears that the HAs from swine may recognize both α (2,3) and α (2,6) linkages.[46] After viral attachment to the target cells, the HA2 subunit located at the membrane-proximal helix-rich stem region mediates virus entry into the target cell by fusion of the viral envelope and the cellular membrane via the fusion peptide at the N-terminus of HA2.[45] The HA2 N-terminal fusion peptide contains the highly conserved sequence among all IAVs, and neutralizing epitopes targeting this region have been identified.[47]

As the main region for binding cellular receptors, RBD is located at the membrane-distal end (HA1) of each HA monomer, spanning less than 300 aa (from residues around 105 to residues around 319, depending on different HAs). The receptor-binding site is composed of three structural elements, including the 190-helix (HA1 188–190), the 130-loop (HA1 134–138) and the 220-loop (HA1 221–228), as well as several conserved residues at the W153, H183 and L194 positions.[45] The RBD of HA is the primary target of neutralizing antibodies, based on which anti-IAV vaccines can be designed and developed.[48]

Current influenza vaccines and disadvantages

The latest influenza pandemic caused by 2009 swine-origin IAV H1N1, coupled with the continuing threat of the highly pathogenic avian influenza A virus H5N1, makes it essential to develop efficacious vaccines to prevent future influenza pandemics and epidemics.

Conventionally, influenza vaccines have been prepared on the basis of inactivated virus, in which viruses are grown in embryonated chicken eggs and then inactivated with formalin and purified for use with appropriate adjuvants. However, these vaccines are mainly administered parenterally, limiting their ability to induce high immunity and efficacy in preventing heterologous virus infection.[49] Therefore, live-attenuated, cold-adapted influenza vaccines have been produced to increase immunogenicity. When injected intramuscularly into mice, these live influenza virus-based vaccines induced greater protection than those based on the inactivated virus.[50] Unfortunately, a safety concern arose from the possibility of recovering virulence or the potential re-assortment of the viruses with circulating influenza viruses. Although other available influenza vaccines, including those based on DNA, protein and viral vectors, as well as virus-like particles, may induce immune responses and/or protection against IAV infection,[51,52] their capacity to elicit highly potent and broad immune responses and protection is greatly restricted.

These disadvantages have made it essential to explore alternative novel approaches for future vaccine development. Preference will be given to those vaccines that are capable of stimulating broad neutralizing antibody responses and cross-protective immunity against divergent IAVs, including the pandemic strains of 2009 H1N1 and highly pathogenic strains of H5N1.

Discovery of the neutralizing epitopes in the RBD of IAV

Neutralizing antibodies play critical roles in protecting against IAV infection. The neutralizing antibodies could be induced by virus infections or raised by vaccination with specific antigens. The produced antibodies may neutralize various viruses by first recognizing and targeting specific positions (epitopes) of viral antigens. Currently, a number of neutralizing epitopes have been identified by binding of the neutralizing antibodies to the exposed loops surrounding the receptor binding site of HA protein and interfering with virus attachment, or selecting antibody escape mutants.[53]

Using genome-fragment phage display libraries, a group from the US Food and Drug Administration was able to map the epitopes of the neutralizing monoclonal antibody (mAb) FLD21.140 into two aa clusters in the RBD, 121-SWS-123 and 164-YNNT-167, the highly conserved sequences among clades 1, 2, 5, 6 and 8 of H5N1 viruses.[48] Using yeast surface display of neutralizing mAb NR2728 with a full-length H5 HA, researchers identified a conformation-dependent neutralizing epitope in the interface overlapping the receptor binding site of HA1. Another conformational epitope adjacent to the RBD was identified by mapping mAb S139/1 with HA. This mAb showed

cross-reactive neutralization and HA inhibition against particular strains of H1, H2, H3 and H13 subtypes of IAVs.[54]

Conformational neutralizing epitopes covering residues 125–129 and 157–169 at the apex of the RBD were identified by co-crystal structure of the 1918 HA with 2D1 mAb from a survivor of the 1918 Spanish flu. These epitopes were conserved in pandemic viruses causing the 1918 Spanish flu and 2009 swine-origin IAV.[55] Studies on the HA of Qinghai-type influenza H5N1 virus revealed other antigenic epitopes at the globular head region of H5 HA containing residues 113, 117, 118, 120 and 123.[56] Neutralizing epitopes at residues 260 and 261 of RBD were also identified using H5N1-specific neutralizing monoclonal Ab 9F4. These residues are well-conserved across different clades of H5N1 strains.[57]

In addition to the above neutralizing epitopes identified by antibody binding assays, several immunodominant epitopes were identified by selection of escape mutants using neutralizing mAbs. These neutralizing epitopes cover residues at positions 138, 140, 155, 159, 189, 194, and 218 of RBD.[58] Other conserved neutralizing epitopes in the head region of HA1 were identified from naturally occurring antibodies F045-092 and F026-427.[59] The neutralizing epitopes at residues 178–181 and 227–239 of the receptor binding site of HA globular region were identified by two human neutralizing antibodies, B-1 and D-1, to H3N2 derived from different donors. These neutralizing epitopes are proven to be highly conserved in human H1N1, swine-origin pandemic H1N1, human H5N1 and avian H5N1 strains.[60] It was also reported that a neutralizing mAb, CH65, was capable of recognizing the receptor-binding pocket of IAV H1N1 HA, indicating that broad neutralization of IAV could be achieved by antibodies targeting the RBD pocket.[61]

Prospective development of novel influenza vaccines based on the neutralizing epitopes in RBD of the IAV HA1

Identification of the above neutralizing epitopes in the RBD region brings hope for the development of innovative vaccines because the identified epitope regions may be applied as novel vaccine targets. Vaccines based on these neutralizing epitopes could induce neutralizing antibodies against a broad spectrum of IAV strains. Studies on the development of such vaccines have been initiated, and some of them have shown promising results.[62]

It is reported that an *E. coli*-expressed recombinant protein, HA_{63-286}-RBD, containing HA-RBD of the A/H1N1/2009 influenza virus, is immunogenic with the capacity to trigger neutralizing antibodies and induce protective activity in the tested ferret model.[63] Studies have shown that recombinant vaccines targeting the globular head RBD region fused to flagellin of *Salmonella typhimurium fljB* gene induced protective immunity to H1N1 and H5N1 infections in challenged mice.[62] It was demonstrated that a recombinant oligomeric protein covering H5N1 (A/Vietnam/ 1203/2004) HA1-RBD neutralizing epitopes elicited potent neutralizing antibodies in the vaccinated mice against homologous and heterologous H5N1 strains and protected ferrets from homologous and heterologous H5N1 challenge.[48] Previous studies by the same group also demonstrated the protective immunity of vaccines containing H1N1 (A/California/07/2009) HA1-RBD region against challenge of H1N1 pandemic influenza virus. A report by Prabakaran and colleagues indicated that a recombinant baculovirus-expressed trivalent vaccine covering the major neutralizing epitopes in the RBD of H5N1 effectively neutralized viruses from clades 1, 2.1, 2.2, 4, 7 and 8 of H5N1 and protected 100% of the mice against challenge with three different clades (clade 1.0, clade 2.1 and clade 7.0) of H5N1 strains. Our recent studies also revealed that a recombinant HA1-Fdc vaccine containing RBD neutralizing region induced high titers of antibodies that cross-neutralized clades 0, 1, 2.2 and 2.3.4 of H5N1 strains and completely protected vaccinated mice against high-dose lethal challenge of different strains of H5N1 covering clades 0, 1 and 2.3.4.[64]

The above findings further support our hypothesis that the neutralizing epitopes in the RBD of IAV HA are attractive targets for development of universal influenza vaccines, which can be used to prevent future influenza pandemics or epidemics caused by emerging or re-emerging IAV strains.

RBDS IN THE G PROTEINS OF HEV AND NIV AS TARGETS FOR VACCINE DEVELOPMENT

HeV and NiV are emerging zoonotic viruses discovered during outbreaks in Australia in 1994 and in Malaysia in 1998, respectively. As newly defined viruses, HeV and NiV belong to the family Paramyxoviridae and genus Henipavirus.[5] The broad species tropism and the ability to cause severe diseases in both humans and animals distinguish HeV and NiV from other zoonotic viruses. Symptoms of HeV and NiV infection of humans can be respiratory, including hemorrhage and edema of the lungs, or encephalitic, resulting in meningitis. The principal natural reservoirs for HeV and NiV are pteropid fruit bats; however, recent evidence of henipavirus infection has been found in a wider range of species, including flying foxes, humans, cats, horses, rabbits and laboratory rodents[6] (Figure 4). Because HeV and NiV can potentially cause significant morbidity and mortality in humans, with consequent major economic and public health impact, they are classified as biological safety level-4 pathogens.[65]

Both HeV and NiV have non-segmented, negative-stranded RNA genosomes consisting of transcription units encoding six major structural proteins, including nucleocapsid protein (N), phosphorprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and large protein (L).[66] The G protein, which contains a length of 602 aa, is responsible for virus binding to the receptor and entry into the host cell.

While the G protein has neither HA nor neuraminidase activities, it plays an important role in HeV and NiV binding to ephrin-B2 and ephrin-B3, which are members of a large family of important signaling proteins involved in cell–cell interactions.[67] Binding of RBD in the G protein with its receptor facilitates F protein-mediated viral fusion with the target cell. Mutations of some residues in the RBD of the G protein significantly impaired viral binding and fusion activities.[68] The G protein of HeV or NiV is comprised of a short cytoplasmic tail, a transmembrane domain, a stalk region and a globular head domain[68] (Figure 2C). The globular head region of the G protein contains the minimal RBD (aa 439–468)[68] (Figure 2C). The G protein of HeV or NiV appears to be the dominant target antigen for neutralizing antibodies.[69] While no vaccines are available for the prevention of henipavirus disease in humans, several G protein-based henipavirus vaccine candidates have been tested in three different animal models.[70] Two of them using ALVAC canarypox as vector showed protection against NiV infection in pigs. A recombinant soluble G protein-based vaccine adjuvanted with CpG was shown to induce strong immune responses in a ferret NiV infection model, which could protect cats from NiV infection.[71]

The first human neutralizing mAb, m101, with exceptional potency against HeV and NiV, was identified in 2006.[72] A panel of 17 alanine-scanning mutants of the G protein of HeV was tested for binding ability to the human neutralizing antibodies m101 and m102.4. It was found that the binding of HeV G mutants D260A, G439A,

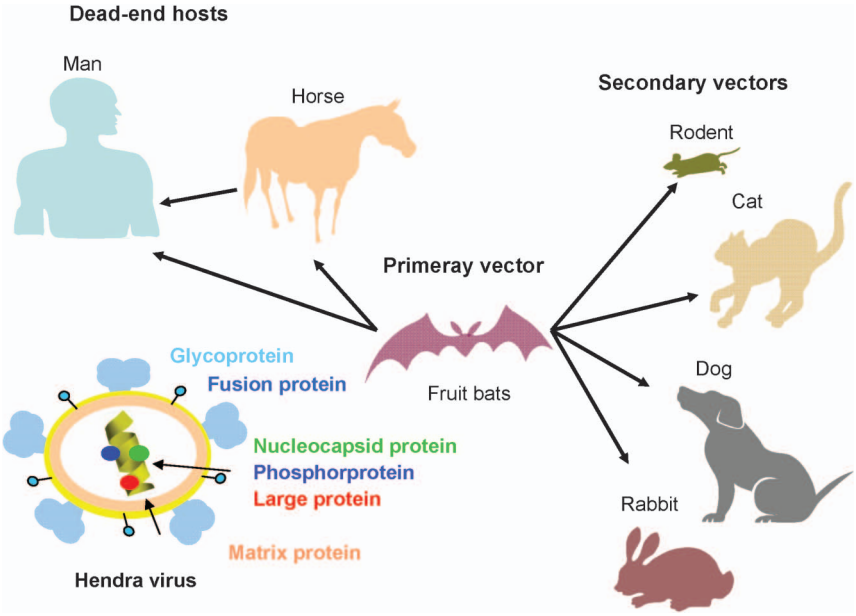

Figure 4 The structure and natural reservoirs of HeV. The principal natural reservoirs for HeV are fruit bats. Recent evidence of Hendra infection has indicated that flying foxes can also be natural reservoirs. The secondary vectors can be laboratory rodents, cats, dogs and rabbits. The dead-end hosts are horse and man. HeV has six major structural proteins. They are nucleocapsid protein (N), phosphorprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and large protein (L). HeV, Hendra virus.

K443A and K465A to both m101 and m102.4 was almost completely eliminated, while the binding activity of mutants G449A and D468A was significantly decreased.[73] These results suggest that the epitopes of the neutralizing antibodies overlap the RBD of HeV or NiV G proteins and that some discontinuous residues located in the RBD are critical for HeV or NiV binding to its receptors ephrin-B2 and ephrin-B3.[74] Based on these findings, the design of an RBD-based henipavirus vaccine to prevent HeV or NiV infection is very feasible.

CONCLUSION AND PROSPECTS

The use of RBD of spike proteins for developing antiviral vaccines to prevent epidemics of emerging or re-emerging viral infectious diseases is attracting more research interest. The common features of RBDs of spike proteins of SARS-CoV, influenza virus and Hendra virus include: (i) being located in the spike proteins; (ii) mediating binding of the virus to the target cell via the interaction between viral spike protein and the receptor on the target cell; (iii) having a length ranging from 30 to 215 aa; and (iv) containing discontinuous neutralizing epitope(s).

With its relatively conserved sequences, RBD plays a critical role in the elucidation of antiviral immune response and protective immunity. The best example is the RBD-based SARS vaccine. It is a safe vaccine candidate and has been shown to induce the most effective immune response and protective immunity against SARS-CoV. Thus, it is expected that an RBD-based SARS vaccine will be one of the first SARS vaccines going to clinical trials.

To design vaccines with the ability to induce highly potent and cross-reactive neutralizing antibodies with high binding affinity, we recommend the following strategies: (i) construct an immunogen containing the discontinuous sequences of bonding set and/or induced epitopes; (ii) induce conformational changes by mutation or glycan shield of the non-neutralizing immunodominant epitopes from the immunogen in order to reduce the immunogenicity of these epitopes; and (iii) add an immunopotentiation motif to the immunogen in order to enhance the immunogenicity of the RBD-based vaccine.

Similar to the RBD of SARS-CoV spike protein, the IAV HA protein RBD spans about 215 aa (aa 105–319) with three conserved structural elements[45] and contains primary neutralizing antibody epitopes that serve as important targets for influenza vaccine development.[48] A number of broadly cross-reactive neutralizing mAbs, such as NR2728 (146), S139/1,[54] B-1 and D-1,[61] were identified to specifically bind to the conformation-dependent neutralizing epitopes in the interface overlapping with, or adjacent to, the RBD in HA1. Our recent studies have shown that vaccination of mice with the fusion protein HA1-Fdc, which consists of H5N1 HA1, a trimeric motif foldon and a human immunoglobulin G F*c* fragment, resulted in induction of potent neutralizing antibody responses and complete cross-clade protection against H5N1 infection,[64] including elucidation of neutralizing mAbs whose epitopes were mapped to RBD (unpublished data). All these findings support the feasibility of developing RBD-based influenza vaccines. It is anticipated that these RBD-based influenza vaccines could be effective against divergent IAV strains, including those that may cause future influenza pandemics or epidemics.

HeV and NiV, which belong to the family Paramyxoviridae and genus Henipavirus,[5] are emerging zoonotic viruses that caused outbreaks in humans in Australia and resulted in high mortality. The G protein of HeV or NiV contains the minimal RBD overlapping about 30 aa (aa 439–468) in its globular head domain.[68] The first human neutralizing antibody, m101, is highly potent in neutralizing both HeV and NiV,[71] and its epitope overlaps the minimal RBD in the G protein, suggesting the potential of developing RBD-based henipavirus vaccine to prevent HeV or NiV infection.

Besides the viruses mentioned above, the strategy of designing RBD-based vaccines can also be applied to the development of vaccines against other emerging or re-emerging viruses with identified receptor(s) and RBDs, such as Zaire Ebolavirus and Lake Victoria Marburgvirus.[75] Although the receptors for some viruses have not

been clearly defined, the sites in the spike proteins of these viruses for binding to the cellular cofactors that facilitate viral entry may also be used to design vaccines able to induce antibodies to block viral entry or neutralize viral infectivity. For example, entry of hepatitis C virus (HCV) into the hepatocyte requires at least four cellular cofactors, including CD81,[76] the tight junction proteins claudin-1 and occludin,[77] and scavenger receptor BI.[78] It is expected that the sites in the E1 and E2 proteins of HCV for binding to the above cofactors or putative HCV coreceptors may serve as targets for development of preventive vaccines against HCV in the near future.

ACKNOWLEDGMENTS

We thank Ms Qian Wang for preparing Figure 1. This study was supported by grants from the National Basic Research Program of China (#2012CB519001) to Shibo Jiang, and NIH of the United States (R01 AI68002) to Shibo Jiang and (R03 AI088449) to Lanying Du.

1 Drosten C, Gunther S, Preiser W *et al*. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003; **348**: 1967–1976.
2 Rota PA, Oberste MS, Monroe SS *et al*. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003; **300**: 1394–1399.
3 Zhong NS, Zheng BJ, Li YM *et al*. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *Lancet* 2003; **362**: 1353–1358.
4 Palese P. Influenza: old and new threats. *Nat Med* 2004; **10**: S82–S87.
5 Rockx B, Bossart KN, Feldmann F *et al*. A novel model of lethal Hendra virus infection in African green monkeys and the effectiveness of ribavirin treatment. *J Virol* 2010; **84**: 9831–9839.
6 Williamson MM, Hooper PT, Selleck PW *et al*. Transmission studies of Hendra virus (equine morbillivirus) in fruit bats, horses and cats. *Aust Vet J* 1998; **76**: 813–818.
7 Marshall E, Enserink M. Medicine. Caution urged on SARS vaccines. *Science* 2004; **303**: 944–946.
8 He YX, Li JJ, Heck S, Lustigman S, Jiang SB. Antigenic and immunogenic characterization of recombinant baculovirus-expressed severe acute respiratory syndrome coronavirus spike protein: implication for vaccine design. *J Virol* 2006; **80**: 5757–5767.
9 Tseng CT, Sbrana E, Iwata-Yoshikawa N, Newman PC, Garron T, Atmar RL *et al*. Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PLoS ONE*. 2012; **7**: e35421.
10 Weingartl H, Czub M, Czub S *et al*. Immunization with modified vaccinia virus Ankara-based recombinant vaccine against severe acute respiratory syndrome is associated with enhanced hepatitis in ferrets. *J Virol* 2004; **78**: 12672–12676.
11 He YX, Zhou YS, Liu SW *et al*. Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. *Biochem Biophys Res Commun* 2004; **324**: 773–781.
12 He YX, Zhu QY, Liu SW *et al*. Identification of a critical neutralization determinant of severe acute respiratory syndrome (SARS)-associated coronavirus: Importance for designing SARS vaccines. *Virology* 2005; **334**: 74–82.
13 Du LY, He YX, Wang YJ *et al*. Recombinant adeno-associated virus expressing the receptor-binding domain of severe acute respiratory syndrome coronavirus S protein elicits neutralizing antibodies: implication for developing SARS vaccines. *Virology* 2006; **353**: 6–16.
14 He Y, Li J, Li W *et al*. Cross-neutralization of human and palm civet severe acute respiratory syndrome coronaviruses by antibodies targeting the receptor-binding domain of spike protein. *J Immunol* 2006; **176**: 6085–6092.
15 Du LY, Zhao GY, Lin YP *et al*. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS-CoV infection. *J Immunol* 2008; **180**: 948–956.
16 Du L, Zhao G, Lin Y *et al*. Priming with rAAV encoding RBD of SARS-CoV S protein and boosting with RBD-specific peptides for T cell epitopes elevated humoral and cellular immune responses against SARS-CoV infection. *Vaccine* 2008; **26**: 1644–1651.
17 Du L, Zhao G, Chan CC *et al*. Recombinant receptor-binding domain of SARS-CoV spike protein expressed in mammalian, insect and *E. coli* cells elicits potent neutralizing antibody and protective immunity. *Virology* 2009; **393**: 144–150.
18 Du L, He Y, Zhou Y *et al*. The spike protein of SARS-CoV: a target for vaccine and therapeutic development. *Nat Rev Microbiol* 2009; **7**: 226–236.
19 Kuiken T, Fouchier RA, Schutten M *et al*. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* 2003; **362**: 263–270.
20 Normile D. Infectious diseases—mounting lab accidents raise SARS fears 5. *Science* 2004; **304**: 659–661.
21 Dimitrov DS. The secret life of ACE2 as a receptor for the SARS virus. *Cell* 2003; **115**: 652–653.
22 Li WH, Moore MJ, Vasilieva N *et al*. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* 2003; **426**: 450–454.
23 Gu J, Korteweg C. Pathology and pathogenesis of severe acute respiratory syndrome. *Am J Pathol* 2007; **170**: 1136–1147.
24 Ding YQ, He L, Zhang QL *et al*. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. *J Pathol* 2004; **203**: 622–630.
25 Peiris JSM, Lai ST, Poon LL *et al*. Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 2003; **361**: 1319–1325.
26 Buchholz UJ, Bukreyev A, Yang LJ *et al*. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. *Proc Natl Acad Sci USA* 2004; **101**: 9804–9809.
27 Zhi Y, Kobinger GP, Jordan H *et al*. Identification of murine CD8 T cell epitopes in codon-optimized SARS-associated coronavirus spike protein. *Virology* 2005; **335**: 34–45.
28 Du LY, Kao RY, Zhou YS *et al*. Cleavage of spike protein of SARS coronavirus by protease factor Xa is associated with viral infectivity. *Biochem Biophys Res Commun* 2007; **359**: 174–179.
29 Li F, Li WH, Farzan M, Harrison SC. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* 2005; **309**: 1864–1868.
30 Liu S, Xiao G, Chen Y *et al*. Interaction between heptad repeat 1 and 2 regions in spike protein of SARS-associated coronavirus: implications for virus fusogenic mechanism and identification of fusion inhibitors. *Lancet* 2004; **363**: 938–947.
31 Wong SK, Li WH, Moore MJ, Choe H, Farzan M. A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J Biol Chem* 2004; **279**: 3197–3201.
32 Xiao X, Chakraborti S, Dimitrov AS, Gramatikoff K, Dimitrov DS. The SARS-CoV S glycoprotein: expression and functional characterization. *Biochem Biophys Res Commun* 2003; **312**: 1159–1164.
33 Jeffers SA, Tusell SM, Gillim-Ross L *et al*. CD209L (L-SIGN) is a receptor for severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci USA* 2004; **101**: 15748–15753.
34 Han DP, Lohani M, Cho MW. Specific asparagine-linked glycosylation sites are critical for DC-SIGN- and L-SIGN-mediated severe acute respiratory syndrome coronavirus entry. *J Virol* 2007; **81**: 12029–12039.
35 Bonavia A, Zelus BD, Wentworth DE, Talbot PJ, Holmes KV. Identification of a receptor-binding domain of the spike glycoprotein of human coronavirus HCoV-229E. *J Virol* 2003; **77**: 2530–2538.
36 Kubo H, Yamada YK, Taguchi F. Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino-acids of the murine coronavirus spike protein. *J Virol* 1994; **68**: 5403–5410.
37 He YX, Zhou YS, Siddiqui P, Jiang SB. Inactivated SARS-CoV vaccine elicits high titers of spike protein-specific antibodies that block receptor binding and virus entry. *Biochem Biophys Res Commun* 2004; **325**: 445–452.
38 Chen ZW, Zhang LQ, Qin CA *et al*. Recombinant modified vaccinia virus Ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. *J Virol* 2005; **79**: 2678–2688.
39 Liu J, Stevens DJ, Haire LF *et al*. Structures of receptor complexes formed by hemagglutinins from the Asian Influenza pandemic of 1957. *Proc Natl Acad Sci USA* 2009; **106**: 17175–17180.
40 Dawood FS, Jain S, Finelli L *et al*. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med* 2009; **360**: 2605–2615.
41 Peiris M, Yuen KY, Leung CW *et al*. Human infection with influenza H9N2. *Lancet* 1999; **354**: 916–917.
42 Fouchier RA, Schneeberger PM, Rozendaal FW *et al*. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci USA* 2004; **101**: 1356–1361.
43 Chutinimitkul S, Payungporn S, Chieochansin T *et al*. The spread of avian influenza H5N1 virus; a pandemic threat to mankind. *J Med Assoc Thai* 2006; **89** Suppl 3: S218–S233.
44 Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. *Nature* 2009; **459**: 931–939.
45 Stevens J, Blixt O, Tumpey TM *et al*. Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science* 2006; **312**: 404–410.
46 Rogers GN, Paulson JC, Daniels RS *et al*. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 1983; **304**: 76–78.
47 Prabhu N, Prabakaran M, Ho HT *et al*. Monoclonal antibodies against the fusion peptide of hemagglutinin protect mice from lethal influenza A virus H5N1 infection 156. *J Virol* 2009; **83**: 2553–2562.
48 Khurana S, Verma S, Verma N *et al*. Bacterial HA1 vaccine against pandemic H5N1 influenza virus: evidence of oligomerization, hemagglutination, and cross-protective immunity in ferrets. *J Virol* 2011; **85**: 1246–1256.
49 Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H. PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants. *Vaccine* 2009; **27**: 6276–6279.
50 Harris K, Ream R, Gao J, Eichelberger MC. Intramuscular immunization of mice with live influenza virus is more immunogenic and offers greater protection than immunization with inactivated virus. *Virol J* 2011; **8**: 251.
51 Landry N, Ward BJ, Trepanier S *et al*. Preclinical and clinical development of plant-made virus-like particle vaccine against avian H5N1 influenza. *PLoS ONE* 2010; **5**: e15559.

52 Xu K, Ling ZY, Sun L *et al*. Broad humoral and cellular immunity elicited by a bivalent DNA vaccine encoding HA and NP genes from an H5N1 virus. *Viral Immunol* 2011; **24**: 45–56.
53 Khurana S, Suguitan AL Jr, Rivera Y *et al*. Antigenic fingerprinting of H5N1 avian influenza using convalescent sera and monoclonal antibodies reveals potential vaccine and diagnostic targets. *PLoS Med* 2009; **6**: e1000049.
54 Yoshida R, Igarashi M, Ozaki H *et al*. Cross-protective potential of a novel monoclonal antibody directed against antigenic site B of the hemagglutinin of influenza A viruses. *PLoS Pathog* 2009; **5**: e1000350.
55 Xu R, Ekiert DC, Krause JC *et al*. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. *Science* 2010; **328**: 357–360.
56 Rudneva IA, Kushch AA, Masalova OV *et al*. Antigenic epitopes in the hemagglutinin of Qinghai-type influenza H5N1 virus. *Viral Immunol* 2010; **23**: 181–187.
57 Oh HL, Akerstrom S, Shen S *et al*. An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. *J Virol* 2010; **84**: 8275–8286.
58 Kaverin NV, Rudneva IA, Govorkova EA *et al*. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. *J Virol* 2007; **81**: 12911–12917.
59 Ohshima N, Iba Y, Kubota-Koketsu R *et al*. Naturally occurring antibodies in humans can neutralize a variety of influenza virus strains, including H3, H1, H2, and H5. *J Virol* 2011; **85**: 11048–11057.
60 Yamashita A, Kawashita N, Kubota-Koketsu R *et al*. Highly conserved sequences for human neutralization epitope on hemagglutinin of influenza A viruses H3N2, H1N1 and H5N1: implication for human monoclonal antibody recognition. *Biochem Biophys Res Commun* 2010; **393**: 614–618.
61 Whittle JR, Zhang R, Khurana S *et al*. Broadly neutralizing human antibody that recognizes the receptor-binding pocket of influenza virus hemagglutinin. *Proc Natl Acad Sci USA* 2011; **108**: 14216–14221.
62 Liu G, Tarbet B, Song L *et al*. Immunogenicity and efficacy of flagellin-fused vaccine candidates targeting 2009 pandemic H1N1 influenza in mice. *PLoS ONE* 2011; **6**: e20928.
63 Aguilar-Yanez JM, Portillo-Lara R, Mendoza-Ochoa GI *et al*. An influenza A/H1N1/2009 hemagglutinin vaccine produced in *Escherichia coli*. *PLoS ONE* 2010; **5**: e11694.
64 Du L, Leung VH, Zhang X *et al*. A recombinant vaccine of H5N1 HA1 fused with foldon and human IgG Fc induced complete cross-clade protection against divergent H5N1 viruses. *PLoS ONE* 2011; **6**: e16555.
65 Khetawat D, Broder CC. A functional henipavirus envelope glycoprotein pseudotyped lentivirus assay system. *Virol J* 2010; **7**: 312.
66 Wang L, Harcourt BH, Yu M *et al*. Molecular biology of Hendra and Nipah viruses. *Microbes Infect* 2001; **3**: 279–287.
67 Bossart KN, Crameri G, Dimitrov AS *et al*. Receptor binding, fusion inhibition, and induction of cross-reactive neutralizing antibodies by a soluble G glycoprotein of Hendra virus. *J Virol* 2005; **79**: 6690–6702.
68 Bishop KA, Stantchev TS, Hickey AC *et al*. Identification of Hendra virus G glycoprotein residues that are critical for receptor binding. *J Virol* 2007; **81**: 5893–5901.
69 Bossart KN, Geisbert TW, Feldmann H *et al*. A neutralizing human monoclonal antibody protects African green monkeys from Hendra virus challenge. *Sci Transl Med* 2011; **3**: 105ra103.
70 Pallister J, Middleton D, Wang LF *et al*. A recombinant Hendra virus G glycoprotein-based subunit vaccine protects ferrets from lethal Hendra virus challenge. *Vaccine* 2011; **29**: 5623–5630.
71 Mungall BA, Middleton D, Crameri G *et al*. Feline model of acute Nipah virus infection and protection with a soluble glycoprotein-based subunit vaccine. *J Virol* 2006; **80**: 12293–12302.
72 Zhu Z, Dimitrov AS, Bossart KN *et al*. Potent neutralization of Hendra and Nipah viruses by human monoclonal antibodies. *J Virol* 2006; **80**: 891–899.
73 Zhu Z, Bossart KN, Bishop KA *et al*. Exceptionally potent cross-reactive neutralization of Nipah and Hendra viruses by a human monoclonal antibody. *J Infect Dis* 2008; **197**: 846–853.
74 Bonaparte MI, Dimitrov AS, Bossart KN *et al*. Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. *Proc Natl Acad Sci USA* 2005; **102**: 10652–10657.
75 Kondratowicz AS, Lennemann NJ, Sinn PL *et al*. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. *Proc Natl Acad Sci USA* 2011; **108**: 8426–8431.
76 Pileri P, Uematsu Y, Campagnoli S *et al*. Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938–941.
77 Liu S, Yang W, Shen L *et al*. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* 2009; **83**: 2011–2014.
78 Scarselli E, Ansuini H, Cerino R *et al*. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 2002; **21**: 5017–5025.
79 Yang ZY, Werner HC, Kong WP *et al*. Evasion of antibody neutralization in emerging severe acute respiratory syndrome coronaviruses. *Proc Natl Acad Sci USA* 2005; **102**: 797–801.
80 Jones IM, Reay PA, Philpott KL. Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. *EMBO J* 1986; **5**: 2371–2376.