University of Groningen

Neutralizing antibodies a protective shield against influenza infection

***Student*:** Violeta Stojanovska
2nd year Medical Pharmaceutical Sciences
***Supervisor*:** Anke Huckriede
Department of Medical Microbiology

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Abstract

Introduction: Neutralizing antibodies have the ability to bind to antigens and to block their biological function. By targeting the surface antigens in viruses such as hemagglutinin (HA), influenza infection can be neutralized.

Recent findings: With phage display libraries and single plasma cell isolation technique, neutralizing antibodies that cross-react with different subtypes of influenza were isolated. They target conserved regions in the hemagglutinin and block the binding of the virus to the receptors or the fusion of the virus with host cell membranes. When administrated in organisms, they elicit significant protection against influenza infection.

Conclusion: Neutralizing antibodies that attack preserved antigenic epitopes in all subtypes of influenza can provide broad-spectrum of neutralization against influenza infection and can be used for passive immunization of the population or for developing of universal vaccines.

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I. Introduction

Efficient influenza protection and a successful eschew of pandemic outbreak are the two main global concerns of the public health each year. Usually the clinical course of the infection is mild or even symptomless, but the problem starts when influenza infects vulnerable groups of the population such as elderly people and children. They can suffer from severe respiratory syndrome and secondary bacterial pneumonia (Nicholson, 2003). WHO every year registers 250 000 to 500 000 deaths due to seasonal influenza, imposing considerable economic burden worldwide (*WHO 2009*). There are two main types of influenza viruses A and B that are related to epidemics. Influenza B virus can cause morbidity and mortality in humans, but it was not associated with pandemic outbreaks because formation of new strains is constrained due to its limited host range in humans and seals (*Thomson 2004*). On the contrary, influenza A virus is the most dangerous type of influenza, and has been responsible for three pandemics at least in the past century. This is related to various biological hosts of the virus and the continuous change by antigenic drift and shift (*Nicholson 2003*). Hemagglutinin is one of the transmembrane glycoproteins, responsible for initiation of virus infection by recognizing the sialic acid receptor on the surface of the cells in the upper respiratory tract and mediating fusion of the virus with the host cell membranes (*Julien 2012*). When viruses invade the body, the immune system recognizes them as a 'non-domestic' and starts producing matching antibodies against the antigens of the intruder such as the hemagglutinin of the influenza virus. However, the rapid and continuous change in the virus genome so called drift and the recombination of different subtypes known as shift, result in subsequent change of the hemagglutinin molecule, making it unrecognizable for the immune system and the previously produced antibodies are ineffective against the virus. To date, 17 different types of hemagglutinin have been identified in influenza A virus. Further they are divided in two groups on the basis of phylogenetic analyses of their sequences. The group 1 consists of: H1, H2, H5, H6, H11, H12, H13, H16, H17 and the following subtypes: H3, H4, H7, H10, H14, H15 form the group 2 of influenza A viruses (*Julien 2012; Lee PS 2012*).

Preventive vaccination so far is the most efficient measure of influenza control. Each year influenza vaccines are reformulated by forecasting which influenza viruses will circulate in the upcoming season. The trivalent vaccine usually gives protection against the human subtypes of influenza A (H1 and H3) and against one strain of influenza B. The newly introduced quadrivalent vaccine (*Lee SW 2012*) provides additional protection against another strain of influenza B. Still the problem with the pandemics and severe morbidity from influenza is not solved. The possible cause is that the strains included in the vaccine can't elicit formation of broad-spectrum neutralizing antibodies against the other subtypes that are not included in the vaccine. The recently reported antibodies have the ability to neutralize different strains of influenza viruses by recognizing conserved region in hemagglutinin. These antibodies can be used to fight against influenza outbreaks as passive immunotherapy. The aim of the essay is to explore and understand in which way cross-protective antibodies can neutralize many different subtypes of influenza. Briefly we will explore the sources for neutralizing antibodies, the mechanism of neutralization and the predicted prophylactic and therapeutic efficacy.

II. Isolation of neutralizing antibodies

When virus invades the organism, a set of immune cells, part of the adaptive immune system work together to recognize the antigens of the virus, to present it to the B cells so they can start producing compatible antibodies. These antibodies continue to persist in the body for shorter or longer period of time (*Amanna 2007*) and when the same antigen is presented again in the body they react usually by neutralization. The problem with influenza virus is the genetic instability and continuous mutations that results in viruses with novel antigens on the surface. Already present antibodies can't recognize the new antigen and formation of new ones has to begin. The biggest concern are people with immunodeficiencies, elderly and small children because their immune system can't protect them in adequate way from severe clinical course of influenza virus infection.

Neutralizing antibodies can block the virus infectivity in many ways such as binding to receptors, blockage of fusion with the host cells or prevention of uncoating of the virus genome (*Rhorer 2009*). Usually antibodies recognize only one antigenic determinant, but some of them can recognize more than one antigenic determinant, which is known as cross-reactivity. In the last years there is progress in identification of broad-spectrum neutralizing antibodies which can recognize conserved regions in the hemagglutinin that are also presented in the other subtypes of influenza viruses. With the technological progress, tracking the broad-spectrum neutralizing antibodies became true with the processes of high throughput technologies. Methodologies commonly used nowadays are hybridoma technique, phage display libraries and single plasma cell antibody isolation. The hybridoma technique is based on forming hybrid cell lines by fusion of human B cells with immortalized cell lines that can grow indefinitely like cancer myeloma cells. Sometimes there is a problem in finding a suitable human myeloma cell line. That can be overcome by activation and immortalization of human B cells with Epstein Barr virus (EBV) infection and addition of Toll like receptor 9 (TLR9) agonist for co-stimulation of the B cells. After successful transformation of B cells, they are fused with myeloma cells for stable production of antibodies, which are further tested for their affinity and viral neutralization (*Gorny 2012*). This is a relatively simple procedure which enables continuous production of antibodies. However screening large number of B cells is needed to obtain antibodies with desired activity.

Phage display libraries are based on displaying peptides and proteins on the surface of bacteriophages (*Rader 1997*). Briefly, large gene libraries are constructed by isolation or synthesizing immunoglobulin variable V_h-V_l gene segments. Than are cloned into the phage in order to display either the antigen binding fragment (Fab) or the single chain variable antigen fragment (scFvs). Afterwards, selection of specific antibody is done by panning and screening of the library. During panning phages are incubated with antigen or antigens of interest and only the matched ones are kept for further screening. These polyclonal mixtures need to be converted into monoclonal antibodies. That is done by infecting *E.coli* with the selected phages, consequent plating on agar and picking of single colonies. The method of human phage display libraries was used to obtain several neutralizing antibodies with broad spectrum activity against different subtypes of influenza. F10, PN-SIA49 antibodies are successful in neutralization of group 1 influenza A viruses (*Sui 2009*, *de Marco 2012*) but C05 and S139/1 have even broader ability to neutralize viruses from the two phylogenic groups of influenza (*Ekiert 2012, Lee PS 2012*). So far only

CR9114 antibody presented from Dreyfus *et al*. presented ability to neutralize viruses from the two groups of influenza viruses and the two antigenic strains of influenza B (*Dreyfus 2012*).

The method of single plasma cell isolation can generate antibodies from single human B cell. As described by Corti et al. B cells can be obtained from donors one week after immunization with trivalent vaccine or one week after diagnosed infection with influenza. The B cells are sorted and plated, and after four days supernatants are collected and tested for antibodies against different antigens. From efficient antibodies the variable immune genes are retrieved by RT-PCR, then cloned and expressed in eukaryotic cell lines like 293F cells. With this method F16 antibody was isolated from 104 000 screened plasma cells and showed incredible ability to cross-react with hemagglutinins from group 1 and group 2 of influenza viruses (*Corti 2012*). The previous findings show that cross-protective neutralizing antibodies, although rare, still can be formed in the human organism as a response to influenza virus.

Hybridoma technique is still used for isolation of human monoclonal antibodies because it is relatively simple and inexpensive, but the low fusion efficiencies with cell lines limits the efficacy of the method. With phage display libraries there is a possibility to mix-and-match the heavy and the light chains of the antibodies from different B cells and to produce completely new ones (*Glokler 2010*). However expression of antibodies on non-eukaryotic cells can bias the characteristics of the antibody. Single plasma cell isolation antibody technique seems to be the most strategic research method in discovery of naturally formed cross-protective antibodies. The choice of method for isolation neutralizing antibodies usually depends on the researcher's expertise, interest and finances. For authentic isolation of broad spectrum neutralizing antibodies, single plasma cell isolation antibody technique seem to be the favorite due to its ability to screen large number of clones and to isolate naturally formed cross-protective antibodies in humans against influenza viruses.

III. Targets for neutralization in influenza virus

The influenza virus is part of the orthomyxoviridae family and is divided in three types A, B and C. This classification is made in accordance with differences in the type-specific integral antigens such as matrix protein (M1) and nucleoprotein (NP). External presenting antigens are hemagglutinin (HA) and neuraminidase (NA) and they show much more variations and are used for subclassification of the influenza A viruses (*Nicholson 2003*). To date 17 subtypes of HA (*Tong 2012*) as previously described are discovered and divided in two groups. The changes in the viral genome that occur by antigenic drift and/or shift usually modify the antigenic presentation of HA, making it unrecognizable to the protective mechanisms of the human body (*Sui 2009*). That constitutes HA as the most important and challenging target for neutralization by antibodies.

HA is composed of three identical monomers that represent the precursor protein known as HA0. For activation of the HA, cleavage between HA1 and HA2 subdomains needs to be done by endoproteases (*Lamb 1996*). Without this conformational change, the HA can't recognize the sialic acid receptors in the upper respiratory tract nor can it provide fusion with the host endothelial cell. The structure of the HA can be divided in two topographic parts, the 'head' and the 'stem' region. The 'head' or the membrane distal globular part is important for recognition and binding of the sialic acid receptors

and almost exclusively is composed of the HA1 subdomain. Mutations in the genome of influenza most often leads to changes in the globular part of the HA, which makes it also the most variable region in the hemagglutinin. When this region is targeted by neutralizing antibodies, successful neutralization of different subtypes of the virus is not observed, because this region changes constantly (*Lee 2012, Ekiert 2012, de Marco 2012*). However, the 'head' region also needs to have a constant, unchangeable region that can recognize the sialic acid receptors in humans. In accordance to this, presence of a non-variable region is necessary for a successful receptor binding of the hemagglutinin to the host cell. This region is presented as a pocket and therefore it not easily accessible to the immune cells. The recently presented neutralizing antibodies C05 and S139/1 are successful recognizers of the shallow receptor binding pocket and provide effective neutralization to most influenza A subtypes (*Ekiert 2012, Lee, 2012*).

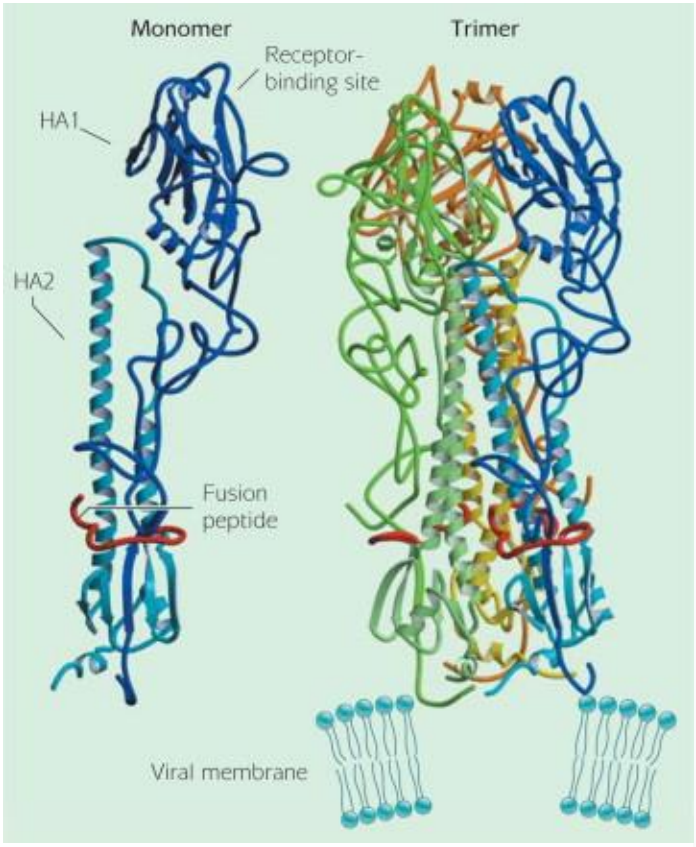

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Figure 1. **Three dimensional representation of the hemagglutinin**. On the left panel is representated one monomer of HA, with the HA1 (dark blue) and the HA2 (light blue) subdomains. The distal globular part, known as the 'head' contains the receptor binding site on the top and the proximal part known as the 'stem' region contains the fusion peptide (red). On the right panel is presented the whole molecule of hemagglutinin as a trimer. Different colors represent different monomers (green, orange and blue)

The 'stem' region presents the membrane proximal part and is formed by the HA2 segment (known as F subdomain) and a small portion of the HA1 segments (known as F' subdomain). It has the characteristics of a stable, non-variable part of the HA molecule, which contains the fusion peptide, important for membrane fusion in the acidic endosome of the host cells (*Skehel 2000*). It represents the

most attractive target for the neutralizing antibodies, because recently reported efficient neutralization of many influenza subtypes including influenza A and influenza B viruses, was by recognition of the 'stem' region of the HA (*Ekiert 2009, Sui 2012, Corti 2011*). Sui *et al*. describe that the target of the F10 neutralizing antibody in the stem region is the hydrophobic pocket that is formed after cleavage of HA into the HA1 and HA2 subdomain and is located just below the receptor binding head region. By interaction with this stem pocket, the fusion peptide is locked, and the structural reorganizations in the HA required for membrane fusion are inhibited. This antibody showed only group 1 influenza A specificity. Later Corti *et al*. described that the F16 antibody is sharing the same binding site with F10 antibody, with different type of interaction with the 'stem' pocket. By incorporation of only one heavy chain complementarity determining region (HCDR) instead of three in the stem binding site, broader spectrum of neutralization was achieved and subtypes from group 2 influenza A viruses were also neutralized. Recently was presented the CR9114 antibody that targets the stem region by using three HCDRs domains and has the ability to neutralize influenza A and B viruses together, which makes it the most potent cross-protective neutralizing antibody (*Dreyfus 2012*).

Targeting the non-variable, highly conserved epitopes on the hemagglutinin molecule that are shared between many subtypes of influenza virus leads to successful neutralization of influenza viruses. From the previously described epitopes, we can say that selection of the stem region as a target for neutralizing antibody gave better results than targeting the receptor binding site in the cross-protective activity of the antibody with subsequent neutralization of the two types of influenza A and B.

IV. Underlying mechanism of virus inactivation

Antibodies can neutralize the influenza virus infectivity in several ways and two main mechanisms are involved in inhibition of virus activity: inhibiton of the receptor binding or blockage of the fusion. They can interfere with the binding of the virus to the sialic acid receptors on the host cell, or can block the uptake into cells by locking of the fusion peptide and the subsequent conformational changes of the HA (*Rhorer 2009*).

At the top of the 'head' region on the HA molecule, there is a shallow depression, which is the known receptor binding domain for sialic acid terminal residues of the glycoproteins and the glycolipids presented on the endothelial cells from the upper respiratory tract. The neutralizing antibodies that target the globular head region neutralize the virus infectivity by direct competition with the receptors of the host cell. Then the receptor binding pocket is occupied with the antibody and the virus can't attach to the cells. In two recent studies were introduced an efficient targeting of the shallow receptor binding pocket by an insertion of a single complementary determining region (CDR) of the antibody into the pocket (*Ekiert 2012, Lee 2012*). The S139/1 antibody inserts the HCDR2 region into the pocket and blocks the binding to the host receptors (*Lee 2012*). The C05 antibody incorporates efficiently into the receptor binding pocket by insertion of a loop from the HCDR3 region, without contacting many of the surrounding variable parts. It shows direct competition with the sialic acid binding receptor domain, although they have different interactions with the receptor binding pocket (*Ekiert 2012*).

Fusion of the virus with the host cell is an important process for efficient infectivity of the influenza virus. When the virus attaches to the sialic acid receptors of the host cell, the virus become

engulfed by the cell membrane and subsequently the virus envelope and the membrane of the host cell become continuous, which results in mixing the both compartments. This is a pH-dependent process because low pH induces structural conformational changes in the HA, and the fusion peptide is uncovered and can interact with the host cell (*Sammalkorpi 1997*). Inhibition of this process leads to successful neutralization of the virus infectivity and can be obtained by at least three different mechanisms (*Corti 2011, Barbey-Martin 2002*). One involves stabilization and resistance to cleavage of the precursor HA0. Inhibited formation of HA1 and HA2 leads to inactive HA (*Corti 2011*). In the second one the cleavage of the HA antigen occurs, but the conformational changes are inhibited, and subsequently the fusion peptide is not exposed to the target membrane (*Corti 2011*). The third one involves cleavage and conformational changes in the HA molecule, but afterwards the exposed fusion peptide is blocked by the light chain of the neutralizing antibody (*Barbey-Martin 2002*). Usually the neutralizing antibodies that target the 'stem' region such as F16 and CR9114, include all three mechanisms for virus neutralization (*Corti 2011, Dreyfus 2012*). The F10 antibody inhibits the conformational changes in the HA molecule, but can neutralize only the group 1 subtypes of influenza A virus.

Neutralization mechanism that involves targeting of the receptor binding site seems tricky, because the receptor binding pocket is shallow and the structure of the surroundings is highly variable and that can jeopardize, in future the cross-reactive protection due to antigenic changes in these particular parts. The 'stem' region is shown as a being strictly conserved in many subtypes of influenza and can give better results in broad neutralization. So far the most potent CR9114 antibody with neutralization activity against influenza A and B also targets the 'stem' region (*Dreyfus 2012*). Sometimes this region seems unavailable for antibodies because it is hidden by the globular 'head' region, and then the accessibility is limited depending on the HA coverage of the virus surface. Also even the virus, has accomplished the contact with the host cell by attaching to the receptors; blockage of the subsequent fusion is as powerful neutralizing mechanism as the inhibition of receptor binding to the target cell.

V. Efficacy of nAbs in influenza prevention/therapy

Neutralization of the virus infectivity by broad-spectrum antibodies showed surprisingly better results in *in vivo* animal studies as compared with the *in vitro* experiments such as the hemagglutination inhibition (HI) assay and the microneutralization tests (MNT) (*Dreyfus 2012*). Here raises the question; do *in vitro* experiments truly represent the *in vivo* activity of the neutralizing antibodies. The basis of the HI assay is prevention of red blood cell hemagglutination. If neutralizing antibodies that prevent attachment of the virus are present, the hemagglutinin binding site will be inactivated and no lattice formation between the influenza viruses and the red blood cells will be observed. Microneutralization is another test to assess the antibody affinity to the receptor binding site of the HA in influenza virus. Shorty, in this test cell lines like MCDK are incubated with suspension made of viruses and antibodies. Afterwards the results are interpreted, based on the cytopathic effect. If the virus receptors are blocked by the antibodies, the cells will be viable and no damage will be observed. These tests are widely used for investigation of the immune response to vaccination against influenza viruses and therefore are used

in laboratories for credible selection of cross-protective antibodies (*Grund 2010*). When the antibodies neutralize the virus by binding to other epitope on the hemagglutinin, than to the receptor region standard *in vitro* tests can be irrelevant. Corti *et al.* overcome this problem by performing inhibition of test based on syncytia formation, to re-check the efficacy of their antibody. In this technique transfection of hemagglutinin in particular cell line was done and the cells were further incubated with the antibodies of interest. No visible formation, of multinuclear cells goes in favor for effective blockage of the fusion between the viruses and the host cell. Although the standard *in vitro* techniques can fail in their goal, clearly they are important part in the research process to narrow the list of useful antibodies and to continue the work with the efficient ones. Improvement or involvement of other *in vitro* techniques would make this process more effective.

Mouse models are the most common organisms used for *in vivo* experiments. They share 95% of the genome with humans, they are small and easy to handle. The mice models have been1 used for decades as an infection model for influenza, because they show good prediction of the human response to influenza infection and vaccination (*Bouvier 2010*). That makes them appropriate and cheap model for studying the protective efficacy of neutralizing antibodies. In previous studies it was shown that doses in a range of 1 to 10 mg/kg, provide nearly 100% protection against infection with human (H1, H2, H3) and avian (H5) subtypes of influenza virus (*Ekiert 2012, de Marco 2012, Sui 2009, Corti 2011*). Antibody suspension is given as a single dose 24 hours prior to inoculation with lethal dose of the virus. For therapeutic treatment the dosage regimen is different and 15 mg/kg of antibodies is a standard baseline dose, because is easily achievable dose in humans. Single dose of antibodies is given in the first, second or third day of infection and subsequently two weeks observation period is conducted to check the mice survival and weight loss. The results showed 80-100% protection against lethal infection (*Ekiert, 2012, de Marco 2012, Sui 2009, Corti, 2011*) and potent suppression of the viral replication in the lungs evaluated by measuring the viral titers in the lungs four days after infection. Presented prophylactic and therapeutic efficacy in mice underline the importance of developing anti-influenza strategies based on passive immunization. Still the passive immunization is not the best cost-effective method to stop the influenza infection. Firstly the production of antibodies takes some time and lots of money and secondly the amount of virus particles decreases rapidly by three days after onset in experimental infection model (*Lau 2010*). In accordance with this, passive immunization treatment is useful in the first 72 hours; because afterwards virus is eliminated from the body and main concern then are secondary bacterial infections such as pneumonia. Prophylaxis with antibodies can't be used as a broad-protective treatment. When administrated as a treatment, the serum half-time of the antibodies is between 2 and 20 days (*Hinton 2006*), they are expensive for production and can be used only in particular cases, such as for people at high risk of infection like elderly and children, or when there is an insufficient time for the body to develop own immune response. It should be reconsidered the cost-effectiveness of neutralizing antibodies as a prophylactic and therapeutic treatment. Induction of cross-protective neutralizing antibodies by universal vaccines against all subtypes of influenza viruses in humans would be the most ideal protection from epidemics and pandemics.

VI. Discussion

The remarkable progress over the past years with regard to the role of broad-spectrum neutralizing antibodies in protection against influenza leaves us with a number of possibilities. They can be used as a novel passive immunization treatment against influenza infection, but the highest impact on the public health would be achieved, if these antibodies can be elicit in the organism by suitable universal vaccine. So far, the isolated F16 cross-protective neutralizing antibody from few vaccinated or infected individuals with single plasma cell isolation (*Corti 2012*) lead us to conclusion that immune system in response to conserved structures in the antigen can produce such antibodies. Still needs to be investigated why these antibodies are only found in few people, in order to give us a solid base for further developing of universal vaccines. Partially, this can be explained by the different individual characteristics of the B cells or by the multiple exposures to the influenza antigens. Utilization of single plasma cell isolation method should be imperative for the discovery of naturally produced broad-spectrum neutralizing antibodies, which can be used for immunoprophylaxis.

Constant variation of the HA molecule, challenges the immune system to produce each time a new compatible antibodies. Targeting the highly conserved regions in the HA, which are also vital for the infectivity of the virus leads to efficient targeting and blockage of the virus activity. The neutralizing antibodies that target the 'stem' region are more effective than the antibodies that recognize the binding receptor part of the HA, due to the position of the fusion peptide in the membrane proximal part of the HA which has the characteristics of non-variable part. By targeting this region the fusion of the virus with the host is interrupted and the possibility to inhibit this process in several ways, as indicated earlier, makes this region more susceptible to the broad-spectrum neutralizing antibodies.

When administrated in the mice, the neutralizing antibodies efficiently protect the organism from influenza infection. These promising results show that the cross-protective neutralizing antibodies are seen as a possible future treatment of the influenza infection. However, the use of neutralizing antibodies in case of epidemics or pandemics would be unimaginable because, firstly large amounts of antibodies need to be produced and secondly their production is expensive. More promising implication of these antibodies would be for discovery of universal vaccine that can elicit broad-spectrum protection against all subtypes of influenza viruses. To date, the presentation of HA epitopes on virus-like particles showed promising strategy to elicit production of broad spectrum antibodies, but the influenza virus neutralization was missing in *in vivo* experiments (*Schneemann 2012*), which means that the tolerance mechanisms of the body also need to be determined besides the characteristics of the proposed vaccine.

In conclusion, highly conserved regions of the hemagglutinin antigen of influenza viruses form the bases for development of broad-spectrum neutralizing antibodies that can provide cross-protection against different subtypes of influenza viruses in the organism. The gained knowledge about successful targeting of conserved antigens on the surface of variable viruses and their subsequent neutralization is used intensively for designing novel treatments for diseases caused by other highly variable viruses such as HIV, hepatitis C virus and human papilommavirus.

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