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# The essentiality of alpha-2-macroglobulin in human salivary innate immunity against new H1N1 swine origin influenza A virus

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## Abstract

A novel strain of influenza A H1N1 emerged in the spring of 2009 and has spread rapidly throughout the world. Although vaccines have recently been developed that are expected to be protective, their availability was delayed until well into the influenza season. While anti-influenza drugs such as neuraminidase inhibitors can be effective, resistance to these drugs has already been reported. Although human saliva was known to inhibit viral infection and may thus prevent viral transmission, the components responsible for this activity on influenza virus, in particular, influenza A swine origin influenza A virus (S-OIV), have not yet been defined. By using a proteomics approach in conjunction with beads that bind alpha 2,6-sialylated glycoprotein, we determined that an alpha-2-macroglobulin (A2M) and a A2M-like protein are essential components in salivary innate immunity against hemagglutination mediated by a clinical isolate of S-OIV [San Diego/01/09 (SD/H1N1-S-OIV)]. A model of an A2M-based "double-edged sword" on competition of alpha 2,6-sialylated glycoprotein receptors and inactivation of host proteases is proposed. We emphasize that endogenous A2M in human innate immunity functions as a natural inhibitor against S-OIV.

### Keywords

Proteomics; alpha 2,6-sialylated glycoproteins; alpha-2-macroglobulin; Salivary innate immunity; H1N1 swine origin influenza A virus

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A novel influenza A H1N1 virus, termed influenza A swine origin influenza A virus (S-OIV) [1], combines a triple assortment first identified in 1998 - including human, swine and avian influenza - with two new pig H3N2 virus genes from Eurasia, themselves of recent human origin. Although the full clinical spectrum of the illness has not been fully delineated, there are indications that it can cause serious illness in certain patients, especially those younger than 50 years of age who have not been exposed to similar viruses and those with underlying respiratory, cardiac, neurological and immunosuppressive disorders. The influenza A H1N1 strain that was used in the development of the trivalent vaccine planned for the 2009–2010 season is immunologically quite distinct from influenza A S-OIV and does not produce protective immunity against this new strain of virus. Influenza A H1N1 S-OIV can be treated with the influenza neuraminidase inhibitors including oseltamivir (Tamiflu) and zanamivir (Relenza) [2,3]. Recently, the neuraminidase inhibitor resistant strains of H1N1 S-OIV have been reported. In this study, we attempted to define the endogenous molecules that function as natural inhibitors in human innate immunity against S-OIV infection with the hope that this knowledge would contribute to the development of antiviral agents with new mechanisms of action. Most importantly, the use of endogenous molecules for treatments of viral infection is fully consistent with evolutionary (Darwinian) medicine, which supports the notion that innate antimicrobials and innate antimicrobial resistance mechanisms have co-evolved, resulting in a host-virus balance that has shaped the existing repertoire of innate antimicrobials in humans [4]. Furthermore, if these endogenous molecules can efficiently counteract various subtypes of influenza virus, their broad-spectrum antiviral activities may overcome the drawbacks of vaccines, which are strain-specific, and as has been demonstrated this year pose logistical challenges in terms of rapid production and widespread availability. Although the respiratory tract is the main site of replication for influenza virus, the virus is able to colonize and persist in oral cavity and this cavity is a potentially important route for viral transmission.

## Human saliva abolishes S-OIV-induced hemagglutination

We first examine if human saliva exerts inhibitory effect on virus-induced hemagglutination. A H1N1 S-OIV San Diego/01/09 (SD/H1N1-S-OIV) was propagated in Madin-Darby canine kidney (MDCK) cells. Whole saliva was collected from three healthy volunteers (Supplementary Materials and methods). Non-aggregated guinea pig erythrocytes in phosphate buffered saline (PBS) settled to the v-bottom 96-well microtiter plates where they form a round pellet. Hemagglutination occurred when SD/H1N1-S-OIV [32 hemagglutinating units (HAU)] bound to the erythrocytes and prevented the erythrocytes from forming the pellet. To examine if human saliva inhibits hemagglutination, guinea pig erythrocytes were mixed with SD/H1N1-S-OIV in the presence of human whole saliva (Fig. 1). The saliva reduced SD/H1N1-S-OIV-induced hemagglutination titer by approximately 16-fold, in agreement with previous findings that human saliva and salivary gland extracts possess the capacity to inhibit hemagglutination by influenza viruses including swine influenza virus [5–8]. To examine if saliva itself has hemagglutination activity, we incubated erythrocytes with saliva alone in the absence of SD/H1N1-S-OIV. As shown in Fig. 1 lane 5, no hemagglutination activity was observed.

## The essentiality of alpha 2,6-sialylated glycoproteins in human saliva against S-OIV

Influenza hemagglutinin (HA) mediates viral attachment to sialylic acid cell receptors and virus entry into target cells [9]. The cleavage of HA0 (75kDa) into HA1 (55kDa)/HA2 (25kDa) by host proteases activates virus infectivity [10,11] and is important for influenza virus pathogenicity [12,13]. It has previously been shown that guinea pig erythrocytes express alpha 2,6-sialylated glycoprotein receptors [14]. Most human influenza isolates

preferentially bind to an alpha 2,6-sialylated glycoprotein receptor, although influenza A (H1N1) 2009 HA might be less stringent in its binding specificity and also can bind to a range of alpha 2,3-sialylated glycoprotein receptors [15]. It has been reported that several salivary proteins can inhibit the hemagglutination of erythrocytes. These proteins including MUC5B, scavenger receptor cysteine-rich glycoprotein 340 (salivary gp-340), histatins, and human neutrophil defensins (HNPs) function as endogenous components in human innate immunity against influenza virus infection [16]. MUC5B counteracted influenza virus by presenting a sialic acid ligand for the viral HA [16]. Although these proteins have antiviral activities, their essentiality in the ability of human saliva to inhibit hemagglutination has not been demonstrated. For example, the gp-340 did contribute to some of the antiviral activity of saliva, but activity remained after removal of gp-340 [17]. To determine the essentiality of salivary proteins in innate immunity against S-OIV, alpha 2,6-sialylated glycoproteins in whole saliva were removed by *Sambucus nigra* (SNA) lectin coated beads (Supplementary Materials and methods) before incubation of saliva with erythrocytes and SD/H1N1-S-OIV. As shown in Fig. 1A, lane 4 and B, the inhibitory effect of whole saliva on SD/H1N1-S-OIV-induced erythrocyte hemagglutination was noticeably diminished after removal of alpha 2,6-sialylated glycoproteins. Western blot analysis validated that a SNA bead-bound alpha 2,6-sialylated glycoprotein with a molecular weight greater than 100 kDa was completely removed from whole saliva (Fig. 2A). These data demonstrated that the alpha 2,6-sialylated glycoproteins are an essential component in salivary innate immunity that contributes to the disruption of S-OIV-induced erythrocyte hemagglutination.

## Identification of alpha 2,6-sialylated glycoproteins as alpha-2-macroglobulin (A2M) and A2M-like protein

In-gel trypsin digestion was conducted to extract proteins in a major band (>100 kDa) in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicated in Fig. 2A lane 3. A nano liquid chromatography linear trap quadruple mass spectrometry (Nano-LC-LTQ MS/MS) analysis was employed to sequence the tryptic digests (Supplementary Materials and methods). Two proteins (A2M; 163.3 kDa; Accession Number: P01023 and A2M-like protein; 161.1 kDa; Accession Number: A8K2U0) were identified from this major band. An internal peptide (AYIFIDEAHITQALIWLSQR; 1054–1073 amino acid residues) of A2M (Fig. 2B) and an internal peptide (YATTAYVPSEEINLVVK; 1251–1267 amino acid residues) of A2M-like protein are presented (Fig. 2C). Seventy-six internal peptides derived from A2M and two internal peptides derived from A2M-like protein were fully sequenced by Nano-LC-LTQ MS/MS analysis (Supplementary Table 1).

## A2M reduces the virus-induced hemagglutination and viral infectivity

To verify the essentiality of A2M in human saliva against SD/H1N1-S-OIV, a human native A2M protein was added into an A2M-removed (SNA bead-unbound) fraction of saliva. As shown in Fig. 3A, the addition of A2M (75 μg) significantly restored the reduction of SD/H1N1-S-OIV-induced hemagglutination caused by absence of A2M in saliva. To examine if A2M itself has antiviral activity, A2M (75 μg) was added into a mixture of guinea pig erythrocytes and SD/H1N1-S-OIV. Data in Fig. 3A showed that A2M inhibited the hemagglutination medicated by SD/H1N1-S-OIV. By contrast, the addition of BSA (75 μg) did not display any inhibitory effect on hemagglutination, indicating that the effect was not simply nonspecific protein-mediated. To determine if A2M possesses the ability to reduce the viral infectivity, MDCK cells were infected with SD/H1N1-S-OIV in the presence of A2M or BSA. The presence of A2M (75 μg) considerably decreased the cell death (10.19 ± 5.89%) caused by virus infection compared to that of the same amount of BSA (40.05 ± 3.91%) (Fig. 3B). These results clearly demonstrated the antiviral activity of human A2M.

## A2M is an alpha 2,6-sialylated glycoprotein and protease inhibitor that may function as a “double-edged sword” against S-OIV infection

A2M is not only present in human saliva [18,19], but also predominantly in nasal secretions [20], lung lavages [21] and blood [22,23]. Purified equine A2M has been shown to be a potent inhibitor of both hemagglutination and infection of influenza viruses containing the H2 and H3 subtype HA [24]; the latter being introduced into the human virus population in 1968 as the Hong Kong strain (H3N2), demonstrating that A2M is an endogenous molecule that has broad-spectrum anti-influenza activity. In addition, the N-glycanase-released glycans of human and equine A2M was capable of inhibiting influenza virus [A/Memphis/102/72(H3N2)] adsorption to erythrocytes [22], suggesting that surface glycans including 2-6-linked sialic acid residues of A2M may be at least partially responsible for the antiviral activity of A2M. Furthermore, it has been reported that A2M can interact and capture virtually any protease whether self or foreign, suggesting a function as a unique "pan-protease inhibitor" [25]. A2M has a peptide stretch, called the “bait region” which contains specific cleavage sites for different proteases [25]. When a protease cleaves the bait region, a conformational change is induced in A2M that traps the protease. As shown in Fig. 4, the A2M may have bi-functionality: the 2-6-linked sialic acid residues block influenza virus infection by competition of alpha 2,6-sialylated glycoprotein receptors on the host, and bait region inactivates the host proteases to prevent virus entry into the host cells. Although data in this study did not provide evidence for the interaction of A2M with various host proteases, it is worthwhile to evaluate the *in vivo* potency of A2M against SD/H1N1-S-OIV in animals that contain various proteases with a positive [11,12] or negative [26] effect on viral infection.

The A2M and anti-HA antibody may compete for binding to alpha 2,6-sialylated glycoprotein receptors. If A2M directly binds to antibody epitopes or creates steric hindrance by binding to a site in close proximity to epitopes, it will decrease the potency of curative antibodies circulating in our body. To explore the possibility, we added purified A2M into an immunoreaction of anti-HA serum to HA in Western blot analysis (Supplementary Fig. 1). The specific antiserum to HA was produced in mice immunized with an inactivated *Escherichia coli* (*E. coli*) over-expressing HA, but not green fluorescent protein (GFP) or CAMP factor as described in Supplementary Materials and methods. The addition of 75 μg of A2M or BSA (a control protein) did not alter the binding of anti-HA serum to HA (Supplementary Fig. 1) despite that the same amount of A2M efficiently reduced virus-induced hemagglutination and viral infectivity (Fig. 3). Although the result suggests that A2M may not influence the binding of anti-HA antibody to HA, we cannot rule out the possibility that not all the binding sites for SD/H1N1-S-OIV HA are saturated by A2M. In addition, if the ability of A2M to inactivate proteases contributes more to the inhibition of viral infection than dose the ability of A2M to bind alpha 2,6-sialylated glycoprotein receptors, A2M may eventually diminish the viral infectivity and could potentiate the efficacy of neutralizing anti-HA antibodies. Thus, it would be interesting to investigate the impact of A2M on anti-influenza vaccines in the future.

The A2M-like protein fits the biological characteristics of A2M: 1) high conservation in amino acid sequence including most of cysteine positions with A2M; 2) a putative central bait region; 3) a classic thiol ester sequence. A2M-like protein shares approximately 40% amino acid identity with A2M. The protein has been identified as a novel protease inhibitor in human epidermis and expressed predominantly in the granular layer at the apical edge of keratinocytes [27]. The binding of A2M-like protein to SNA-coated beads suggests that A2M-like protein is an alpha 2,6-sialylated glycoprotein. In addition, our data demonstrate for the first time that A2M-like protein is an essential component in salivary innate immunity against SD/H1N1-S-OIV-induced hemagglutination. Seventy-six internal peptides

of A2M were sequenced whereas only two internal peptides of A2M-like protein were identified. The distinction may be due to the difference in amino acid sequences that influence the sensitivity to trypsin digestion and/or differential protein abundance in human saliva. Future works will determine the abundances of A2M and A2M-like proteins in various human body fluids and investigate if the deficiency of A2M or A2M-like protein in humans is a cause of influenza virus infection or correlates with the severity of this infection. In humans, the α1-antitrypsin (A1AT) deficiency is a hallmark of the development of early-onset pulmonary emphysema [28,29]. Augmentation therapy using purified A1AT such as human plasma-pooled Prolastin® has been used in clinics for treatment of chronic obstructive pulmonary disease [30]. Thus, it is valuable to assess whether purified A2M can be used in augmentation therapy to limit influenza virus infection. Recently, Elledge and his colleagues have identified the interferon-inducible transmembrane (IFITM) proteins in human cells as natural antiviral proteins that restrict an early step in H1N1 influenza viral replication [31]. The finding provided a mechanism explaining how cells eliminated viruses once they got infected. Congruently, the secretory A2M and A2M-like proteins may be released by host cells to attenuate the infectivity of H1N1 influenza virus before virus comes into contact with cells.

## Significance and conclusion

This study has potentially broad implications because 1) proteomics in conjunction with lectin-coated beads provides a simple method to identify receptor-targeted inhibitors against S-OIV infection; 2) the method can be applied to the identification of novel viral inhibitors from various biological samples; 3) exploring the function of endogenous molecules such as A2M and A2M-like protein as natural viral inhibitors opens a new area of research on salivary innate immunity against influenza virus infection; 4) inactivating influenza virus in the oral cavity could reduce human-to-human transmission; 5) developing A2M-derived antiviral therapeutics (such as glycanor protease-based inhibitors) may be useful for a combinational therapy with current antiviral drugs; 6) most importantly, the use of endogenous molecules as natural antiviral agents is concordant with evolutionary medicine and could provide a new set of tools for fighting antiviral resistance.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Abbreviations

| | |
|---|---|
| **A1AT** | α1-antitrypsin |
| **A2M** | alpha-2-macroglobulin |
| **ACP** | acid phosphatase |
| **BR** | bait region |
| **BSA** | bovine serum albumin |
| **CFU** | colony forming units |
| **CID** | collision-induced dissociation |
| ***E. coli*** | *Escherichia coli* |
| **GFP** | green fluorescent protein |
| **HA** | hemagglutinin |

| | |
|---|---|
| **HAU** | hemagglutinating unit |
| **HNPs** | human neutrophil defensins |
| **IFITM** | interferon-inducible transmembrane |
| **IPTG** | isopropyl b-D-thiogalactopyranoside |
| **MDCK** | Madin-Darby canine kidney |
| **MEM** | minimum essential medium |
| **NA** | neuraminidase |
| **Nano-LC-LTQ MS/MS** | nano liquid chromatography linear trap quadrupole mass spectrometry |
| ***P. acnes*** | *Propionibacterium acnes* |
| **PBS** | phosphate buffered saline |
| **PVDF** | polyvinylidene fluoride |
| **SE** | standard error |
| **S-OIV** | swine origin influenza A virus |
| **SD/H1N1-S-OIV** | San Diego/01/09 S-OIV |
| **SNA** | *Sambucus nigra* |
| **SDS-PAGE** | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **UV** | ultraviolet |

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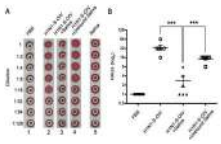

**Figure 1.**

Blockage of the inhibitory effect of human whole saliva on SD/H1N1-S-OIV-induced erythrocyte hemagglutination after removal of alpha 2,6-sialylated glycoproteins. (A) A representative result from six independent hemagglutination activity inhibition assays was shown. The hemagglutination of 50 µl of 0.5% guinea pig erythrocytes in PBS occurred at room temperature for 40 min (lane 1; PBS). The hemagglutination activity inhibition assay was performed by mixing 50 µl of 0.5% guinea pig erythrocytes in PBS with 25 µl of each dilution (as indicated) of SD/H1N1-S-OIV alone (32 HAU), (lane 2; H1N1-S-OIV) or with 25 µl of each dilution of human whole saliva (1 µg/µl) (lane 3; H1N1-S-OIV+Saliva). The alpha 2,6-sialylated glycoproteins in human whole saliva were removed by using SNA lectin-coated beads. After removal, the bead-unbound fraction of saliva (Unbound Saliva) was used for hemagglutination activity inhibition assay. The 50 µl of 0.5% guinea pig erythrocytes in PBS with 25 µl of two-fold serial dilution (1–128) of SD/H1N1-S-OIV (32 HAU) in the presence of 25 µl of two-fold serial dilution (1–128) of unbound saliva (1 µg/µl) (lane 4; H1N1-S-OIV/Unbound Saliva). The 50 µl of 0.5% guinea pig erythrocytes in PBS mixed with 50 µl of each dilution of human whole saliva in the absence of SD-H1N1-S-OIV served as (lane 5; Saliva) an additional control. (B) The inhibition of hemagglutination activity was expressed as $\log_2$ of HAUs and calculated from six independent experiments. ***, $P < 0.001$ by Student's t-test.

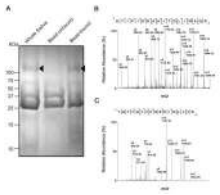

**Figure 2.**

Validation of the removal of alpha 2,6-sialylated glycoproteins from whole saliva by western blot and mass spectrometric identification of alpha 2,6-sialylated glycoproteins. (A) The alpha 2,6-sialylated glycoproteins in human whole saliva bound to SNA lectin-coated beads were dissociated by heating at 100°C in SDS-PAGE sample buffer. Proteins (50 μg) in whole saliva (lane 1), bead-unbound (lane 2) and bound (lane 3) fractions were subjected to a 12 % SDS-PAGE and stained with Coomassie blue. Arrowheads indicated that a protein above 100 KDa in whole saliva was completely removed by SNA lectin-coated beads. Tryptic digests of a protein band indicated in (A) were subjected to Nano-LC-LTQ MS/MS. Two sequenced peptides [AYIFIDEAHITQALIWLSQR (B) and YATTAYVPSEEINLVVK (C)] are presented and assigned as internal peptides of A2M (P01023) and A2M-like protein (A8K2U0), respectively. The m/z value of each “y” and “b” ion in collision-induced dissociation (CID) spectra was indicated. All sequenced peptides were shown in supplementary Table 1.

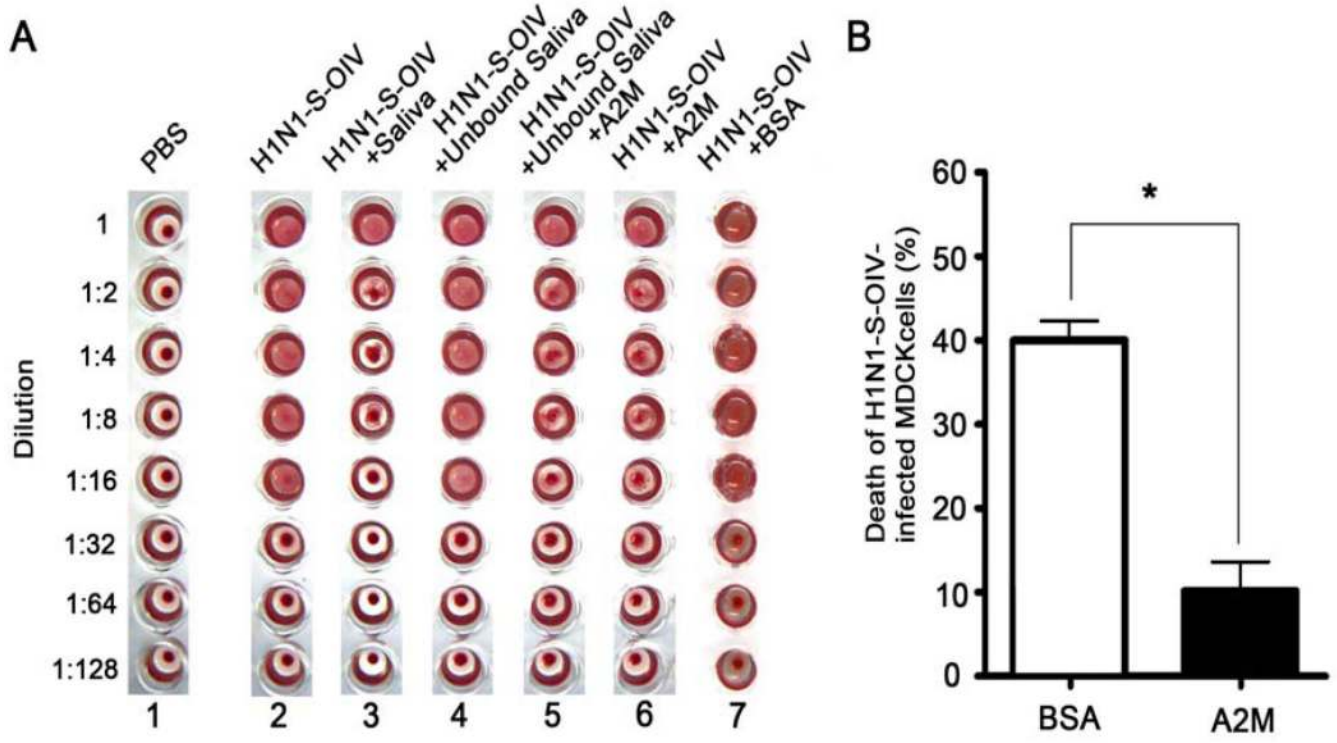


**Figure 3.**

Restitution of the diminished anti-hemagglutination activity of bead-unbound fraction of saliva by adding purified A2M and examination of the potency of A2M in reduction of hemagglutination and viral infectivity. (A) The hemagglutination activity inhibition assay was performed as described in Fig. 1. The guinea pig erythrocytes in PBS (lane 1; PBS) were mixed with SD/H1N1-S-OIV (32 HAU) (lane 2; H1N1-S-OIV) in the presence of human whole saliva (lane 3; H1N1-S-OIV+Saliva), unbound saliva (lane 4; H1N1-S-OIV/Unbound Saliva), unbound saliva plus purified A2M (75 μg) (lane 5; H1N1-S-OIV/Unbound Saliva/A2M), purified A2M (75 μg) alone (lane 6; H1N1-S-OIV/A2M) or BSA (75 μg) (lane 7; H1N1-S-OIV/BSA). A representative of three separate experiments with similar results was shown. (B) To test the effect of A2M on viral infectivity, a 1/100 dilution of SD/H1N1-S-OIV (32 HAU) plus 75 μg of A2M or BSA was added into confluent MDCK cells in a 96-well plate for 1 h before adding virus growth medium. After overnight culture, the cell viability was determined by an acid phosphatase (ACP) assay as described in Materials and methods. Data represent the mean ± standard error (SE) of three independent experiments. *, $P < 0.05$ by Student's t-test.

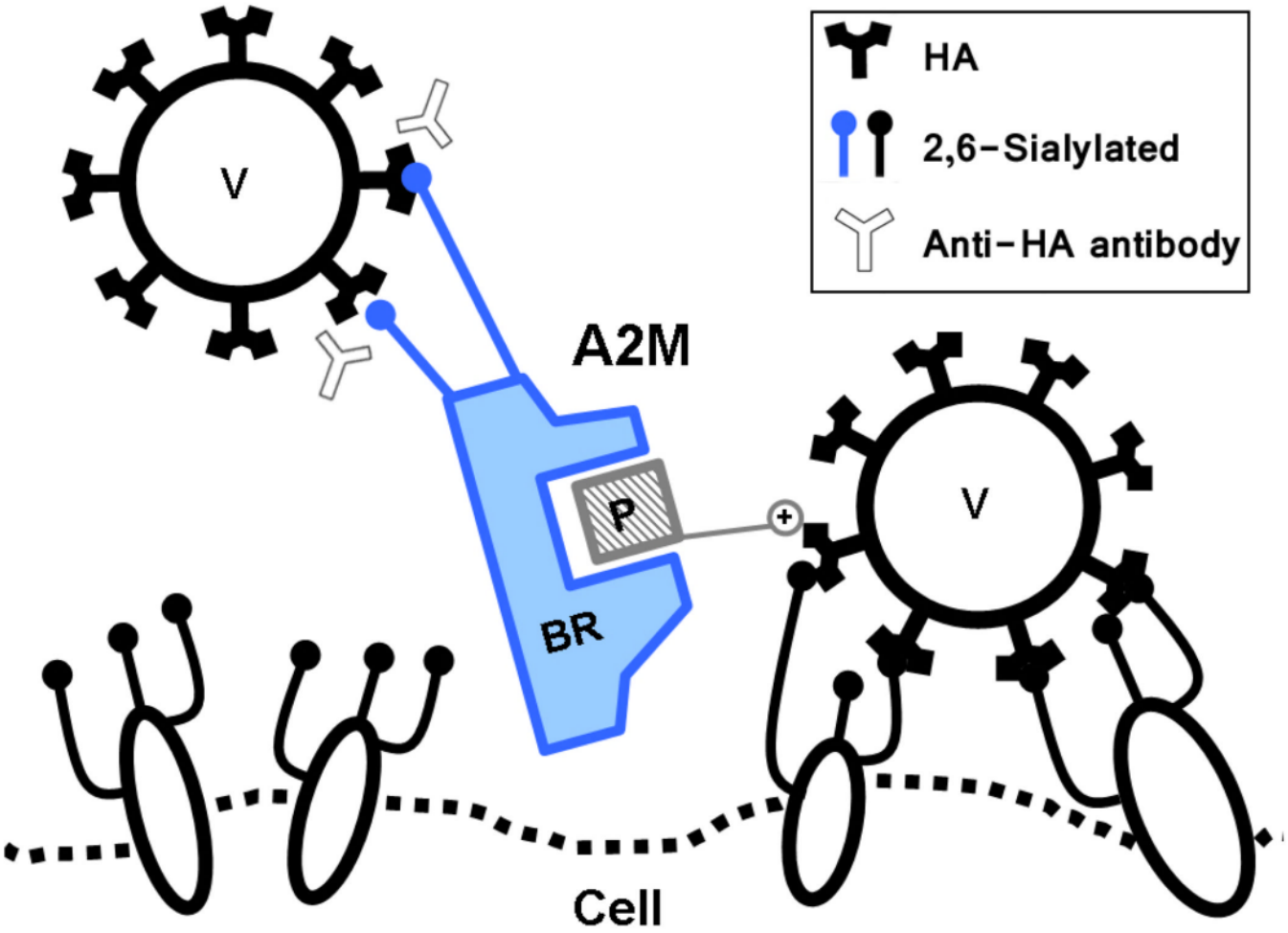


**Figure 4.**
Scheme of A2M functions as a "double-edged sword" on the blocking and inactivating influenza virus infection. Activation of human influenza virus HA was mediated by host proteases (P) and attachment to cells was via cell-surface alpha 2,6-sialylated glycoprotein receptors (2,6-sialyated). The 2,6-sialyated residues of A2M obstruct influenza virus (V) infection by competing with alpha 2,6-sialylated glycoprotein receptors on the host, and the bait region (BR) of A2M traps and inactivates the host proteases that disrupt the viral infectivity. The binding of A2M to HA may influence the recognition of HA by anti-HA antibody. Both activities of A2M (binding of alpha 2,6-sialylated glycoprotein receptors and inactivation of host proteases) may impact the efficacy of antiviral vaccines.