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# Limited effect of recombinant human mannose-binding lectin on the infection of novel influenza A (H7N9) virus *in vitro*



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## ABSTRACT

Mannose-binding lectin (MBL), a pattern-recognition molecule in serum, recognizes specific hexose sugars rich in mannose and N-acetylglucosamine on bacterium, yeasts, viruses as well as apoptotic cells. It has been well-identified that MBL has antiviral effects via binding to seasonal influenza H1 and H3 subtype viruses. Influenza A (H7N9) virus, a novel reassortant virus to human population, possesses the surface hemagglutinin (HA) and neuraminidase (NA) genes from duck and wild-bird influenza viruses and internal genes from poultry H9N2 viruses. As of Dec 7th, 2014, a total of 467 human infections and 183 fatal cases have been identified. Here, recombinant human (rh) MBL was tested for its binding and effects on hemagglutination inhibition (HI) and NA activity inhibition (NAI) of avian H7N9, H9N2 and human H3N2 viruses. We discovered that rhMBL exhibited a strong binding to H7N9 virus as human H3N2 did at high virus titers. However, it performed a significantly weaker HI activity effect on H7N9 comparing to those of H3N2 and H9N2, even at a much higher concentration ( $3.67 \pm 0.33$  vs.  $0.026 \pm 0.001$  and  $0.083 \pm 0.02$   $\mu\text{g/mL}$ , respectively). Similarly, minor NAI effect of rhMBL, even at up to 10  $\mu\text{g/mL}$ , was found on H7N9 virus while it displayed significant effects on both H3N2 and H9N2 at a lowest concentration of  $0.0807 \pm 0.009$  and  $0.0625$   $\mu\text{g/mL}$ , respectively. The HI and NAI effects of rhMBL were calcium-dependent and mediated by lectin domain. Our findings suggest that MBL, the host innate molecule, has differential interference effects with human and avian influenza virus and limited antiviral effect against H7N9 virus.

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## 1. Introduction

Host innate immunity plays a critical role in the early phase of infection. This first-line defense against pathogens is mediated by a variety of pattern-recognition molecules including collectins, toll-like receptors and ficolins as well as inflammatory cytokines and type I interferon or macrophages and natural killer cells. Mannose-binding lectin (MBL) is one of collectins circulating in the serum and synthesized by liver. It consists of collagenous domains and

carbohydrate recognition domains (CRD). The CRDs recognize sugars including D-mannose, N-acetylmannosamine, N-acetylglucosamine and L-fucose on the surface of many pathogens in a calcium-dependent manner [1]. Previous studies showed that MBL can bind to a range of clinically relevant microorganisms such as *Staphylococcus aureus*, *Candida Albicans* [2], HIV, SARS-CoV, Ebola virus, HSV, influenza virus [3–6]. The binding of MBL to microorganisms is presumed to induce MBL conformational changes that allow the molecule to initiate viral neutralization or kill virus via opsonization or complement activation [7].

Influenza A virus, a segmented single-stranded negative-sense RNA virus, belongs to *orthomyxoviridae* and is subtyped according to the antigenic properties of their envelope glycoproteins, HA and NA. Currently, 16 HA subtypes and 9 NA subtypes circulate in birds. Among them, only seasonal H1N1 and H3N2 viruses circulate in human population [8]. Occasionally, some subtypes of avian

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influenza A virus can jump into human and cause diseases with a range of clinical symptoms and outcomes, such as conjunctivitis, mild upper respiratory tract disease, as well as severe pneumonia and death [9–12]. Viral HA and NA assist virus binding, entry and releasing during infection cycle. Their potential N-linked glycosylation sites (NGS) can be glycosylated, which might allow their binding to host MBL. It has been found that the glycan at residue 165 in H3N2 HA was of high-mannose and MBL neutralized viral infectivity via it. Many lines of evidences have shown that the MBL plays an important role in fighting against seasonal flu [13–15]. However, little is known about the interactions between avian influenza virus and the innate molecules. Avian influenza H7N9 virus is novel to human population [16,17], which contains the surface HA and NA genes from duck and wild-bird influenza viruses and internal genes from poultry H9N2 viruses. Unlike other H7 viruses that generally cause mild symptoms such as conjunctivitis or influenza-like illness (except one fatal case infected with H7N7 in Netherlands in 2003), H7N9 virus usually results in severe pneumonia or respiratory failure in human. Here, we examined the interactions of MBL with avian influenza virus H7N9, H9N2 and human virus H3N2. Furthermore, we studied the molecule mechanisms for them by structure modeling.

## 2. Materials and methods

### 2.1. Virus

The vaccine strain A/Anhui/1/2013(H7N9) (NIBRG-268) was obtained from National Institute for Biological Standards and Control (UK), namely H7N9Vac. The virus bears the HA and NA of A/Anhui/1/2013(H7N9) and internal genes of A/Puerto Rico/8/1934 (PR8, H1N1); A/Brisbane/10/2007(H3N2) was named as H3N2WT in the study; H9N2 virus, a reassortant bearing the HA, NA from A/Hongkong/33982/2009(H9N2) and internal genes of PR8, was named as H9N2RG. The reassortant H7N1<sub>HA1</sub> HA+PR8 NA was with HA of A/Anhui/1/2013 and seven genes of PR8, which is rescued as previously reported [18]. H7N9Vac, H3N2WT and H7N1<sub>HA1</sub> HA+PR8 NA were propagated in 9–11-day-old embryonated chicken eggs, H9N2RG was grown in Madin-Darby canine kidney (MDCK) cells (ATCC, USA) with Modified Eagle's Medium (invitrogen, USA) containing 2 µg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma, USA). Virus stocks were purified by adsorption to and elution from turkey red blood cells (TRBCs) and stored at –80°C until use [19]. Virus titer was determined by titration in MDCK cells and the tissue culture infectious dose affecting 50% of the cultures (TCID<sub>50</sub>) is calculated by the Reed–Muench formula [20].

### 2.2. Detection of MBL binding to influenza virus

Recombinant human MBL (rhMBL) was purchased from Sino Biological Inc (Beijing, China). Ninety-six-well plates were coated with  $2 \times 10^5$  TCID<sub>50</sub> influenza virus at a volume of 100 µl/well for overnight at 4 °C, then were blocked for 1 h with 1% Bovine Serum Albumin (BSA, Roche, Switzerland) at 37 °C. Different concentrations of rhMBL (0, 1, 3, 5, 7 µg/mL) were added and incubated for 1 h at 37 °C. The virus-dose dependent binding assay was conducted as that wells were precoated with  $2 \times 10^2$ ,  $2 \times 10^3$ ,  $2 \times 10^4$  and  $2 \times 10^5$  TCID<sub>50</sub> influenza viruses per well. Then 3 µg/mL rhMBL was added and incubated for 1 h at 37 °C. The binding was detected by the biotinylated human MBL pAb (0.2 µg/mL) (R&D, USA), followed by streptavidin-horseradish peroxidase (HRP) (1:200) (R&D, USA) and tetramethylbenzidine substrate solution (BD, USA), the reaction was stopped by 2 M H<sub>2</sub>SO<sub>4</sub> and the Optical Density (OD) at 450 nm was measured by ELISA reader (Perkin-Elmer, USA). The wells coated with 10 µg/mL mannan from

*Saccharomyces cerevisiae* (Sigma, USA) or coating buffer (Kirkegaard & Perry Laboratories, USA) were used as positive control and negative control respectively. The test was performed in duplicates and in three independent experiments, absorbance from negative control was subtracted and results were normalized to positive control, data was expressed as a relative absorbance value using mean  $\pm$  SEM (%).

### 2.3. Hemagglutination inhibition (HI) assays

HI assay was performed in V-bottom 96-well plates as previously described [20]. Briefly, 25 µL influenza virus (4HAU) was mixed with 25 µL rhMBL of different concentrations diluted in Hank's Balanced Salt Solution (HBSS) containing 1.26 mM Ca<sup>2+</sup> for 1 h at 37 °C, then 50 µL 1% TRBC was added to the mixture and incubate at room temperature for 30 min. For HI reverse assay: rhMBL was diluted in HBSS containing 10 mM EDTA or 10 mg/mL mannan, then incubated with 4 HAU of influenza virus. The results were expressed as the minimum inhibitory concentration (MIC) of rhMBL that exhibited HI effect.

### 2.4. Neuraminidase activity inhibition (NAI) assays

Influenza virus NA activity was measured by ELISA in which peanut agglutinin conjugated with HRP was used to detect β-D-galactose-N-acetylglucosamine exposed after removal of sialic acid from fetuin [21]. Appropriate amounts of virus in Dulbecco's 1X PBS with CaCl<sub>2</sub> and MgCl<sub>2</sub> (Life Technologies, USA) were used to perform the NAI assays. Different concentrations of rhMBL were diluted in HBSS containing Ca<sup>2+</sup> and mixed with influenza virus in a total volume of 100 µL and preincubated at 37 °C for 1 h, and then transferred to wells precoated with fetuin (Sigma, USA) and incubated at 37 °C for 4 h. After washing, 100 µL of HRP-labeled peanut lectin (3 µg/mL) was added and after 1 h at room temperature, the wells were washed and o-phenylenediamine dihydrochloride in citrate buffer was added, reaction was stopped by 2 M H<sub>2</sub>SO<sub>4</sub>, and the OD at 492 nm was measured. The wells only with virus were used as the positive control, the OD of wells with HBSS used as a negative control was subtracted. Results were expressed as relative NA activity (%) calculated as the OD of the tested wells with virus and rhMBL divided by the OD of the wells with only virus.

**Table 1**

Distances from the potential N-linked glycosylation sites (NGS) to receptor binding domain or NA activity region (Å).

NGS	Protein	Distances from the NGS to functional region (Å)		
		H3N2WT	H9N2RG	H7N9Vac
63	HA	27.3	–	–
95	HA	–	23.2	–
122	HA	26.9	–	–
128	HA	–	17.6	–
126	HA	24.3	–	–
133	HA	16	–	–
144	HA	18.9	–	–
165	HA	24.1	–	–
198	HA	–	16.2	–
240	HA	–	–	37.3
246	HA	22.2	–	–
86	NA	29.4	29.4	30
146	NA	20.8	21.2	28.9
200	NA	18.1	18.4	18.4
234	NA	29.4	29.5	–
329	NA	26.5	–	–
402	NA	22.4	22.7	–

–: Denotes the absence of NGS in the corresponding virus.

**Table 2**

HI titer of rhMBL against influenza A viruses.

	Concentrations inhibiting hemagglutination of IAV by rhMBL ( $\mu\text{g/mL}$ )			
	H3N2WT	H9N2RG	H7N9Vac	H7N1 <sub>HA1 HA+PR8 NA</sub>
MBL	$0.026 \pm 0.005$	$0.083 \pm 0.02$	$1.42 \pm 0.239$	$3.67 \pm 0.33$
+EDTA <sup>a</sup>	>0.5	ND	ND	ND
+mannan <sup>b</sup>	>0.5	ND	ND	ND

Results are expressed as mean  $\pm$  SEM of three independent experiments. ND: not determined.H3N2WT: A/Brisbane/10/2007(H3N2) wild-type; H9N2RG: virus with the HA, NA from A/Hongkong/33982/2009(H9N2) and internal genes from A/Puerto Rico/8/1934(H1N1); H7N9Vac: virus with the HA, NA from A/Anhui/1/2013(H7N9) and internal genes from A/Puerto Rico/8/1934(H1N1); H7N1<sub>HA1 HA+PR8 NA</sub>: virus with HA from A/Anhui/1/2013(H7N9) and other seven genes from A/Puerto Rico/8/1934(H1N1).<sup>a</sup> The HI test was performed in the presence of 5 mM EDTA and rhMBL of different concentrations. The maxim concentration of MBL for testing is 0.5  $\mu\text{g/mL}$ .<sup>b</sup> The HI test was performed in the presence of 5 mg/mL mannan and rhMBL of different concentrations. The maxim concentration of MBL for testing is 0.5  $\mu\text{g/mL}$ .

## 2.5. Structure modeling

The HA and NA 3D structures were predicted by using the homology modeling method of SWISS-MODEL [22]. The modeling structures, corresponding templates and identities are shown in Supplementary Table S1. Potential NGS were identified with the NetNGlyc 1.0 server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) using artificial neural networks that examine the context of Asn-Xaa-Ser/Thr sequences. The predicted NGS include 86, 146, 200, 234, 329 and 402 of NA, 79, 105, 138, 141, 142, 149, 160, 181, 206, 249 and 262 of HA (H3N2 numbering) (Table 1). The NGS at the modeling structures were highlighted in Fig. S1 with Pymol (DeLano Scientific). The trimer structure of MBL was assembled using MBL crystal structure (PDB code: 1HUP) with PISA [23] which generate the oligomeric forms of protein according to the symmetry information. To quantify the distance between RBD/NA activity region and each NGS, we employed the average Euclidean distance of the center (C alpha atom) of every RBD/NA activity region and the center (C alpha atom) of NGS.

## 2.6. Statistical analysis

Differences between groups were tested using non-parametric Kruskal–Wallis analysis of variance (ANOVA) for multiple comparisons using IBM SPSS Statistics 20.0 (IBM, USA) and  $p < 0.05$  was considered statistically significant.

## 3. Results

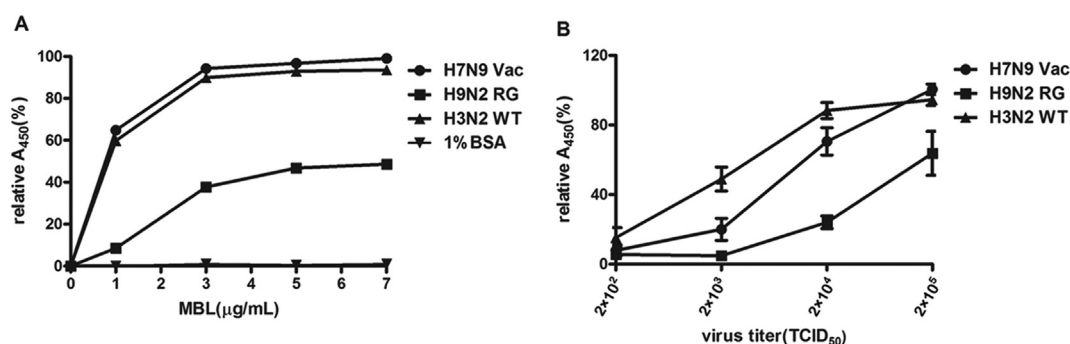
### 3.1. Binding of rhMBL with seasonal and avian influenza viruses

The ELISA results showed that rhMBL bound both seasonal and avian influenza viruses *in vitro*. Incubation with increasing

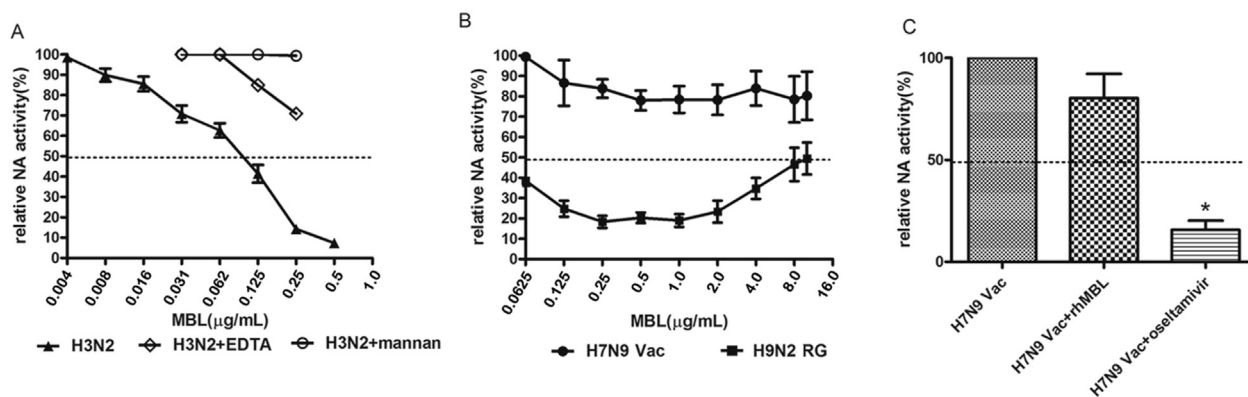
concentrations of rhMBL resulted in elevated levels of MBL bound to immobilized influenza virus, reaching a plateau at 3  $\mu\text{g/mL}$  (Fig. 1A). Then, 3  $\mu\text{g/mL}$  rhMBL was used to determine the affinity to increasing amounts of virus, revealing the virus-dose dependent binding feature (Fig. 1B). High binding of rhMBL to the human H3N2 virus might be due to a relatively more NGS in its HA and NA (Table S2), in addition to the glycans attached at the site of 165 as previously reported [14]. Beyond our expectation, H7N9 virus exhibited stronger binding than H9N2 virus, reaching a comparable level as human H3N2 at higher titers of  $2 \times 10^4$  and  $2 \times 10^5$ , although fewer NGS was observed in avian H7N9 than avian H9N2 (Table S2). The differential binding of rhMBL to avian virus might result from the types of oligosaccharide attached and further investigations are needed. The finding demonstrated here implied a possible antiviral effect initiated by rhMBL at early stage of novel avian virus infection since naïve host lacked the pre-existed cross-reactive or specific anti-HA or anti-NA antibodies.

### 3.2. Weak HI interference with H7N9 virus by rhMBL

Initially, we discovered that the lowest concentration for rhMBL ( $\mu\text{g/mL}$ , mean  $\pm$  SEM) to abolish virus-mediated TRBC agglutination was  $1.42 \pm 0.239$ ,  $0.083 \pm 0.02$ ,  $0.026 \pm 0.005$  for H7N9vac, H9N2RG and H3N2WT (Table 2), respectively. The HI effect could be reversed in the presence of 5 mM EDTA or 5 mg/mL mannan, which indicates that HI was calcium-dependent and mannan-inhibitable. We then used a H7N1 reassortant obtained HA from H7N9 and other seven segments from PR8 to exclude a possible hemadsorption by N9 reported elsewhere [24]. The MIC against the H7N1 was  $3.67 \pm 0.33$   $\mu\text{g/mL}$  (Table 2), which was remarkably higher than those against H3N2 or H9N2 virus, indicating a weaker HI effect of rhMBL on H7.



**Fig. 1.** Binding of rhMBL to purified influenza A virus. A. The dose-dependent binding of rhMBL to influenza virus. Wells were precoated with  $2 \times 10^5$  TCID<sub>50</sub> influenza virus H7N9Vac (●), H9N2RG (■), H3N2WT (▲), 1% BSA (▼). B. The bindings of rhMBL to influenza virus increase with viral titer. Wells were precoated with  $2 \times 10^2$ ,  $2 \times 10^3$ ,  $2 \times 10^4$ ,  $2 \times 10^5$  TCID<sub>50</sub> influenza viruses H7N9Vac (●), H9N2RG (■) and H3N2WT (▲), 3  $\mu\text{g/mL}$  rhMBL was used to detect the binding. Levels of rhMBL bound to immobilized influenza virus were detected by ELISA, as described in Materials and methods. The absorbance from negative control was subtracted and the results were normalized to positive control, the mannan. Data are expressed as mean  $\pm$  SEM (%) from three independent experiments.



**Fig. 2.** Inhibition of influenza virus Neuraminidase activity by rhMBL. A. Inhibition of H3N2 NA activity by rhMBL. Virus was incubated with increasing concentrations of rhMBL ( $\blacktriangle$ ), or with EDTA at final concentration of 5 mM ( $\diamond$ ), mannan at final concentration of 5 mg/mL ( $\circ$ ). B. Inhibition of H7N9, H9N2 NA activity by rhMBL. H7N9Vac ( $\bullet$ ) and H9N2RG ( $\blacksquare$ ) were incubated with increasing concentrations of rhMBL. NA activity was measured by ELISA, as described in Materials and methods. C. The effects of 10  $\mu$ g/mL rhMBL and 25  $\mu$ M oseltamivir on NA activity of H7N9Vac. Results were expressed as the OD of the tested wells with virus and rhMBL divided by the OD of the wells only with virus. The dashed line represents 50% of the original NA activity. Data are expressed as mean  $\pm$  SEM of three independent experiments. \* $p < 0.05$ .

### 3.3. NAI of H3N2 and H9N2 by rhMBL not H7N9 virus

As shown in Fig. 2A, NA activity of H3N2 was inhibited by rhMBL in a dose-dependent manner, and the  $IC_{50}$  was  $0.0807 \pm 0.009$   $\mu$ g/mL. Similarly, the rhMBL, at a concentration of 0.0625  $\mu$ g/mL, could inhibit the NA activity of H9N2 (Fig. 2B). Nevertheless, the relative NA activity of H7N9 remained above 50% in the presence of increasing concentration of rhMBL even at up to 10  $\mu$ g/mL. By contrast, the positive control, oseltamivir (Roche, Switzerland) at the concentration of 25  $\mu$ M (equivalent to 7.109  $\mu$ g/mL), could significantly inhibit the NA activity of H7N9 (Fig. 2C). Furthermore, the inhibition of H3N2 NA activity by rhMBL could also be abolished in the presence of 5 mM EDTA or 5 mg/mL mannan, suggesting that the NAI was calcium-dependent and mediated by lectin domain.

### 3.4. Steric interference between MBL and NGS in HA and NA head

RBD in HA, including the motifs of 190-helix, 130- and 220-loop, is one of important functional domains [25]. NA activity region is composed of 8 functional residues (R118, D151, R152, R224, E276, R292, R371 and Y406) and 11 framework residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294 and E425) [18]. To elucidate a possible interference between MBL and the NGS around the functional motifs: RBD in HA or the NA activity domain, we compared NGS distribution in them by structure modeling (Fig. S1). We also obtained the trimer form of MBL using the crystal structure (PDB code: 1HUP) and measured the radius of MBL CRD which is at least 30 Å. Although there are indications that trimers of MBL are not biologically active and at least a tetramer form is needed for activation of complement [26], 30 Å can be regarded as the minimum value. We subsequently calculated the average distances between the NGS located around RBD or NA activity region (Table 1) and speculated that their distance within the size of human MBL CRD, 30 Å, might affect the functions of HA or NA. As listed in Table S2 and Fig. S1, only one conserved NGS on HA head was detected at position 240 in the avian H7N9 while different pattern was found in H3 and H9 (63, 122, 126, 133, 144, 165, 246 for H3; 95, 128, 198 for H9). The distances of NGS located in H3 and H9 from its individual RBD all are within 30 Å while it is of 37.3 Å at 240NGS in H7N9. Furthermore, the NGS at residue 165, 246 [14,27] were indeed associated with the sensitivity of human H3N2 virus to MBL and 144 for 2009pdm H1N1 [28]. Notably, additional NGS at Site 133 in HA emerged in some H7N9 strain (4 out of 269) and the effect of MBL on it needed further tests. Similarly, only three NGS at 86, 146

and 200 on NA head were found in H7N9 and additional sites including 239, 329 and 402 in H3N2 and 239 and 402 in H9N2. Even the distances of NGS in H7N9 NA were less than 30 Å, we could not detect any NAI effect of MBL on it. The findings might attribute to the absence of effective NGS adjacent to NA functional region, such as 239 and 402 in both H3N2 and H9N2 virus, or different glycan type attached.

## 4. Discussion

Influenza A virus circulating in animal reservoirs is a continual cause for public health concern. In addition to the ever-present threat of seasonal influenza, we also face the threats from novel viruses such as H5N1 and H7N9, 2009pdm H1N1 virus in recent years. Since the viruses can escape from the protection of anti-HA or anti-NA antibodies due to antigenic shift, the innate immunity in naïve host is crucial for battling against newly-emerging viruses.

MBL, a pattern-recognition molecule in innate immunity, is known as a  $\beta$  inhibitor for seasonal influenza A virus. The average concentration of MBL in healthy human plasma (aged 18–87years) is  $1.72 \pm 1.51$   $\mu$ g/mL [29], with about 30% of individuals present MBL levels below 0.5  $\mu$ g/mL [30]. The antiviral activity of MBL against seasonal influenza A virus is via its interactions with viral HA or NA by blocking viral entry, fusion or releasing [15,31]. MBL could neutralize the virus either in a complement dependent or independent manner [13,15]. However, the effects of MBL may vary depending on specific strains. It also plays an important role in modulating inflammation and has been reported to contribute to deleterious inflammatory response to pdmH1N1 and a H9N2 avian isolate (A/Quail/Hong Kong/G1/97) [32].

Here, we found differential binding of rhMBL to human influenza H3N2 and avian H7N9, H9N2 viruses. Specifically, rhMBL exhibited significant HI activity against H3N2 and H9N2 virus at a relatively low concentration ( $0.026 \pm 0.005$ ,  $0.083 \pm 0.02$ , respectively), while its HI activity on H7N9 virus was around  $3.67 \pm 0.33$   $\mu$ g/mL, reaching the upper limit in plasma of healthy population. In contrast, for NAI on H7N9 virus, the rhMBL showed little effect even at a high concentration as 10  $\mu$ g/mL. As MBL is supposed to display a steric interference with HA or NA when binding to the specific hexose sugars across or adjacent to RBD in HA or activity region in NA, the limited impact of MBL on H7N9 might result from its property of few NGS adjacent to functional region on the HA and NA. Of note, a serial of pathotypings including higher virus load, excess chemokine/cytokine response and



functional impairments of B and T cells have been observed in H7N9 infection cases, particularly in fatal cases [33,34]. The aberrant inflammatory response in H7N9-infected animals could be reversed partially by anti-C5a, indicating a hyperactivated complement mediated [35]. Therefore, the strong MBL-H7N9 virus interaction whereas limited effects on viral HA-receptor binding or NA-mediated releasing, might amplify immune dysfunctions *in vivo* and confer clinical severity of H7N9 infection via activating complement pathway and further investigations are needed.

### Conflict of interest

None reported.

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### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2015.01.070>.

### Transparency document

The transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrc.2015.01.070>.

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