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Recombinant chimeric lectins consisting of mannose-binding lectin and L-ficolin are potent inhibitors of influenza A virus compared with mannose-binding lectin

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ABSTRACT

MBL structurally contains a type II-like collagenous domain and a carbohydrate recognition domain (CRD). We have recently generated three novel recombinant chimeric lectins (RCL), in which varying length of collagenous domain of mannose-binding lectin (MBL) is replaced with that of L-ficolin (L-FCN). CRD of MBL is used for target recognition because it has a broad spectrum in pathogen recognition compared with L-FCN. Results of our study demonstrate that these RCLs are potent inhibitors of influenza A virus (IAV). RCLs, against IAV, show dose-dependent activation of the lectin complement pathway, which is significantly higher than that of recombinant human MBL (rMBL). This activity is observed even without MBL-associated serine proteases (MASPs, provided by MBL deficient mouse sera), which have been thought to mediate complement activation. These observations suggest that RCLs are more efficient in associating with MASP-2, which predominantly mediates the activity. Yet, additional serum further increases the activity while RCL-mediated coagulation-like enzyme activities are diminished compared with rMBL, suggesting reduced association with MASP-1, which has been shown to mediate coagulation-like activity. These data suggest that RCLs may interfere less with host coagulation, which is advantageous to be a therapeutic drug. Importantly, these RCLs have surpassed rMBL for anti-viral activities, such as viral aggregation, reduction of viral hemagglutination (HA) and inhibition of virus-mediated HA and neuraminidase (NA) activities. These results are encouraging that novel RCLs could be used as anti-IAV agents with less side effect and that RCLs would be suitable candidates in developing a new anti-IAV therapy.

1. Introduction

IAV is an RNA virus whose surface is enveloped with glycoproteins containing neuraminidase (NA) and hemagglutinin (HA), which have glycosylation sites [1]. IAV infection is a common infection that could result in fatal complications, even in individuals who are appeared to be healthy [2,3]. Mortality and hospitalization are estimated to exceed annually 30,000 and 200,000, respectively in the United States alone [2]. Prevention is currently relied upon immunization, however vaccines are less effective in elderly and are not approved by the FDA for infants younger than 6 months of age [3,4]. Resistance to antiviral agents has developed in seasonal and pandemic IAV strains [2,5]. Thus, there is a need for new effective anti-IAV therapeutics.

The first line of host defense is the innate immunity, which recognizes pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors and soluble molecules that include lectins [6]. One such lectin is MBL, which is primarily synthesized in the liver and circulates in the blood [7–9]. MBL belongs to the collectin family that is structurally characterized by consisting of a type II-like collagenous domain at N-terminus, followed by a neck region and a carbohydrate recognition domain (CRD) at C-terminus [10]. The collectin family includes lung surfactant protein (SP)-A and SP-D [11]. These surfactant proteins have anti-viral functions [12–17] and mice lacking SP-A or SP-D have increased susceptibility to AIV infection [14,18].

In early 1990, MBL was identified as a β-inhibitor, which had been discovered as an IAV inactivating serum factor in the 1940s

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[19]. Since then, many studies have described MBL's anti-IAV functions, including inhibition of viral hemagglutination, inhibition of HA and NA and viral neutralization [20–23]. MBL activates complement via the lectin complement pathway, which is a key biologic function along with other complement pathways, such as the classical and the alternative complement pathway. The classical complement pathway is mediated by C1rs proteases, which are replaced by MASP-1, MASP-2 and/or MASP-3, in the lectin complement pathway [24]. Both pathways cleave C4 and C2 to generate the C3 convertase, C4bC2a [25,26].

MBL–MASP complex also initiates coagulation via thrombin-like activity [27,28]. Coagulation is a primitive yet effective host defense mechanism. For example, tachylectins in horseshoe crab hemolymph provide immune protection by clotting lipopolysaccharide and β-glucan, pattern recognition molecules of pathogens (PAMPs of Gram negative bacteria and fungus, respectively) [29].

The collectin family also includes L-FCN and H-FCN, which are also circulating serum pattern recognition molecules of innate immune system [30]. Like MBL, both FCNs contain the collagenous domain while CRD is replaced with fibrinogen-like domain, which preferentially recognizes acetylated molecules and sialic acid [31,32]. In contrast, MBL's target recognition is broad, including mannose, which is widely expressed on many pathogens [33]. It has been shown that other chimeric lectins consisting of MBL-CRD and the collagenous domain of SP-D gain anti-IAV activities, such as viral aggregation and inhibition of HA, NA and viral infectivity [22,34].

We have previously generated three RCLs consisting of L-FCN and MBL, in which various lengths of the collagenous domain were replaced with that of L-FCN [23]. Previous characterization study has demonstrated that these RCLs are either comparable to or surpassed rMBL for several biologic activities, including their binding to Nipah, Hendra and Ebola viruses [23]. Here, we further characterized biologic activities of these recombinant lectins against IAV using *in vitro* system and will discuss our findings.

2. Materials and methods

2.1. Recombinant chimeric lectins

Chimeric lectins were produced as previously described [23]. In this study, these lectins are named RCL1, RCL2 and RCL3, corresponding to L-FCN/MBL126, L-FCN/MBL76 and L-FCN/MBL64, respectively in the previous publication. All RCLs have MBL-CRD while MBL-collagenous domain was replaced with 126, 76 or 64 amino acids of L-FCN's collagenous domain, resulting in total amino acid length of 251, 255 or 254 for RCL1, RCL2 or RCL3, respectively. Thus, overall amino acid length is similar while RCL1 has the longest L-FCN collagenous domain followed by RCL2 and then RCL3. The junction of two proteins in RCL2 is located at the middle of a putative MASP-binding domain.

2.2. Virus preparations

IAV (A/Phillipines/82(H3N2)) was prepared as previously described [35]. Briefly, IAV was grown in the chorioallantoic fluid of chicken eggs and purified on a discontinuous sucrose gradient (Sigma–Aldrich, St. Louis, MO). Virus stocks were dialyzed against PBS (Sigma–Aldrich, St. Louis, MO) and aliquots were stored at −80 °C. HA titers were determined by titration with human type O, Rh^- red blood cells (RBCs) in PBS.

2.3. MBL binding assay

This assay was performed using previously described methods with a minor modification [36]. IAV concentration was arbitrary defined as 1000 U/ml, which was determined to be optimal for many *in vitro* studies based on dose response experiments. Briefly, 96 well plates were coated with mannan (Sigma–Aldrich, St. Louis, MO) or IAV and then blocked. Following wash, the wells were incubated with indicated concentrations of recombinant lectins. After wash, bound MBL was detected by mouse anti-hMBL monoclonal Ab (2A9, a gift from Dr. Gregory Stahl) [37], followed by alkaline-phosphatase conjugated anti-mouse Ab (Promega, Madison, WI) and pNTP substrate (Sigma–Aldrich, St. Louis, MO). Reaction was read at 415 nm using SpectraMax M5 (Molecular Devices, Sunnyvale, CA) and expressed as OD 415 nm reading. Assays were performed in triplicates and were repeated at least twice.

2.4. Mouse sera

MBL null mice were previously generated and fully backcrossed onto C57Black/6J [36,38]. Sera were collected and stored at −80 °C prior to the study. All animal experiments were performed under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, MA.

2.5. Assays of the lectin complement activity

The lectin pathway assay was performed with a minor modification of previously described method [36]. Briefly, 96 well plates were coated with mannan or IAV as above. After wash and block, the wells were incubated with various concentrations of lectins with or without 1% MBL null sera (MASP source) diluted in a binding buffer, 10 mM Tris, pH 7.8, 10 mM $CaCl_2$, 1 M NaCl (all chemicals were purchased from Sigma–Aldrich, St. Louis, MO). After wash, the wells were incubated with human C4 and incubated at 37 °C. After wash, the wells were incubated with rabbit anti-hC4c Ab (Dako, Carpinteria, CA) followed by biotin-conjugated anti-rabbit Ab, alkaline phosphatase-conjugated biotin–avidin (ABC-AP system, Vector Labs, Burlingame, CA) and then with pNTP (Sigma–Aldrich, St. Louis, MO). The plates were read at 415 nm. Binding activity was expressed as OD 415 nm reading. Pooled human serum with known MBL concentration and C4 activity, which was arbitrarily defined as 1000 U/ml (State Serum Institute, Denmark), was used to generate a standard curve on mannan-coated wells. Assays were performed in triplicates and were repeated twice.

2.6. Assay of thrombin-like and factor Xa-like activities

These activities were assayed using previously described methods [39]. Briefly, 384 well plates were coated with mannan or IAV as above. After wash, the wells were incubated with various concentrations of lectins with or without 1% MBL null mouse serum or 1% MASP-1/3 null mouse serum (MASP source) [40] diluted in the binding buffer. After wash, wells were incubated with rhodamine 110-thrombin substrate (R22124, Invitrogen, Carlsbad, CA) or amino-4-methylcoumarin acetate (AMC)-factor Xa substrate (222F, American Diagnostica Inc., Stamford, CT) and read at 500 nm excitation/520 nm emission or 360 nm excitation/440 nm emission, respectively, using SpectraMax M5. The results were expressed as arbitrary units (AUs). Assays were performed in triplicates and were repeated twice.

2.7. Viral neutralizing assay

The assay was performed as previously described [41]. Briefly, viruses were pre-incubated with lectins and washed and then incubated with Madin-Darby Canine Kidney (MDCK) cells. Infection was assayed by FITC-conjugated anti-IAV antibody (Ab) (Millipore, Billerica, MA). Virus neutralizing activity as %inhibition

was calculated by the formula: FFC in test samples/FFC in saline control × 100. Data from 5 experiments were combined.

2.8. HA and HA inhibition assay

The assay was previously described [42]. Briefly, virus was incubated with lectins at various concentrations and assayed for HA titer using human type O, Rh^- RBCs. HA inhibition of IAV by recombinant lectins at 2 μg/ml was measured in round-bottom 96-well plates (Serocluster U-Vinyl plates; Costar, Cambridge, MA). HA inhibition was detected as the formation of a RBC pellet. To enable graphical comparisons of HA inhibition, data were mathematically converted and expressed as the number of HA units inhibited by the lectins. Four experiments were combined.

2.9. NA inhibition assay

The assay was performed using Amplex Red Neuraminidase Assay kit (A22178, Molecular Probes, CA), according to the manufacture's instructions. IAV (500 U/ml) was mixed with recombinant lectins at 2 μg/ml and Amplex Red reagents in 40 μl reaction volume. Assay was performed in triplicate. Reaction was read at 530 nm excitation/590 nm emission using SpectraMax M5. The results were expressed as %inhibition calculated by the formula: [(IAV − (IAV + recombinant lectins)) × 100]/IAV.

2.10. Viral aggregation assay

The assay was previously described [43]. Briefly, at time 0, viral suspensions were mixed with 800 ng/ml of lectins in PBS^{++} in a final volume of 1.0 ml. Under continuous stirring, the light transmission was monitored at excitation/emission 350 nm during 12 min using an SLM/Aminco 8000C (SLM Instrument, Urbana, IL) spectrofluorometer. A decline in light transmission correlates with viral aggregation. Results are expressed as percent of control light transmission (virus without lectins). Six experiments were combined.

2.11. Statistical analysis

All data were analyzed by ANOVA or Wilcoxon/Kruskal–Wallis tests depending on data distribution using JMP software (SAS institute Inc., Cary, NC). p values less than 0.05 was considered to be significant.

3. Results

3.1. RCLs bind IAV as efficient as rMBL

We first confirmed RCLs' binding ability to IAV by comparing to mannan because all RCLs have MBL-CRD, which preferentially recognizes mannan [23]. Therefore, mannan was used as a positive control throughout the investigation. All RCLs bound to mannan at comparable efficiency as rMBL in a dose dependent manner (Fig. 1A). Similarly, all RCLs bound to IAV in a dose dependent manner although RCL1 and RCL3 demonstrated significantly more efficient binding at 10 ng/ml compared with RCL2 and rMBL (Fig. 1B). Thus, all RCLs bound to mannan and IAV in a dose response manner, demonstrating that all RCLs were biologically functional for the target binding activity.

3.2. RCLs activate the lectin complement pathway more efficiently than rMBL

On mannan, rMBL showed C4 deposition in a dose dependent manner only when MASPs (MBL null serum) were supplied as expected (open circles vs. closed circles in Fig. 2A). In contrast, all RCLs even without MASPs showed significant C4 deposition activity. C4 deposition activity by RCLs alone was significantly ($p < 0.0001$) higher than that of rMBL even at 10 ng/ml ($p < 0.05$, 0.005 and 0.0001 for RCL2, RCL1 and RCL3, respectively). At 100 ng/ml, C4 deposition activity of RCL2 was highest followed by RCL3 and then RCL1 ($p < 0.005$ for both RCL4 vs. RCL3 and RCL3 vs. RCL1) (Fig. 2A). Addition of MASPs significantly ($p < 0.0001$) increased C4 deposition activity reaching to plateau at 10 ng/ml. All RCLs demonstrated comparable activities and were significantly stronger than rMBL at 10 ng/ml (Fig. 2A).

On IAV, C4 deposition activity of RCLs alone was also observed while it was not detectable by rMBL alone (Fig. 2B). Similar to their activity on mannan, all RCLs alone activated C4 even at 1 ng/ml ($p < 0.005$ and 0.05 for RCL2 and RCL 3, respectively and no significance for RCL 3) (Fig. 2B). Once again, addition of MASP significantly ($p < 0.0001$) enhanced the C4 deposition activity even at 1 ng/ml on IAV, unlike on mannan (Fig. 2A vs. B) and reached to plateau at 10 ng/ml. At 1 ng/ml, C4 deposition activity by RCLs with MASPs was significantly ($p < 0.005$) stronger than rMBL/MASP complex (Fig. 2B).

Taken together, these results demonstrated that RCLs were more efficient in activating the lectin pathway mediated C4 deposition on both mannan and IAV.

3.3. rMBL strongly activates coagulation enzyme-like activities compared with all RCLs

On mannan, thrombin-like activity was observed in a dose dependent manner by rMBL when MASP was supplemented as expected (Fig. 3A). Unlike C4 deposition activity, no thrombin-like activity was detected without MASP by any recombinant lectin on both mannan and IAV (Fig. 3A and B). Even when MASP was supplied, only MBL mediated significant thrombin-like activity on mannan (Fig. 3A). In contrast, all recombinant lectins activated

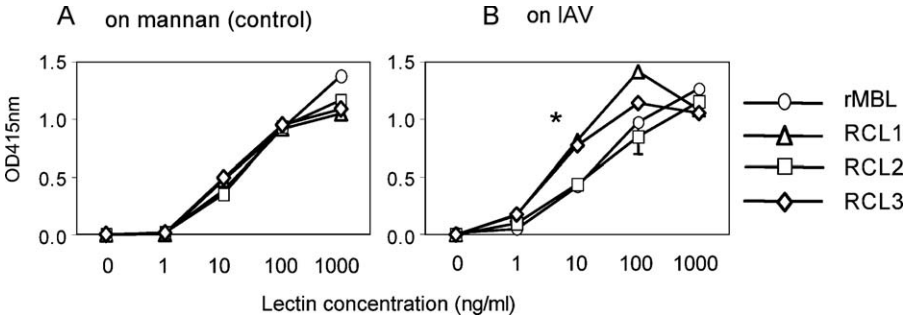

Fig. 1. Lectin binding assay to mannan (A) or IAV (B). Bound lectins on mannan (control) or influenza A virus (IAV) were detected by monoclonal anti-MBL antibody, which detected all RCLs. Open circles, rMBL; open triangles, RCL1; open squares, RCL2; open diamonds, RCL3. Data were expressed as mean ± SD, all of which were smaller than sizes of symbols. $^*p < 0.05$.

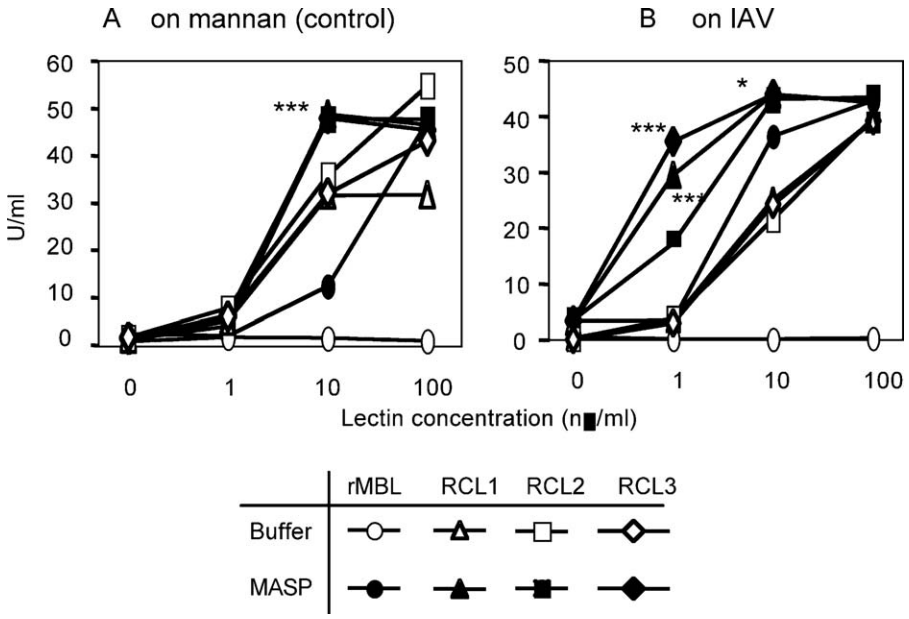

Fig. 2. The lectin complement pathway activation activity on mannan (A) or IVA (B). The lectin pathway activity was assayed as C4 deposition (U/ml) as described in Section 2. Open circles, rMBL; open triangles, RCL1; open squares, RCL2; open diamonds, RCL3. Closed symbols were with MBL null sera to supply MASP. C4 deposition activities (U/ml) were expressed as mean ± SE, most of which were smaller than sizes of symbols. $^{*}p < 0.05$; $^{***}p < 0.0001$.

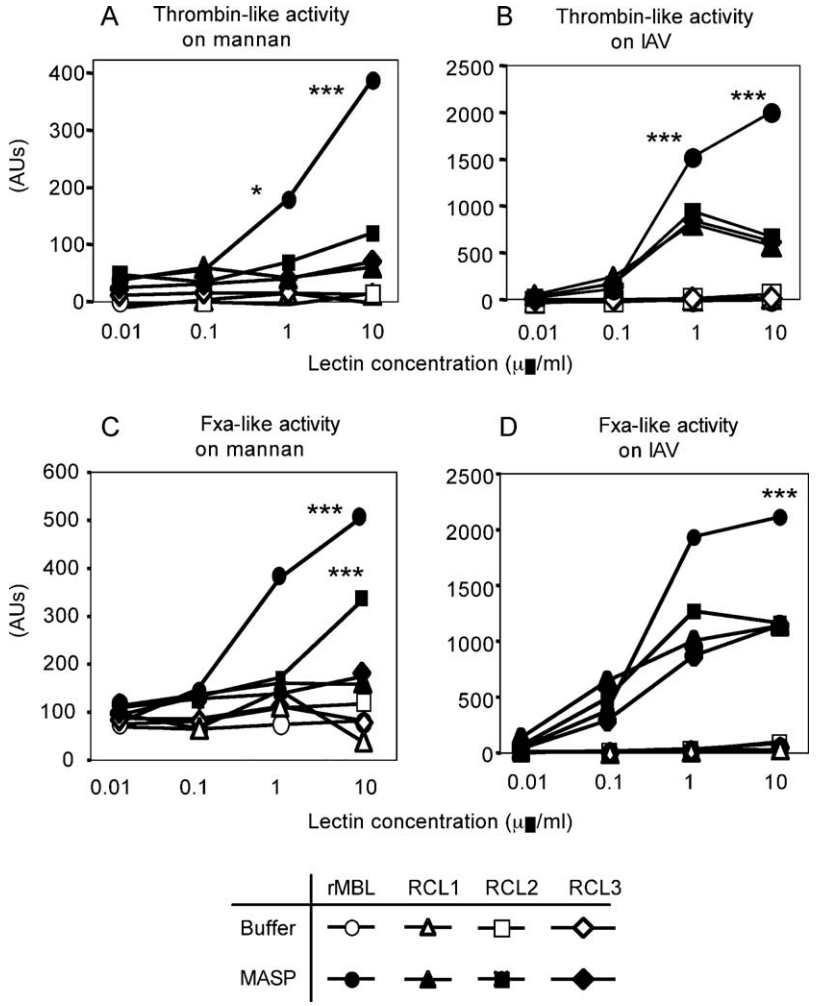

Fig. 3. Thrombin-like activities on mannan (A) or IAV (B) and FXa-like activities on mannan (C) or IAV (D). These activities were assayed using enzyme-specific peptide substrates, which become fluorescent upon enzymatic digestion. Open circles, rMBL; open triangles, RCL1; open squares, RCL2; open diamonds, RCL3. Closed symbols were with MBL null sera to supply MASP. Activities (arbitrary units, AUs) were expressed as mean ± SE, most of which were smaller than sizes of symbols. $^{*}p < 0.05$; $^{***}p < 0.0001$.

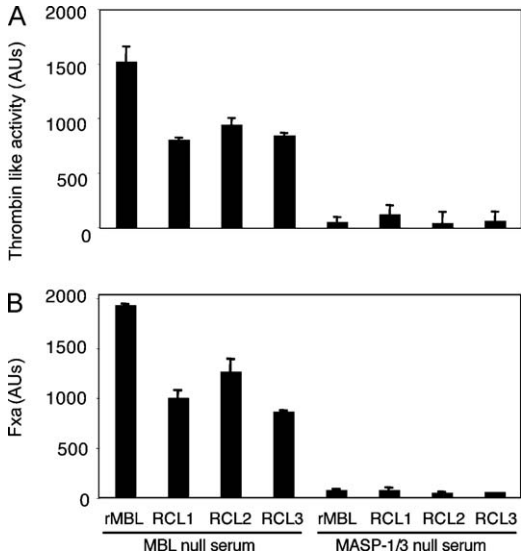

Fig. 4. MASP-1/3 dependent thrombin-like (A) and FXa-like (B) activities. Assays were performed as in Fig. 3. Lectins were used at 1 μg/ml and mixed with 1% of MBL null or MASP-1/3 null mouse sera. Data were expressed as mean ± SE, most of which were smaller than sizes of symbols. The data shown was the representative result of three experiments.

thrombin-like activity on IAV at 1 and 10 μg/ml. rMBL-mediated activity was significantly stronger than that of RCLs (Fig. 3B).

Similar to thrombin-like activity, when MASP was supplied rMBL and RCL2 significantly activated FXa-like activity on mannan compared with RCL1 and RCL3. However, RCL2 showed the activity only at 10 μg/ml while rMBL was significantly active even at 1 μg/ml (Fig. 3C). Once again, all recombinant lectins activated FXa-like activity on IAV at as low as 0.1 μg/ml when MASP was supplied. However, MBL-mediated activity was significantly stronger than that of RCLs (Fig. 3D).

For both thrombin-like and FXa-like activities, when MASP-1/3 null mouse serum (MASP-2/sMAP sufficient) was used as MASP source these activities were abrogated to nearly undetectable levels as this was expected from our previous study (Fig. 4) [39].

3.4. RCLs demonstrate efficient anti-IAV biologic functions

All RCLs inhibited MDCK cell infection in a dose dependent manner. The activity was comparable to rMBL (Fig. 5). IC50s (μg/ml ± SE) for RCL1, RCL2, RCL3, and rMBL were 1.12 ± 0.15, 0.72 ± 0.15, 1.09 ± 0.14, and 0.81 ± 0.16, respectively. Thus, all lectins were active in inhibiting IAV infection to MDCK epithelial cells.

In IAV aggregation activity, RCL2 exceeded all other RCLs and rMBL (Fig. 6). IAV aggregation by RCL2 was detected as early as 50 s into incubation (Fig. 6). rMBL aggregated IAV better than both RCL1 and RCL3 after incubation time at 500 and 700 s ($p < 0.05$ for both time points, Fig. 6).

RCLs inhibited HA titers as potent as rMBL. Similar to viral aggregation results, RCL2 significantly ($p < 0.001$) reduced HA titer at 5 μg/ml compared with other RCLs and rMBL (Fig. 7A). RCL2 abolished HA titer at 10 μg/ml while RCL1, RCL3 and rMBL required 20 μg/ml to achieve the same effect (Fig. 7A).

As complementing lectins ability to reduce HA titer, HA inhibition was assayed at 2 μg/ml for all chimeric lectins. The results showed that all RCLs significantly inhibited HA compared with rMBL (Fig. 7B), demonstrating that all RCLs were significantly more efficient in inhibiting IAV-mediated HA.

Another anti-viral activity is inhibition of NA. RCL2 and RCL3 significantly inhibited NA compared with RCL1 and rMBL, the latter two of which showed similar NA inhibitory activity (Fig. 7C). These results suggested that RCL2 and RCL3 were significantly effective in inactivating NA. Taken together, these results further suggest that RCLs, in particular, RCL2 was effective in preventing IAV infection into host cells.

4. Discussion

In this study, we have demonstrated that novel RCLs, in particular, RCL2, is an efficient anti-IAV agent as we summarize our findings in Table 1. RCLs have been generated by replacing various portions of the collagenous domain of MBL with that of L-FCN, thus utilizing MBL-CRD for target recognition. MBL-CRD is chosen based on our understanding that MBL-CRD has broader target recognition than L-FCN, which preferentially recognizes acetylated compounds [44]. These results support our recent findings that these RCLs have a better binding activity against Ebola, Nipah and Hendra viruses [23]. Taken together, these results demonstrate that novel RCLs have broad and potent anti-viral activities compared with its parent rMBL.

Binding ability of all RCLs on mannan (a positive control) was in a dose dependent manner and was comparable to that of rMBL. Thus, RCLs maintain MBL-CRD binding activity and introduction of the collagenous domain of L-FCN does not alter the MBL-CRD function. Similar to on mannan, RCLs bind IAV in a dose dependent manner at comparable levels to rMBL, confirming that MBL-CRD is functional in binding to IAV.

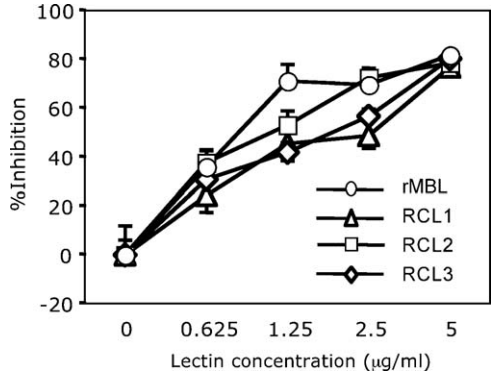

Fig. 5. IAV neutralizing assay. Neutralizing activity was assayed as %inhibition of viral infection to MDCK cells by comparing lectin-pretreatment to no lectin-pretreatment. Open circles, rMBL; open triangles, RCL1; open squares, RCL2; open diamonds, RCL3. Results were expressed as mean ± SE of %inhibition. $^{*}p < 0.05$.

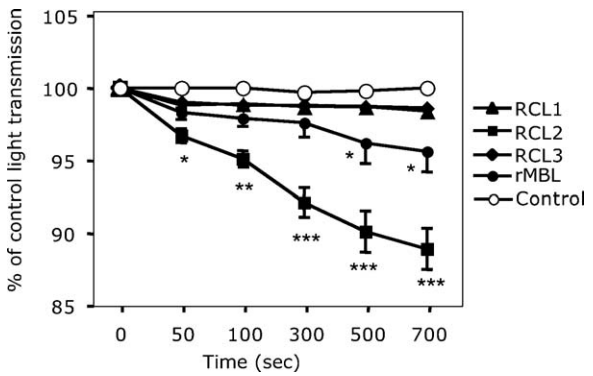

Fig. 6. Viral aggregation assay by lectins. Viral aggregation by lectins was assayed by the reduction of light transmittance and expressed as % of control (saline) light transmittance. Data were expressed as mean ± SE. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0001$.

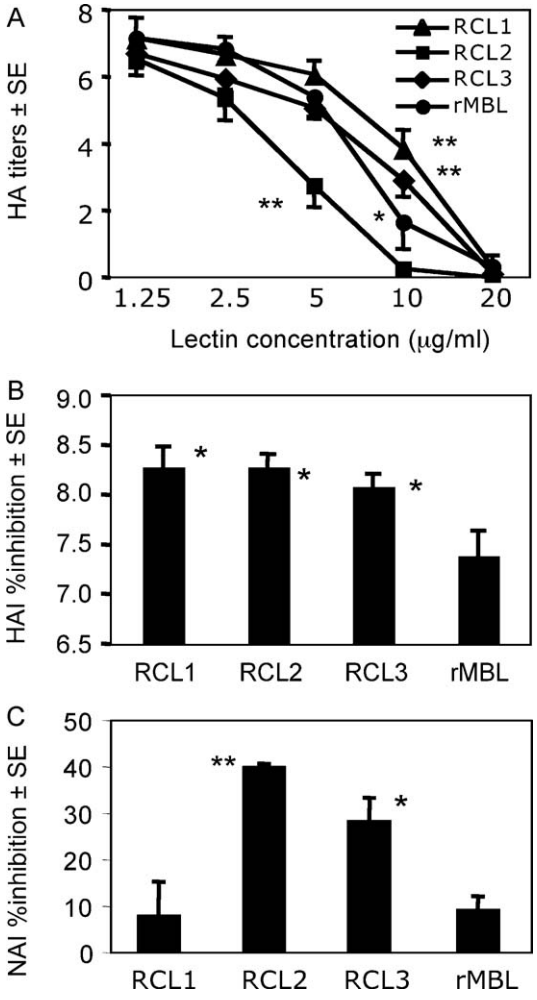

Fig. 7. HA titers and HA inhibition (HAI) and NA inhibition (NAI) by lectins. (A) HA titers were inhibited by lectins. Closed circles, rMBL; closed triangles, RCL1, closed squares, RCL2; closed diamonds, RCL3. The results were expressed as mean ± SE. (B) HAI assay complemented lectin's effects on HA titers in (A). Bars indicated mean ± SE. $^{*}p < 0.05$ against rMBL. (C) NAI assay examined lectin's effect on viral NA activity. Bars indicated mean ± SE. $^{**}p < 0.005$; $^{*}p < 0.05$. Both statistics were against RCL1 and rMBL.

MBL in a complex with MASPs initiates the lectin complement pathway, one of the key functions of MBL. By replacing various portions of collagen domain with that of L-ficolin, all RCLs without MASP activate the lectin complement pathway, which is also observed by rMBL but requires 10 μg/ml, 1000-fold more protein (unpublished observation). Importantly, RCL-mediated lectin complement pathway activation activity is augmented by MASP supplementation, suggesting that the lectin complement pathway activation activity would be efficiently augmented *in vivo*. We have previously shown that the lectin complement pathway activation activity correlates with host protection from bacterial infection, including *S. aureus* infection *in vivo* [36,45]. Taken together, these observations suggest that RCLs are efficient activators of the lectin complement pathway and could be administered with smaller dose than rMBL. Further investigation is required to determine *in vivo* efficiency.

Table 1
Summary of RCL activities.

Activities	Recombinant lectins			
	RCL1	RCL2	RCL3	rMBL
Binding	+	+	+	+
LCP activation activity	++	+++	++	+
Thrombin-like activity	+	+	+	+++
FXa-like activity	+	+	+	+++
Viral aggregation	+	+++	+	+
Reduction of HA titer	+	++	+	+
HA inhibition	++	++	++	+
NA inhibition	+	+++	++	+
Viral infectivity (*in vitro*)	+	+	+	+

Note: LCP, lectin complement pathway; FXa, factor X-activated; HA, hemagglutination; NA, neuraminidase. Activities were scored as follows: +, positive; ++, strong; +++, very strong.

Although SP-A and SP-D belong to the collectin family and share structural and functional similarity with MBL and FCNs these surfactant proteins do not activate the lectin complement pathway [46]. SP-A rather inhibits complement activation [47]. The chimeric protein of SP-D collagenous domain and MBL-CRD has increased anti-viral characteristics compared with its parents [34], however complement activation activity has not been examined. Interestingly, when Wallis and his colleagues introduced MASP binding sequence of MBL/FCN into SP-A the chimeric SP-A became constitutively active in the lectin complement pathway. This is not the case for MBL/FCN chimera because their activities are dose dependent and correlate with ligand binding. Nevertheless, superb lectin complement pathway activities of RCLs may be explained by the idea that altering MASP-binding region might have allowed to increase MASP-2 binding to RCLs and also MASP-2 activity itself because the lectin complement pathway is predominantly mediated by MASP-2 [24].

Conversely, compared with rMBL, all RCLs now have reduced thrombin-like and FXa-like coagulation enzyme activities, which are mediated by MASP-1/3. MASP-1 has been linked to coagulation enzyme-like activities as we also have demonstrated in this study, confirming previous findings of our own and others [28,39]. One can speculate that while all RCLs efficiently bind to MASP-2 their MASP-1 binding is diminished either by competition or by structural conformational change. Another possibility is due to triggering of different amplification loops. It has been proposed that the alternative pathway amplifies the classical pathway and the lectin pathway [48–50]. There could be other such amplification loop as it has become clear that interaction of complement pathway and coagulation pathway is more complex. Further detailed investigations are required to dissect out such pathways and cascades.

The reduced coagulation enzyme-like activities of RCLs are advantageous over rMBL for the following reasons: Blood fluidity is maintained on a fine balance of clotting (coagulation/thrombosis) and bleeding, thus, tip over to one side would trigger coagulation disorders, including disseminated intravascular coagulation [51]. So far, a phase 1 clinical study of rMBL did not result in adverse effect in healthy volunteers [52]. Taken together, our results suggest that RCLs have low coagulation enzyme-like activities, such as thrombin-like and FXa-like activities. This should be advantageous in clinical use, as it would have less risk to trigger coagulation disorder and related side effects.

MBL has been known to neutralize virus as this protein was initially discovered as a serum factor, the β-inhibitor of IAV in 1940s and re-identified to be MBL in 1990s [19]. Viral neutralization is assayed by infectivity of lectin-treated virus to MDCK cells, thus the activity indicates integrity of virus [41]. All RCLs neutralize IAV at similar level to rMBL, suggesting that all RCLs are at least functionally as efficient as rMBL *in vitro*. Further investigations would require assessing these activities in more physiological way, in which primary cells would be used in the presence of serum as well as *in vivo* infection studies.

Further characterization has revealed that these RCLs, in particular, RCL2 surpasses rMBL for important aspects of anti-

IAV functions. For example, aggregation of virus is an efficient anti-viral mechanism as aggregated virus loses infectivity [20]. In regard to this activity, RCL2 surpassed all other lectins, including other two RCLs. RCL2's superb anti-viral activity is also evident in reduction of HA titer. RCL2's activity is further confirmed by HA inhibition, which is an important anti-viral activity as it suggests that RCL2 would inhibit viral attachment to the host cells [20], thereby preventing infection. Similarly, RCL2 inhibits NA, suggesting that RCL inactivate NA that digests sialic acid, which is expressed on intact host cells, and promotes infection [22]. The finding confirms and extends the previous report that MBL not only binds to NA but also inactivates its activity [22]. The RCL2's superior activities may be partly attributed to significantly higher binding affinity to mannan and GlcNAc, another favored MBL-ligand, compared with other lectins [23].

In conclusion, our results demonstrate that RCLs, in particular, RCL2 are more potent anti-IAV reagents than MBL as summarized in Table 1. We also speculate that these RCLs would recognize infected host cells that may be expressing HA and NA. Our studies also provide new insights into understanding how collagenous domains would contribute to biologic functions, such as complement activation and coagulation, which are mediated by MASPs. Further *in vivo* investigation is encouraged to examine their efficiency as a new innate immune therapeutics against IAV infections.

Conflict of interest

All authors have no financial conflict.

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