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Protective role of mouse MBL-C on intestinal mucosa during *Shigella flexneri* invasion

Da-Ming Zuo, Li-Yun Zhang, Xiao Lu, Ying Liu and **Zheng-Liang Chen**

Department of Immunology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, Guangdong, People's Republic of China

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Abstract

Mannan-binding lectin (MBL) is a C-type serum lectin, which is believed to play an important role in the innate immunity against a variety of pathogens. MBL can bind to sugar determinants of a wide variety of microorganisms, neutralize them and inhibit infection by complement activation through the lectin pathway and opsonization by collectin receptors. Given that small intestine is a predominant site of extrahepatic expression of MBL, here we addressed the question whether MBL is involved in mucosal innate immunity. The carbohydrate recognition domain (CRD) genes of mouse MBL-C (mMBL-C) were cloned and expressed in *Escherichia coli*. Recombinant mMBL-C-CRD binds to *Shigella flexneri 2a* in a calcium-dependent manner and that interaction could be blocked by the anti-mMBL-C-CRD antibody. mMBL-C-CRD protein could inhibit the adhesion of *S. flexneri 2a* to intestinal mucosa, while administration of anti-mMBL-C-CRD antibody caused an increased level of bacteria adhesion *in vitro*. Administration of recombinant mMBL-C-CRD protein reduced the secretion of IL-6 and monocyte chemoattractant protein 1 from primary intestinal epithelial cells stimulated with *S. flexneri 2a*. Furthermore, neutralization of MBL activity by anti-MBL-C-CRD resulted in a significant increase in the number of *S. flexneri 2a* that colonized the intestines of BALB/c mice and attenuated the severity of inflammation seen in the areas of bacterial invasion. These findings suggest that mMBL-C may protect host intestinal mucosa by directly binding to the bacteria.

Introduction

Mannan-binding lectin (MBL), a member of collectins which belong to C-type lectin superfamily, is an important pattern-recognition receptor in the innate immune system (1, 2). The overall polypeptide structure of MBL includes a cysteine-rich N-terminal domain, a collagen-like region, a neck region and a C-terminal carbohydrate recognition domain (CRD) (3, 4). The CRD has been shown to bind mannose, mannan, fucose and *N*-acetylglucosamine (4). The cluster of CRDs rendered by oligomerization is thought to be suitable for binding polymeric sugars on microbes (5). Indeed, MBL can selectively recognize sugars presented on pathogens such as bacteria, viruses, yeast and leishmania via the CRD (6). In addition, several lines of evidence suggest that MBL can inhibit influenza A virus infection independent of complement activation (7). Other groups showed that MBL can interfere with the viral attachment to host cells, thus affecting viral spread and release (8–10).

MBL is encoded by a single gene in human, but in rodents it is encoded by two genes, *MBL-A* and *MBL-C* (11, 12). These two forms of rodent MBL bear 50% homology, displaying distinct but overlapping ligand-binding specificity (13). Both MBLs are mainly synthesized by hepatocytes and presented in liver and serum (13). However, extrahepatic expression of MBL was reported in various tissues. Indeed, non-hepatic mouse MBL-C (mMBL-C) expression is highest in the small intestine (14). It is known that intestinal epithelial cells (IECs) can produce cytokines and chemokines that are crucial for the recruitment and activation of immune cells (15, 16). Taken together, these findings indicate that mMBL-C expressed by IECs may contribute to the host defense against intestinal microorganisms.

Shigella are gram-negative bacilli that cause bacillary dysentery or shigellosis in humans, especially in developing countries (17, 18). Interaction between shigella and IECs triggers the important signals for the initiation and amplification of an acute mucosal inflammatory response (19). In this study, we investigated the characteristics of the direct antibacterial activity of mMBL-C independent of complement activation or opsonization following intra-gastric (i.g.) inoculation of invasive *Shigella flexneri 2a* into BALB/c mice.

Correspondence to: Z.-L. Chen; E-mail: zhlchen@fimmu.com

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Methods

Bacterial strains and animals

A lyophilized culture of *S. flexneri 2a* obtained from the Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, Beijing, China, was used throughout these experiments. Streptomycin-resistant mutants (>4 000 μg ml^{-1}) of *S. flexneri 2a* were isolated using the gradient plate technique (20) and then applied on 6-week-old female BALB/c mice (Laboratory Animal Center, Southern Medical University, Guangzhou, People's Republic of China).

Expression of mMBL-C-CRD protein and antibody preparation

The CRD fragment was obtained by PCR from the plasmid containing mMBL-C cDNA cloned from the liver of BALB/c mouse in our laboratory. The target gene was inserted into prokaryotic expression vectors pET-32a and pET-CMP-ketodeoxyoctonate synthetase (CKS) and expressed in *Escherichia coli*. Recombinant CRD protein expressed from pET-32a and pET-CKS was purified by Ni-affinity chromatography or ammonium sulfate precipitation, respectively. Antiserum for CRD was obtained from rabbits immunized with the recombinant CKS-CRD protein expressed by pET-CKS. The specificity of the antibody was verified using the recombinant thioredoxin (Trx)-CRD protein expressed by pET-32a and purified through affinity chromatography on a Protein G-Sepharose column (Pharmacia, GE Healthcare, Freiburg, Germany). The anti-CRD, polyclonal antibody specific for mMBL-C-CRD, purified by protein G chromatography was dialyzed against normal saline overnight at 4°C. Antibody concentrations were determined by measuring the absorbance at 260/280 nm.

Labeling of recombinant protein and S. flexneri 2a

Recombinant Trx-CRD protein solution (1 ml) was prepared at 5.0 mg ml^{-1} in 50 mM carbonate–bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.5). FITC (0.25 mg; Sigma–Aldrich, St Louis, MO, USA) dissolved in dimethyl sulfoxide was then added and incubated for 2 h under constant shaking at room temperature. Free FITC was removed by dialysis for overnight against Tris-buffered saline (TBS) buffer (20 mM Tris–HCl, 1 M NaCl, pH 7.4). Trx expressed by pET-32a vector was labeled with FITC using the same procedure.

Shigella flexneri 2a (1×10^9 ml^{-1}) were washed three times with TBS, suspended in 1 ml of TBS containing 0.5 μg of tetrarhodamine isothiocyanate (TRITC) flurochromes (Sigma–Aldrich) and incubated for 2 h under constant shaking at room temperature.

Binding of recombinant mMBL-C-CRD protein to bacteria

TRITC-*S. flexneri 2a* (1.0×10^6) were incubated with 100 μg of FITC-labeled recombinant Trx-CRD protein for 1 h at 37°C in TBS/EDTA (20 mM Tris–HCl, 10 mM Na_2EDTA, 1 M NaCl, pH 7.4) and TBS/Ca^{2+} (20 mM Tris–HCl, 10 mM $CaCl_2$, 1 M NaCl, pH 7.4) buffer, respectively. FITC-Trx protein was used as a control. For polyclonal antibody blocking study, a group was given 100 μg of anti-CRD antibody and normal rabbit IgG as a control. Fluorescence was visualized under an Olympus BX51 fluorescent microscope.

IEC isolation

IECs were isolated by using a modification of the method of Kristine and co-workers (21). Small intestinal fragments of BALB/c mice were cut into 1-mm fragments and were incubated for 1 h at room temperature on a shaker platform in Ca^{2+}- and Mg^{2+}-free HBSS containing 300 U of collagenase XIa (Sigma–Aldrich) per milliliter, 0.1 mg of dispase I (Sigma–Aldrich) per milliliter, 2% BSA and 0.2 mg of soybean trypsin inhibitor (Sigma–Aldrich) per milliliter. Digested cells were washed three times by centrifugation at 120 × *g* for 3 min in DMEM plus 2% sorbitol. Cells were cultured in 24-well plates. One hour before plating cells, culture surfaces were coated with Matrigel (Sigma–Aldrich). Epithelial cells were cultured in phenol-red-free DMEM with the following additives: 5 mg of insulin (Sigma–Aldrich) per milliliter, 100 μg of heparin (Sigma–Aldrich), 10 ng of epidermal growth factor (Peprotech, Rocky Hill, NJ, USA) per milliliter, 20 mM HEPES, 2 mM glutamine, 100 U of penicillin per milliliter, 100 μg of streptomycin per milliliter, 0.2% D-glucose and 10% fetal bovine serum. Cells were cultured in 5% CO_2 at 37°C with periodic supplementation of medium to maintain a volume of 1 milliliter per well. To confirm the epithelial nature of the cells, expression of cytokeratin was determined by using FITC-labeled monoclonal anti-cytokeratin antibody (Sigma–Aldrich).

Cytokine assay

Shigella flexneri 2a (1×10^8) was incubated with 100 μg of Trx-CRD protein at 37°C for 60 min before adding to cell cultures; meanwhile, the growth medium of the cells was replaced with fresh medium. As a control, *S. flexneri 2a* was given without Trx-CRD protein. For antibody blocking study, a group was given 100 μg of anti-CRD antibody before incubating the bacteria with the cells. The cultures were incubated with bacteria for 24 h in 5% CO_2 at 37°C. Analysis of cytokines from cell culture supernatants was conducted using a mouse inflammation cytometric bead array kit (Bender MedSystems, Vienna, Austria) and on a FACSCalibur flow cytometer. The following cytokines were measured: IL-6, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α).

Adhesion of bacteria to intestinal epithelial tissue

Intestinal tissues were dissected from normal mouse that had been washed with TBS and then the tissues were entirely embedded in optimal cutting temperature (OCT) (Tissue-Tek; Miles, Elkhart, IN, USA) and rapidly frozen on dry ice. Cuts of 5 μm were mounted on poly-lysine-coated glass slides. After warmed to room temperature, slides were incubated with 1×10^8 TRITC-labeled *S. flexneri 2a* for 3 h. Bacteria were incubated with 100 μg of Trx-CRD protein at 37°C for 1 h as a control. For antibody blocking assay, sections were incubated with 100 μg of anti-CRD antibody before the infection. Intestinal epithelial tissue was visualized using FITC-labeled monoclonal anti-cytokeratin

antibody. Fluorescence was visualized under an Olympus BX51 fluorescent microscope.

Immunohistochemistry staining

Tissues were dissected from mice that had been perfused with 20 ml of PBS (140 mM NaC1, 3 mM KC1, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and subsequently with 20 ml of PBS containing 4% PFA. The tissues were then embedded in OCT compound and frozen. Cuts of 5 μm were mounted on poly-lysine-coated glass slides. Endogenous peroxidase activity was quenched by incubating the sections for 30 min with PBS containing 3% H_2O_2. After blocking with 5% non-immune goat serum for 2 h at room temperature, the sections were incubated with anti-CRD diluted with PBS to a concentration of 10 μg ml^{-1} for 1 h at 37°C. After washing with PBS containing 0.05% Tween 20, the sections were incubated with HRP-labeled anti-rabbit IgG polyclonal antibody (Vector Laboratories, CA, USA) for 1 h at 37°C. The chromogenic substrate used was diaminobenzidine (Vector Laboratories). The sections were then counterstained with Mayer's hematoxylin.

Oral infection of mice with S. flexneri 2a

Two days before infection, animals were given 1 mg of erythromycin and 5 mg of streptomycin in 0.1 ml water i.g. and kept abrosia for 1 day (20, 22). As for infection, 10^{11} viable shigella organisms from a 24-h agar culture suspended in 1 ml of normal saline were fed i.g. Mice were given 1 mg of anti-CRD antibody intravenously and 1 mg of the same antibody oral administrated 2 h before the infection. Normal rabbit IgG was used as a control. Sterile water containing 0.1 mg of erythromycin and 4 mg of streptomycin per milliliter were supplied *ad libitum* throughout the entire experiment.

Bacterial counts in tissue samples

Animals were killed by decapitation 48 h after treatment. Tissues were obtained and weighted. The tissues were then treated by homogenating in 1 ml of sterile PBS. The samples were serially diluted before plating on Luria Bertani agar (LB agar) plates containing streptomycin. Colony-forming units were determined from LB plates which were incubated for 12 h at 37°C, and the amount of bacteria was standardized for per gram.

Histopathological analysis

All tissue samples were immediately fixed in 4% PFA, dehydrated and embedded in paraffin. Sequential sections were taken at various levels of the samples. Hematoxylin–eosin staining and immunostaining for TNF-α, IL-6 and MCP-1 were used to evaluate the inflammatory responses. Briefly, histosections were deparaffinized and rehydrated. Endogenous peroxidases were blocked by 0.3% hydrogen peroxide in methanol. Saturation was achieved by incubation in 5% non-immune goat serum. Incubation was then carried out overnight at 4°C with biotin-anti-mouse TNF-α antibody (BioLegend, San Diego, CA, USA), rat anti-mouse IL-6 mAb (BioLegend), biotin-anti-mouse MCP-1 mAb (HyCult Biotechnology, Uden, The Netherlands), respectively. HRP conjugates were added and incubation was carried out for 1 h at 37°C. The chromogenic substrate was diaminobenzidine (Vector Laboratories). The sections were then counterstained with Mayer's hematoxylin.

Assessment of histological score

Mice were scored individually, and histological examination was performed by two independent investigators blinded to the source of treatment (23). Histology was scored as follows:

(1) Epithelium (E): 0, normal morphology; 1, loss of globlet cells; 2, loss of globlet cells in large areas; 3, loss of crypts and 4, loss of crypts in large areas.
(2) Infiltration (I): 0, no infiltrate; 1, infiltrate around crypt basis; 2, infiltrate reaching to lamina muscularis mucosae; 3, extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema and 4, infiltration of the lamina submucosa.

The total histological score represents the sum of the epithelium and infiltration score and ranges from 0 to 8.

Statistical analysis

One-way analysis of variance was used for analysis of the statistical significance of cytokine levels. Student's *t*-test (cytokine levels) was used for two independent group comparisons. Error bars represent the standard error of the mean. $P < 0.05$ was considered to be statistically significant.

The non-parametric Kruskal–Wallis test was used for determination of the statistical significance of differences in bacteria adhesion, histological score and the number of invaded bacteria in intestinal tissue. The Mann–Whitney test was used for two independent group comparisons. A probability of $P < 0.05$ was used to define this significance.

Results

Binding of recombinant mMBL-C-CRD protein to S. flexneri 2a

The binding of FITC-labeled Trx-CRD protein to TRITC-labeled *S. flexneri 2a* is illustrated in Fig. 1. When TBS/Ca^{2+} buffer was used, the bright green fluorescence produced by FITC-labeled protein and aggregation of the bacteria was seen under fluorescent microscopy. However, the binding between Trx-CRD and *S. flexneri 2a* was not detected when the incubation was carried out in TBS/EDTA buffer. It was shown that Trx, expressed by pET-32a, does not bind to *S. flexneri 2a* in TBS/Ca^{2+} buffer (Fig. 1g), indicating that the mMBL-C-CRD protein specifically binds to *S. flexneri 2a* in a calcium-dependent manner, which is consistent with the previous reports (4–6).

We next evaluated whether the polyclonal antibody against mMBL-C-CRD protein prevents the binding between the CRD protein and the bacteria. The affinity-purified antibody against mMBL-C-CRD protein could not bind with recombinant mMBL-A-CRD, as indicated by immunoblotting analysis (Supplementary Figures 1–3, available at *International Immunology* Online). The antibody inhibited effectively the binding of FITC-Trx-CRD protein to *S. flexneri 2a in vitro* (Fig. 1o), whereas normal rabbit IgG failed to do so (Fig. 1j).

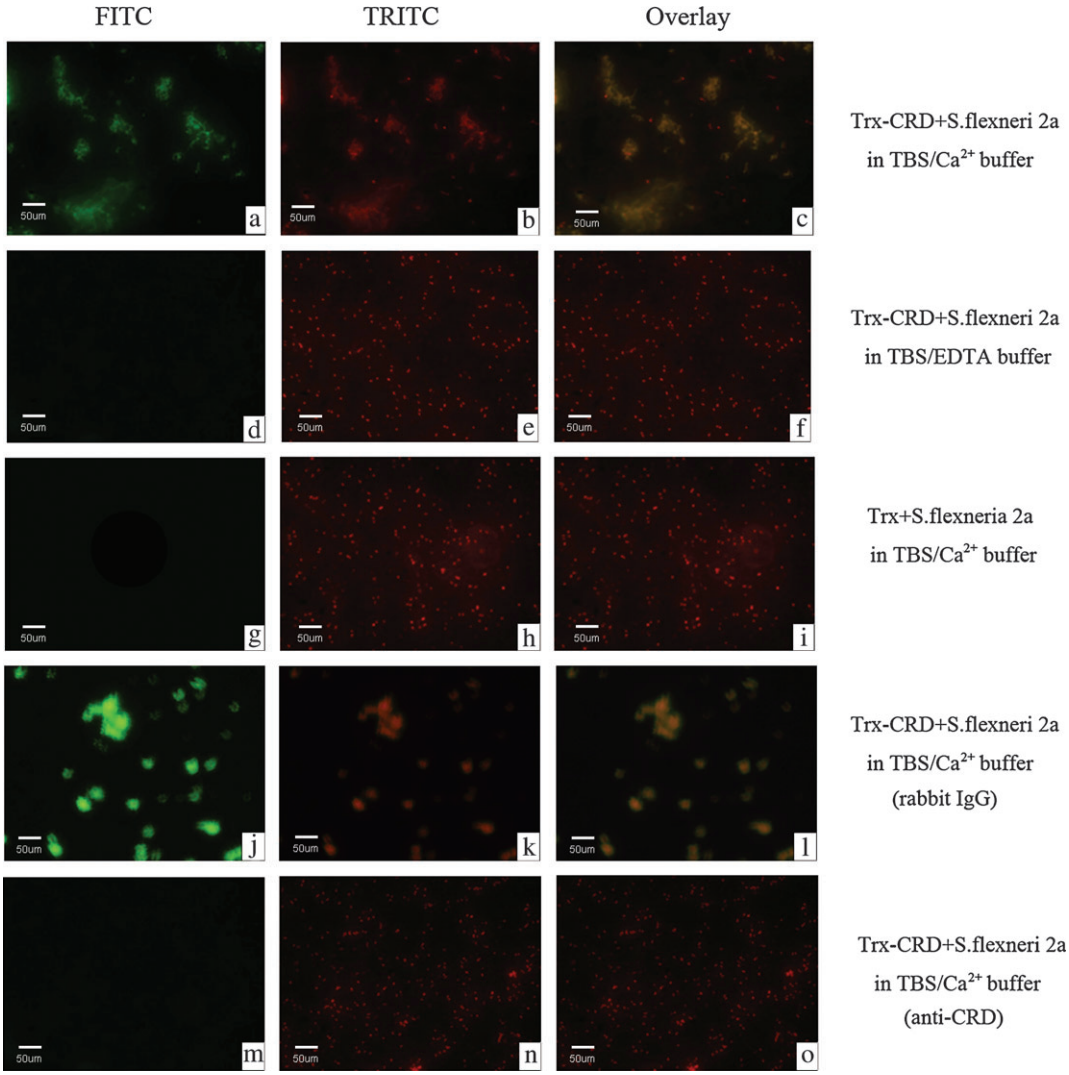

Fig. 1. Binding of mMBL-C-CRD protein to *Shigella flexneri 2a*. FITC-labeled Trx-CRD protein was incubated with TRITC-labeled *S. flexneri 2a* in TBS/Ca^{2+} buffer (panels a, b and c.) and TBS/EDTA buffer (panels d, e and f), respectively. FITC-labeled Trx protein as a negative control was incubated with TRITC-labeled bacteria in TBS/Ca^{2+} buffer (panels g, h and i). FITC-labeled Trx-CRD was incubated with TRITC-labeled *S. flexneri 2a* in TBS/Ca^{2+} buffer in the presence of the neutralizing antibody against mMBL-C-CRD at a concentration of 1 mg ml^{-1} (panels m, n and o). Normal rabbit IgG was used as a control (panels g, h and i).

Immunohistochemical localization of mMBL-C in intestinal tissues

To confirm the mMBL-C expression on intestinal mucosa, immunohistochemical staining was performed using the anti-CRD antibody on PFA-fixed sections obtained from normal BALB/c mice. As shown in Fig. 2, mMBL-C staining was observed in some villous epithelial cells throughout the small intestine, which agrees with the previous report (14). Meanwhile, the results also verified that the antibody can recognize mMBL-C *in vivo*.

mMBL-C reduces the release of pro-inflammatory cytokines from primary IECs induced by S. flexneri 2a

The activation of primary IECs was assessed by measuring cytokine levels in the culture medium. We chose cytokines known to be released by activated epithelial cells, mostly with pro-inflammatory properties. As shown in Fig. 3, *S. flexneri 2a* induced a release of IL-6 and MCP-1, while the secretion of these two cytokines was reduced when recombinant Trx-CRD protein was added to the cultures. *Shigella flexneri 2a* compound with anti-CRD antibody stimulated IL-6 secretion more than the bacteria only; however, there was no significant difference in MCP-1 secretion between these two groups. Surprisingly, no TNF-α was found in this assay.

mMBL-C-CRD protein blocks the adhesion of S. flexneri 2a to intestinal epithelial tissue

To investigate whether mMBL-C can influence the binding of *S. flexneri 2a* to intestinal tissue *in vitro*, horizontal frozen sections of small intestine from mice were incubated with the bacteria. As shown in Fig. 4, Trx-CRD protein blocked the adhesion of TRITC-labeled *S. flexneri 2a* to intestinal epithelial tissues completely. The effect of anti-CRD antibody on

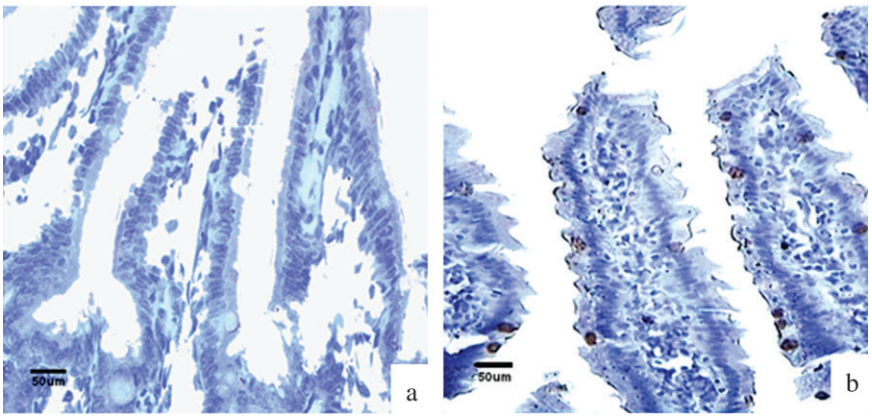

Fig. 2. Immunohistochemical localization of mMBL-C in mouse intestinal tissues. PFA-fixed cryosections of mouse intestine were stained with polyclonal antibody specific for mMBL-C-CRD and counterstained with hematoxylin. Panel (a) was stained with normal rabbit IgG.

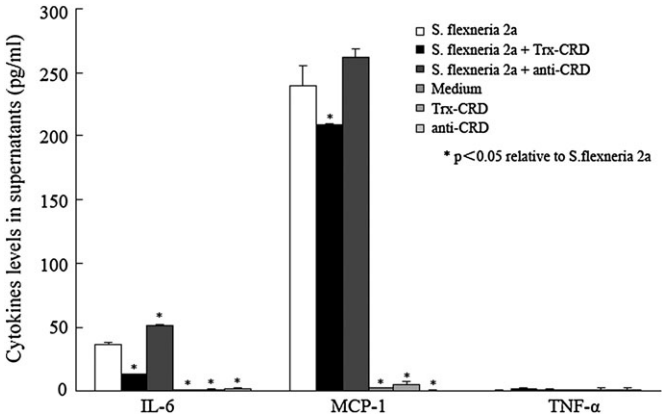

Fig. 3. Results of cytokine assay on the cell culture supernatants of IECs. IECs (5×10^5 ml^{-1}) were incubated for 24 h with *Shigella flexneri 2a* (1×10^8 colony-forming units per milliliter) and same quantity of bacteria with recombinant Trx-CRD protein (100 μg ml^{-1}) or anti-CRD antibody (100 μg ml^{-1}). After incubation, the culture supernatants were collected and cytokine levels were measured (see Methods). The data are given as means ± SDs for each cytokine.

the bacterial adhesion was also investigated. Microscopic count data from counting five fields on three separate slides, which showed that the number of bacteria adhered on the surface of intestinal mucosa when anti-CRD existed is a little more.

mMBL-C on intestinal mucosa prevents against the S. flexneri 2a elicited damage

To test whether mMBL-C can protect intestinal mucosa from bacterial invasion-associated damage, mice were treated with or without anti-CRD antibody prior to *S. flexneri 2a* infection. The intestinal tissue sections stained with hematoxylin and eosin were analyzed by light microscopy. There was only minimal damage in the intestinal morphology in these animals, but no obvious pathologic change was seen in untreated mice or mice that had received normal rabbit IgG. However, the mice receiving anti-CRD antibody before infection displayed more damages in intestinal tissues, as indicated by infiltration of mononuclear leukocytes and polymorphonuclear neutrophils and hemorrhage (Fig. 5). The intestinal morphology appeared normal in mice receiving anti-CRD antibody without bacterial infection (data not shown). Considering that anti-CRD did not influence the complement activation *in vivo* (Supplementary Figure 4, available at *International Immunology* Online), it suggested that the severed bacterial infection may result from that recognition function of MBL-C was blocked.

mMBL-C neutralization is associated with increase of TNF-α expression in mucosal tissues

As shown in Fig. 6, tissue sections were prepared from untreated mice (Fig. 6a), mice treated with either normal rabbit IgG (Fig. 6b) or anti-CRD antibody (Fig. 6d) and subjected to immunohistochemical staining of TNF-α. The untreated animals or those treated with normal rabbit IgG before *S. flexneri 2a* infection showed negative staining for TNF-α in intestinal mucosa. Anti-CRD antibody treatment resulted in a significant increase in the levels of TNF-α. However, there was no staining when using the rat anti-mouse IL-6 mAb or biotin-anti-mouse MCP-1 mAb on the sections from the same intestinal sample (data not shown). Considering that *S. flexneri 2a* can stimulate IL-6 and MCP-1 secretion but no TNF-α from primary cultured IEC *in vitro*, it indicated that the TNF-α may come from infiltrated inflammatory cells.

mMBL-C on intestinal mucosa prevents against the S. flexneri 2a invasion

IECs at mucosal surfaces are the first line of defense against microbial pathogens. The ability of shigella to invade and colonize the intestinal epithelium, in association with an intense inflammatory response leading to the destruction of the colonic mucosa, is central to shigellosis. In order to assess whether mMBL-C expressed from IECs affects the shigella invasion into intestinal mucosa, mice were treated with the antibody against mMBL-C-CRD before the infection of streptomycin-resistant strain of *S. flexneri 2a*. As shown in Fig. 7, no or only a small numbers of streptomycin-resistant *S. flexneri 2a* were recovered from intestinal tissues in untreated mice or mice receiving control IgG. However,

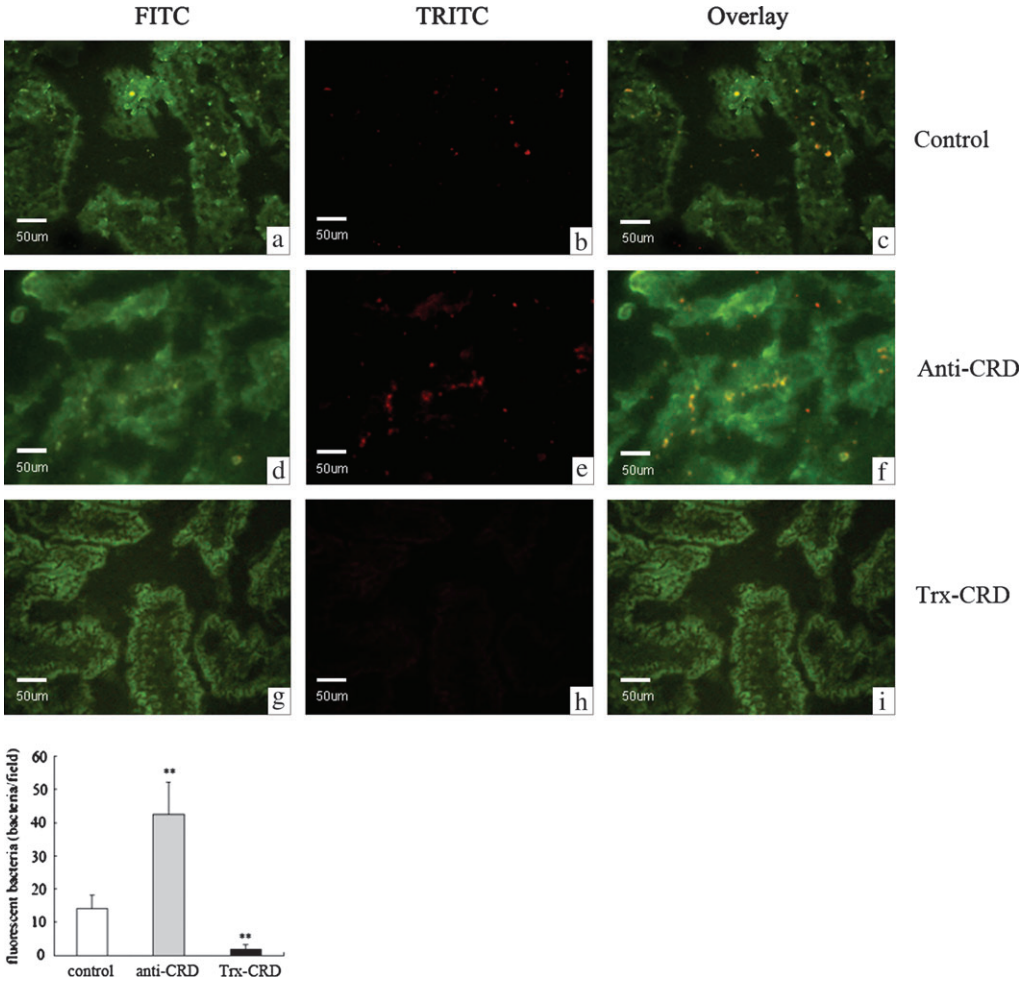

Fig. 4. mMBL-C-CRD protein blocks the adhesion of *Shigella flexneri 2a* to intestinal epithelial tissue. Frozen section of intestinal tissue was incubated with TRITC-labeled *S. flexneri 2a* (a, b and c) for 3 h. For antibody blocking study, the section was incubated with anti-CRD antibody for 1 h before the bacterial incubation (d, e and f). In another group, TRITC-labeled *S. flexneri 2a* was incubated with Trx-CRD for 1 h before adding to frozen sections (g, h and i). After being washed with TBS, fixed with 4% PFA and immunostained with FITC-labeled monoclonal anti-cytokeratin antibody, the slides were analyzed by fluorescence microscope and the amounts of adhesive bacteria were compared by microscopic count data from counting five fields on three slides for each group. The tissue slide treated with anti-CRD antibody displayed more adhesive bacteria, and the adhesive bacteria was significantly decreased when mMBL-C-CRD protein was added (**$P < 0.01$ versus control).

administration of the neutralizing antibody significantly increased the number of bacteria recovered from the intestinal tissues, suggesting that mMBL-C expressed from intestinal epithelium contributes to host defense during *S. flexneri 2a* invasion.

Discussion

The MBL is an evolutionarily conserved host defense protein that acts as a broad-spectrum recognition molecule against a wide variety of infectious agents. Most studies have explored the roles of MBL in relation to the acquisition of an infectious organism (susceptibility) and the nature of the associated clinical course (severity) (24). In the present study, we have demonstrated that mMBL-C directly interacts with *S. flexneri 2a* and that mMBL-C on intestinal villous epithelial cells is involved in protection from bacterial infection. Our results support the well-documented roles of MBL-C in host defense. A number of studies have shown that low levels of MBL contribute to the severity of illness in infections of pathogenic bacteria such as *Neisseria meningitidis*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (25–27). Animal studies also showed that MBL-null mice are indeed highly susceptible to *S. aureus* and *Pseudomonas aeruginosa* systemic infection (28, 29).

Intestinal epithelium allows nutrient and water absorption. It also provides a barrier against infection. IECs at mucosal surfaces are the first line against microbial pathogens (19, 30). Our studies also confirm a previous report of a relatively abundant expression of mMBL-C in small intestine, which may have implications on protective functions of mMBL-C in small intestine (14).

There are multiple mechanisms by which MBL is involved in host defense against pathogen infection. It can kill the pathogens by activating the complement cascade or function as an opsonin to promote phagocytic clearance by

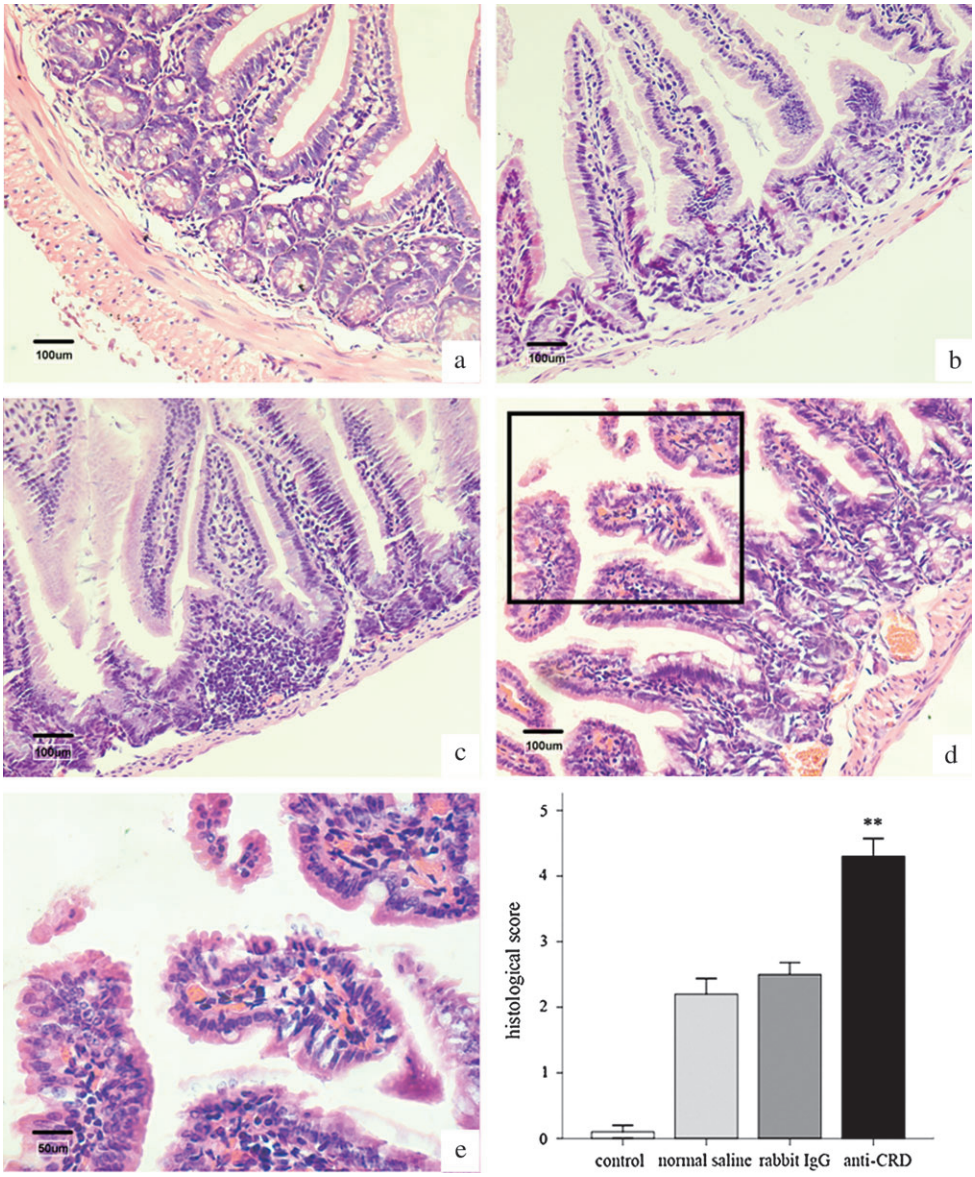

Fig. 5. Neutralization of mMBL-C exacerbates damages in intestinal tissues caused by bacterial infection. Intestinal tissues were isolated 48 h post-infection from mice uninfected (a) or treated with saline (b), normal rabbit IgG (c) or anti-CRD antibody (d) before *Shigella flexneri 2a* infection and subjected to HE staining. Panel (e) is a higher magnification view of the inflammation site indicated by a box in panel (d). Histological scores of each group were determined as described in Methods. (**$P < 0.01$ versus normal rabbit IgG, by Mann–Whitney test).

direct opsonization when binding to lectin receptors on phagocytes. However, human MBL can inhibit type A influenza viral infection and spreading independent of complement activation (7). Furthermore, it has been shown that MBL is capable of blocking interaction of HIV with dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin expressed on dendritic cells, thereby inhibiting the virus transmission (31). In the present study, we have found that mMBL-C can directly bind to *S. flexneri 2a in vitro*, the antibody specific for mMBL-C-CRD is able to interfere the interaction, and mMBL-C can block the adhesion of *S. flexneri 2a* to intestinal tissues *in vitro*.

mMBL-C-CRD decreased pro-inflammatory cytokine production, like IL-6 and MCP-1, from primary IECs stimulated with *S. flexneri 2a*. More importantly, use of the antibody *in vivo* could abolish host defense against *S. flexneri 2a*, as indicated by more damages in IECs, increased the number of invaded bacteria and induced the higher expression of pro-inflammatory cytokine TNF-α. Based on our data, we postulate that MBL may directly inhibit the interaction of microorganism with target cells through its CRD. Supporting evidence also came from the studies of gastrointestinal mucins, which has been shown to compete with underlying epithelium for binding to pathogens, resulting in reduced

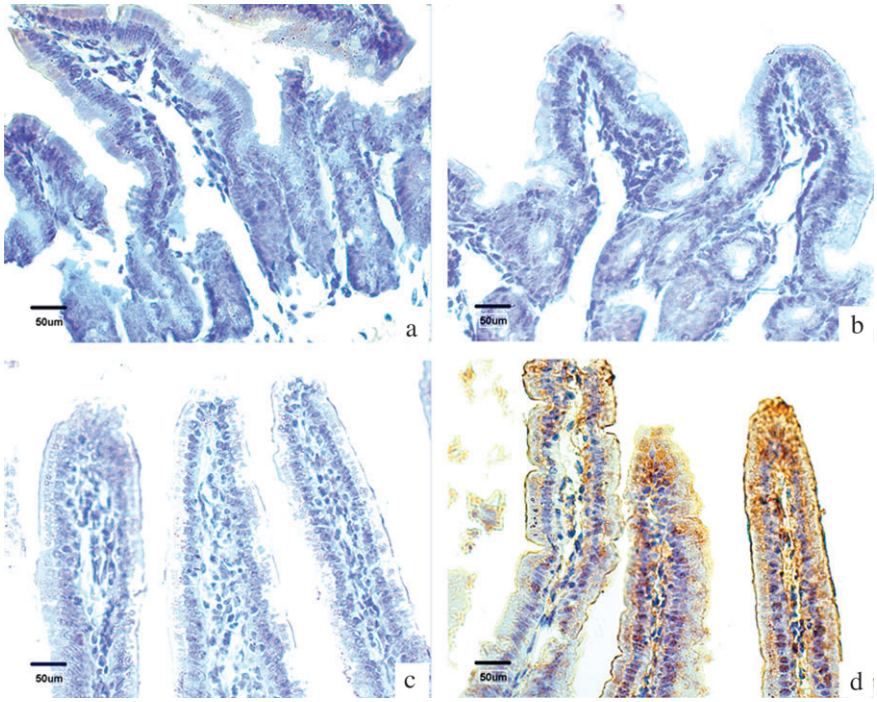

Fig. 6. mMBL-C neutralization increases TNF-α expression in intestinal mucosa following infection. Immunostaining for TNF-α in tissue sections corresponding to samples obtained from loops 48 h after infection with *Shigella flexneri 2a* Panel (a), bacteria only; panel (b), normal rabbit IgG plus bacteria; panel (d), anti-CRD antibody plus bacteria. Panel (c), a negative control is shown, in which the sample is as same as in panel (d) and normal IgG instead of anti-mouse TNF-α antibody was used for staining.

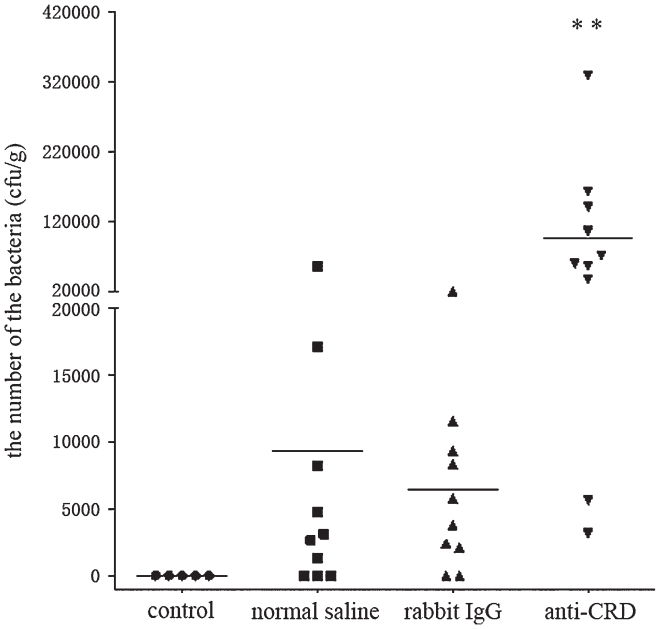

Fig. 7. Blocking of mMBL-C increases the number of invaded bacteria in intestine tissues. Mice were treated with saline, normal IgG and anti-CRD antibody before *Shigella flexneri 2a* infection or left untreated. Colony forming unit (CFU) of bacteria were then counted as described in 'Methods' 48 h post-infection. (**$P < 0.01$ versus normal rabbit IgG, by Mann–Whitney test).

attachment of pathogens to the mucosal IECs for colonization (32, 33). Therefore, it appears that the mechanism of competitive inhibition has been commonly used by the host to protect against invasion of pathogenic microorganisms.

The ability of shigella to invade and colonize the intestinal epithelium is vital to shigellosis. O-specific polysaccharide (O-SP), the outermost domain of the LPS on shigella, has been shown to be both an essential virulence factor mediating invasion of IECs and a protective antigen involved in the primary step of bacteria entry (34). A previous study showed that MBL can bind to the O-SP moiety of Klebsiella O3 LPS (35). It is likely that O-SP is the binding structure on *S. flexneri 2a* for mMBL-C on IECs.

The reproducible shigella infection model has been established in mouse intestinal tract after elimination of the normal enteric flora (20, 22). Although infected mice did not show any gross signs of illness, mesenteric lymph nodes and pathologic changes during various sections of the intestinal tract indicated an establishment of chronic infection. Under most circumstances, the lack of MBL does not predispose the immunocompetent host to bacterial infection. It was in the setting of immune insufficiency that low levels of MBL produced by haplotypes were associated with enhanced susceptibility to infection, as was observed in the setting of cancer chemotherapy (36). Upon antibody blocking *in vivo*, we observed a significant increase in the number of *S. flexneri 2a* in intestinal tissues following infections, as compared with control animals treated with either saline or normal rabbit IgG. In addition, the expression of a pro-inflammatory cytokine

TNF-α was found to be elevated in intestinal mucosa. Considering that *S. flexneri 2a* could not stimulate the TNF-α expression from IECs *in vitro*, we suppose that this cytokine may be secreted by the infiltrated inflammatory cells.

Taken together, our study has demonstrated that mMBL-C likely expressed from IEC's can directly bind to *S. flexneri 2a* and help to prevent acute intestinal damage in mice, supporting the notion that mMBL-C participates in the defense mechanism/or machinery against invaded bacteria and other pathogens. Better understanding of MBL may lead to novel approaches targeting this molecule for improved anti-bacterial host defenses.

Supplementary data

Supplementary figures are available at *International Immunology* Online.

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Abbreviations

CKS	CMP-ketodeoxyoctonate synthetase
CRD	carbohydrate recognition domain
IEC	intestinal epithelial cell
i.g.	intra-gastric
LB	Luria Bertani
MBL	mannan-binding lectin
MCP	monocyte chemoattractant protein
mMBL-C	mouse MBL-C
OCT	optimal cutting temperature
O-SP	O-specific polysaccharide
TBS	Tris-buffered saline
TNF	tumor necrosis factor
TRITC	tetrarhodamine isothiocyanate
Trx	thioredoxin

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