Mannose-Binding Lectin Contributes to Deleterious Inflammatory Response in Pandemic H1N1 and Avian H9N2 Infection

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Background. Mannose-binding lectin (MBL) is a pattern-recognition molecule, which functions as a first line of host defense. Pandemic H1N1 (pdmH1N1) influenza A virus caused massive infection in 2009 and currently circulates worldwide. Avian influenza A H9N2 (H9N2/G1) virus has infected humans and has the potential to be the next pandemic virus. Antiviral function and immunomodulatory role of MBL in pdmH1N1 and H9N2/G1 virus infection have not been investigated.

Methods. In this study, MBL wild-type (WT) and MBL knockout (KO) murine models were used to examine the role of MBL in pdmH1N1 and H9N2/G1 virus infection.

Results. Our study demonstrated that in vitro, MBL binds to pdmH1N1 and H9N2/G1 viruses, likely via the carbohydrate recognition domain of MBL. Wild-type mice developed more severe disease, as evidenced by a greater weight loss than MBL KO mice during influenza virus infection. Furthermore, MBL WT mice had enhanced production of proinflammatory cytokines and chemokines compared with MBL KO mice, suggesting that MBL could upregulate inflammatory responses that may potentially worsen pdmH1N1 and H9N2/G1 virus infections.

Conclusions. Our study provided the first in vivo evidence that MBL may be a risk factor during pdmH1N1 and H9N2/G1 infection by upregulating proinflammatory response.

The pandemic H1N1 2009 (pdmH1N1) influenza A virus has spread globally since the outbreak first started in Mexico in 2009. As of August 2010, the virus had already caused more than 18,449 deaths in at least 214 countries worldwide [1]. The World Health Organization officially announced the step-down of pdmH1N1 to postpandemic phase on 10 August 2010, and the virus was expected to circulate as a seasonal virus in the human population thereafter [2]. Gene segments of the pdmH1N1 virus are derived from multiple lineages, including the Eurasian swine, the classical swine, and the triple reassortant swine lineages. Multiple genetic reassortment events of viral components have taken place and thus gave rise to this novel pandemic virus [3]. Clinical symptoms of pdmH1N1 infection are usually mild, possibly due to the cross-protection offered by memory cytotoxic T lymphocytes established from previous exposure to seasonal influenza [4].

H9N2 avian influenza virus (H9N2/G1) is widely prevalent among poultry in various Eurasian regions, including mainland China and Hong Kong. In 1999 and 2003, H9N2 influenza was reported in 3 children in Hong Kong. All 3 of them developed relatively mild symptoms and recovered within a week [5, 6]. The 6 internal genes of H9N2/G1 virus were found to be related to the highly pathogenic avian influenza virus A/Hong Kong/483/97 (H5N1). Although the H9N2/G1 virus was found mainly in poultry, it has the ability to transmit across species, and with its genetic similarity to the highly pathogenic H5N1, it also poses a threat of becoming pandemic [7].
Mannose-binding lectin (MBL) is a serum protein primarily produced by the liver. It belongs to the collectin family that comprises the collagen-like domain and the carbohydrate recognition domain (CRD). MBL functions as a key pattern-recognition molecule recognizing a wide range of pathogens [8, 9]. Lectin pathway activation [10] and opsonophagocytosis are triggered upon MBL binding to pathogens [11]. While the MBL gene is highly polymorphic in humans, clinical association studies have demonstrated that MBL deficiency was associated with increased susceptibility to certain infections [12, 13].

The antiviral role of MBL in influenza virus infection remains controversial. Previous studies suggested that MBL demonstrates an immunomodulatory role in influenza [23]. Influenza virus–infected epithelial cells and macrophages can initiate a cell-specific response that includes the transcription and release of proinflammatory cytokines and chemokines [19, 20]. Although some studies have indicated that MBL may regulate proinflammatory cytokine and chemokine release from phagocytes in response to bacterial stimulation [21, 22], little is known about its immunomodulatory role in influenza [23].

In the present study, we investigated whether MBL could display any in vitro or in vivo antiviral function toward pdmH1N1 and H9N2/G1 viruses, as well as whether it could modulate the inflammatory response upon infection by these two strains of influenza virus.

MATERIALS AND METHODS

Cells and Viruses

Influenza virus A/California/04/2009 (pdmH1N1) were propagated in embryonated chicken eggs and purified by ultracentrifugation with minor modification of our previous work [4, 24]. Influenza virus A/Quail/Hong Kong/G1/97 (H9N2/G1) was grown in Madin-Darby canine kidney (MDCK) cells with modified Eagle’s medium (Invitrogen) containing 2 μg/mL N-p-Tosyl-l-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (Sigma-Aldrich). Virus stocks were purified by adsorption to and elution from turkey red blood cells and stored at −80°C until use as previously described [25]. The determination of virus titer was performed by titrating virus in MDCK cells, with daily observation of cytopathic effect and confirmation by hemagglutination assay. The tissue culture infective dose affecting 50% of the cultures (TCID₅₀) was calculated by the Reed-Muench formula. Ultraviolet (UV)–irradiated virus was prepared by irradiation with energy of 0.2 J in a UV crosslinker as described previously [26].

Binding of Recombinant Human MBL to Influenza Virus

The binding assay was performed as described previously [12]. In brief, 96-well flat-bottom polystyrene plates (Corning-Costar) were precoated with 100 μL/well of 10², 10³, 10⁴, and 10⁵ TCID₅₀ UV-irradiated influenza viruses or phosphate-buffered saline (PBS). After incubation at room temperature overnight, wells were blocked for 2 hours at room temperature with 1% bovine serum albumin (BSA) in PBS with 0.05% sodium azide. Different concentrations of recombinant human MBL (rhMBL) (0, 0.5, 2, 6, or 8 μg/mL), which was kindly provided by Dr K. Takahashi (Laboratory of Developmental Immunology, Harvard Department of Pediatrics, Massachusetts General Hospital, Boston), were added and incubated overnight at 4°C. Then 100 μL of 0.2 μg/mL biotinylated monoclonal anti-MBL antibody (HYB131-01, Antibody Shop) diluted in PBS with 1% BSA was added into each well. Bound antibody was detected by using horseradish peroxidase–conjugated streptavidin and tetramethylbenzidine substrate solution (R&D Systems). The binding of MBL to influenza virus was evaluated by the absolute absorbance values measured at 450 nm (A₄₅₀).

Mice and Virus Infection

Breeding pairs of MBL wild-type (WT) and MBL knockout (KO) mice on C57B6/J were provided by Dr Takahashi [27]. They were maintained under specific pathogen-free conditions in the animal facilities of the Laboratory Animal Unit, The University of Hong Kong. Female mice were used at 6–10 weeks of age. They were anesthetized and inoculated intranasally with 30 μL of 10³, 10⁴, or 10⁵ TCID₅₀ UV-irradiated influenza viruses or PBS at day 0. Virus-infected or mock-treated mice were weighed daily. All animal care and experiments were conducted in accordance with the Committee on the Use of Live Animals in Teaching and Research guidelines of the University of Hong Kong.

Preparation of Lung Homogenates

Virus-infected or mock-treated mice were sacrificed at days 3, 7, and 14 after infection. The lungs were harvested and homogenized by a tissue homogenizer (Omni International). The homogenates were centrifuged at 2500 rpm for 10 minutes at 4°C. Supernatants were used for virus titer determination and cytokine detection.

Cytokine and Chemokine Detection

Expression levels of interleukin (IL) 1α, IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF) α, interferon (IFN) γ, macrophage inflammatory proteins (MIP)-1α, MIP-1β, monocyte chemotactic protein (MCP)-1, MCP-3, and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) in the lung homogenates were quantitatively determined by flow cytometry–based immunoassay (Mouse Th1/Th2 cytokine 10plex and
Mouse Chemokines 6plex Flowcytomix Multiplex, Bender MedSystems) according to the manufacturer’s protocol. Interleukin 1β and keratinocyte chemoattractant (KC) simplex were purchased separately (Bender MedSystems). In brief, lung homogenates were prepared and processed accordingly. The samples were acquired on a BD LSRII (BD Bioscience), and the amount of cytokine (ng/mL) was calculated by FlowCytomix Pro 2.3 software (Bender MedSystems).

Pulmonary Histopathology
Virus-infected or mock-treated mice were sacrificed at the indicated time point for histopathologic analysis. The lung tissues were fixed in 10% formalin and embedded in paraffin. Five-micrometer-thick, paraffin-embedded sections were cut and stained with hematoxylin and eosin (H&E) to analyze histological lesions. Histopathologic score of lung tissues was examined by a board-certified pathologist blinded to the exposure status. Lung inflammatory changes were graded using a semiquantitative scoring system based on the following parameters: peribronchiolar and bronchial infiltrates, bronchiolar and bronchial luminal exudates, perivascular infiltrates, parenchymal pneumonia, and edema, as previously described [28]. Each parameter was graded on a scale of 0–4 with 0 as absent, 1 as slight, 2 as mild, 3 as moderate, and 4 as severe. The total lung inflammation score was expressed as the sum of the scores for each parameter. The degree of cell infiltration was independently scored on an increasing scale of 0–3 with 0 as no cells, 1 as few cells, 2 as moderate influx of cells, and 3 as extensive influx of cells [29].

Statistical Analysis
Data were expressed as mean (standard error of the mean). Unpaired Student $t$ test in GraphPad Prism 5.0 software (GraphPad) was used for statistical analysis. A $P$ value $<$ .05 was considered significant.

**Figure 1.** Binding of recombinant human mannose-binding lectin (rhMBL) to pdmH1N1 and H9N2/G1 influenza viruses. Binding of MBL to influenza viruses was determined by microtiter capture assay. Wells of plates were coated with influenza viruses, and MBL was incubated overnight in the virus-coated wells. A and D, MBL dose-dependent binding. Incubation with increasing amounts of MBL resulted in increasing levels of detected MBL bound to immobilized influenza virus. In contrast, MBL binding did not increase when the wells were coated with 1% bovine serum albumin (BSA). B and E, Virus dose-dependent binding. The amount of MBL binding was dependent on the amount of influenza virus used to coat the wells. C and F, Binding dependent on the absence of ethylenediaminetetraacetic acid (EDTA). MBL was incubated at 8 µg/mL in microtiter plates coated with 100 µg/mL 1% BSA, mannan, or influenza virus in the absence (control) or presence of EDTA for 2 h. Results are presented as mean ± standard error of the mean and are representative of 3 experiments. A$_{450}$, absolute absorbance values measured at 450 nm; TCID$_{50}$, median tissue culture infective dose.
RESULTS

rhMBL Binds Both pdmH1N1 and H9N2/G1 Viruses

A microtiter capture assay demonstrated that MBL could bind to pdmH1N1 and H9N2/G1 in vitro (Figure 1). The MBL-virus binding occurred in a dose-dependent manner (Figure 1A and 1D). Similarly, increased amount of virus could also result in increased binding by MBL (Figure 1B and 1E). MBL utilizes the CRD to recognize pathogens in a calcium-dependent manner [30]. Further addition of ethylenediaminetetraacetic acid (EDTA) in the assay inhibited the binding of MBL to both strains of influenza virus (Figure 1C and 1F), suggesting that the binding occurred through the CRD of MBL.

Influenza Virus Infection Results in Greater Weight Loss in MBL WT Mice Than in MBL KO Mice

Wild-type and MBL KO mice, 6–10 weeks of age, were infected intranasally with 30 μL of 10^3 TCID₅₀ pdmH1N1 virus or 10^5 TCID₅₀ H9N2/G1 virus. The viral dosage chosen for the experiment was previously demonstrated to be sublethal (data not shown). Mice were inoculated with 30 μL of PBS as the mock treatment. No mice died throughout the 14-day experiment. The body weight of mice, which was a physiological value indicating infection progress and the health of the animals, was recorded daily. Mice receiving mock treatment did not lose body weight, demonstrating the absence of potential harmful effects due to anesthetics and intranasal inoculation (Figure 2A).

Both strains of influenza virus could successfully infect MBL WT and MBL KO mice as evidenced by the significant weight loss in these mice after virus infection. Compared to MBL KO mice, MBL WT mice had more weight loss upon virus infection. For pdmH1N1 virus infection, MBL WT mice showed a significantly greater body weight drop on days 3–12 compared with MBL KO mice (Figure 2B). The mean peak body weight loss observed in the MBL WT mice and MBL KO mice were −23.51% and −17.38%, respectively. For H9N2/G1 virus infection, MBL WT mice only showed a significantly greater weight loss on day 8 when compared with MBL KO mice (Figure 2C). Although similar mean peak weight loss was observed between the MBL WT mice and MBL KO mice after H9N2/G1 virus infection, MBL WT mice recovered more slowly than the KO mice. Collectively, these data suggested that the presence of MBL caused a more severe infection by the pdmH1N1 and H9N2/G1 viruses.

Lung Virus Titer in Infected Mice

Wild-type and MBL KO mice infected with pdmH1N1 or H9N2/G1 virus were sacrificed on days 3, 7, and 14 after infection. Virus titers in lung homogenates were determined by TCID₅₀. As shown in Figure 3, these 2 strains of influenza virus were detectable in the lung homogenates collected from MBL WT and KO mice on day 3 and day 7, confirming viral lung infection. On day 14, titers for both strains of virus were undetectable, which was consistent with the regain of body weight by MBL WT and MBL KO mice and suggested recovery from the infection. For pdmH1N1 virus infection, there was no significant difference in the lung virus titer between MBL KO and MBL WT mice on days 3 and 7 after infection (Figure 3A). In contrast, significantly less virus titer was detected in MBL KO mice on day 7 but not on day 3 after H9N2/G1 virus infection compared to that in MBL WT mice (Figure 3B).
MBL Upregulates the Inflammatory Response to Influenza Virus Infection

To further investigate whether MBL would modify the inflammatory response upon pdmH1N1 and H9N2/G1 virus infection, a panel of 14 proinflammatory cytokines and chemokines were examined in the lung homogenates collected from the infected MBL WT and MBL KO mice. The simultaneous profiling of the cytokines and chemokines was examined by using bead-based suspension array, which could allow the sensitive and specific detection of these proteins in the available amount of lung homogenates.

As shown in Figures 4 and 5, upon pdmH1N1 and H9N2/G1 virus infection, inflammatory response assayed by cytokines and chemokines production was triggered in both MBL WT and MBL KO mice. Except for IL-2, of which the level remained constantly low during the course of experiment, the kinetics of individual proteins were similar in that they were readily detectable on day 3, reached the highest level on day 7, and declined on day 14. Strikingly, MBL KO mice had reduced inflammatory responses during infection. Among the 14 cytokines examined, the majority of them showed significantly lower amounts in MBL KO mice lung homogenates than in MBL WT mice, including IL-1α, IL-1β, IL-6, IL-10, TNF-α, IFN-γ (Figures 4A and 5A), KC, MIP-1α, MIP-1β, MCP-1, MCP-3 and RANTES (Figures 4B and 5B). These results suggested that MBL upregulates the inflammatory response to influenza virus infection, resulting in elevated production of proinflammatory cytokines and chemokines in the MBL WT mice as compared with the MBL KO mice.

More Acute Lung Damage in Infected MBL WT Mice Than in MBL KO Mice

To further confirm the severe inflammatory response in the MBL WT mice compared with MBL KO mice, lung sections were stained with H&E for histological analysis to evaluate inflammation-associated lung damage caused by pdmH1N1 and H9N2/G1 influenza virus infection. In the histological sections of MBL WT mice, more severe lung inflammation and more cell infiltration were observed when compared to that of MBL KO mice on day 7 (Figure 6). Consistent with our cytokine and chemokine data, the pulmonary histological analysis suggested that the MBL WT mice had a more severe inflammatory response upon pdmH1N1 and H9N2/G1 virus infection.

DISCUSSION

Mannose-binding lectin is a pattern-recognition molecule, which provides first line of host defense. Accumulating evidence has suggested that MBL exhibits in vitro anti-influenza virus properties by direct neutralization, inhibiting influenza virus hemagglutination, binding to the influenza virus as an opsonin, and activating the complement system through the lectin pathway [14, 16, 23]. However, these properties vary among different virus strains and subtypes [17, 18]. In this study, we focused on the pandemic influenza A H1N1 virus and avian influenza A H9N2/G1 virus, which are of potential threat to the global community.

We demonstrated that despite these 2 strains of viruses being bound by rhMBL via the CRD of MBL at the physiological level, they infected both MBL WT and MBL KO mice effectively. Our results are consistent with a recent in vitro study by Job et al [18], in which MBL was found to bind to pdmH1N1 fairly in vitro but the virus was resistant to the antiviral activity of MBL. The number and position of potential glycosylation sites on the viral HA globular domain determine the binding affinity between MBL and the virus. Even though MBL can physically bind to the virus, the binding may be insufficient for executing any antiviral function. Arguably, Chang et al [23] recently reported that MBL deficiency increases susceptibility to infection with
influenza A virus Philippine 82 H3N2 (Phil82), which is a human strain. We reconcile with the suggestion that MBL effects would differ depending on strains of influenza A virus and thus MBL causes variable antiviral activities and host responses. The degree of glycosylation on the globular head of the HA molecule is believed to be essential for MBL to exhibit its antiviral properties. For Phil82 virus, the high-mannose oligosaccharide at residue 165 of the HA molecule has already been shown to be crucial for the neutralization by MBL [31]. Although pdmH1N1 virus contains a single potential glycosylation site at the base of the HA globular head (Asn104), it lacks potential glycosylation sites on the globular head region of HA (Asn142, Asn144, Asn172, Asn177, and Asn179) [18]. To our knowledge, binding of MBL on H9N2/G1 virus is not well documented in the literature. Therefore, we analyzed the potential glycosylation sites on HA of H9N2/G1 virus based on an in silico approach as suggested by Job et al [18]. The HA sequence data was retrieved from GenBank (AAF00706.1) and we used NetNGlyc 1.0 server to predict the number of potential glycosylation sites. We found that there was no potential glycosylation sites near residue 165 of the HA molecule of H9N2/G1 virus. We

**Figure 4.** Production of the proinflammatory cytokines and chemokines in the lung homogenates from pdmH1N1 virus infected mannose-binding lectin (MBL) wild-type (WT) and MBL knockout (KO) mice. A, Cytokine profile of the lung homogenates extracted from pdmH1N1 virus–infected MBL WT and MBL KO mice. On day 7, significantly higher amounts of interleukin (IL) 1α, IL-1β, IL-6, IL-10, tumor necrosis factor (TNF) α, and interferon (IFN) γ were found in infected MBL WT mice than in MBL KO mice. On day 14, significantly higher amounts of IL-1α, IL-1β, IL-10, and IFN-γ were found in infected MBL WT mice than in MBL KO mice. B, Chemokine profile of the lung homogenates extracted from pdmH1N1-infected MBL WT and MBL KO mice. On day 7, significantly higher amounts of keratinocyte chemoattractant (KC), monocyte chemotactic protein (MCP)-1, and MCP-3 were found in infected MBL WT mice than in MBL KO mice. On day 14, significantly higher amount of macrophage inflammatory proteins (MIP)-1α and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) were found in infected MBL WT mice than in MBL KO mice. The concentrations of cytokines shown are mean ± standard error of the mean of 5 mice per group and 2 mice per mock treatment group. *P < .05, **P < .01.
speculate that as a result of the absence of potential glycosylation sites near the receptor-binding domain of the HA globular head of both pdmH1N1 virus and H9N2/G1 virus, MBL fails to interfere with the viral binding to target cells despite its ability to bind the virus. This can adequately explain the discrepancy between the present in vivo data and Chang’s study [23].

In this study, MBL WT mice were found to have a more severe disease in terms of greater weight loss and worse lung pathology than MBL KO mice during either pdmH1N1 or H9N2/G1 virus infection. This suggests that MBL may contribute to the disease severity seen in the MBL WT mice. To elucidate the mechanisms, we investigated the immune response, such as production of cytokines and chemokines at various time points during the infection. Most cytokines and chemokines were detected with similar kinetics, with the peak on day 7 following influenza virus infection in both MBL WT and MBL KO mice. These data suggest that the most critical phase of influenza infection occurs around day 7 after infection, and this is
consistent with the result showing the peak of body weight loss around day 7.

Interestingly, we found that MBL contributed to a more severe proinflammatory response by increasing the production of several proinflammatory cytokines, such as IL-1α, IL-1β, IL-6, TNF-α, and IFN-γ. Interleukin 1α and IL-1β are multifunctional proinflammatory cytokines produced readily by influenza-infected leukocytes. They are capable of inducing fever, anorexia, and weight loss [32]. Enhanced production of these cytokines can contribute to the acute lung immunopathology after influenza virus infection in mice [33] and induce gene expression of other cytokines like IL-6 and TNF-α [34, 35]. Despite the abundance of IL-6 following the influenza virus infection, in vivo studies showed that it does not contribute significantly to the pathogenesis of influenza virus infection because the mortality and morbidity observed in mice infected with H5N1 are comparable in both MBL WT and IL-6 deficient mice [36, 37]. Tumor necrosis factor α is readily produced by influenza virus-infected leukocytes and can activate macrophages, stimulate dendritic cell maturation and neutrophils, further enhance the inflammatory response, and activate efficient antigen presentation system in the infected site [20, 38]. Excessive production of TNF-α causes tissue injury, hemorrhagic shock, and death in mice [39, 40]. Interferon γ is also an important proinflammatory cytokine that has different functions, including the activation of macrophages, differentiation of Th1 from T cells, enhancement of antigen presentation, and expression of the chemokine gene [41, 42]. These proinflammatory cytokines are commonly found in the acute-phase response to influenza virus infection and may induce immunity but also cause damage to the host tissue [43]. These cytokines were also increased in our infected murine lung, with significantly higher levels in MBL WT mice than in MBL KO mice on day 7, coinciding with body weight loss and lung histological findings. Mock-treated mice did not show any lung damage. The scores shown are mean ± standard error of the mean of 4 mice per group. *P < .05, **P < .01. Scale bar: 100 μm.

Figure 6. Histological analysis of pulmonary tissues from infected and mock-treated mannos-binding lectin (MBL) wild-type (WT) and MBL knockout (KO) mice. A, Representative pictures (200×) of hematoxylin and eosin–stained pulmonary tissues from mice sacrificed on day 7 after infection. B, Inflammation score of pulmonary tissues from virus-infected MBL WT and MBL KO mice sacrificed on day 7 after infection. C, Cell infiltration score of pulmonary tissues from virus-infected MBL WT and MBL KO mice sacrificed on day 7 after infection. PdmH1N1 and H9N2/G1 virus–infected MBL WT mice were found to have more severe pathological lesions, including more severe lung inflammation and cell infiltration. Mock-treated mice did not show any lung damage. The scores shown are mean ± standard error of the mean of 4 mice per group. *P < .05, **P < .01. Scale bar: 100 μm.
disease course than in the MBL KO mice, including greater body weight loss and more severe lung inflammation.

In addition, we also found that most chemokines, including KC, MIP-1α, MIP-1β, MCP-1, and MCP-3, were elevated in MBL WT mice compared with the MBL KO mice in both pdmH1N1 and G1/97 virus infection. Influenza virus–infected macrophages produced large amounts of these chemokines in vitro [25, 47, 48]. Functionally, these chemokines are important mediators for immune cell activation and chemotactic factors, which recruit leukocytes to the infected sites [49]. This may help account for our histological observation that more inflammatory cell infiltration was observed in MBL WT mice than in MBL KO mice.

The role of MBL in modulating immune responses has also been observed in Staphylococcus aureus infection. It was shown that MBL amplifies the host immune response during S. aureus infection by cooperating with Toll-like receptors 2 and 6 and augments the production of proinflammatory cytokines and chemokines [50]. The observation from our present study prompted us to further investigate whether MBL may also cooperate with other pattern-recognition receptors and thus further amplify the host response during influenza virus infection.

In conclusion, we have shown for the first time that MBL is a risk factor leading to a more severe pdmH1N1 and H9N2/G1 virus infection by upregulating proinflammatory responses.

Notes

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