Mannose Receptor Ligands Regulate the Gene Expression of Toll-like Receptors in Chicken Monocytes

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Pattern recognition receptors play an important role in distinguishing foreign organisms and triggering host defense responses. In this study, we present evidence for differential immune responses in chicken monocytes exposed to the mannose receptor (MR) ligands mannan and horseradish peroxidase (HRP). In our study, mannan significantly up-regulated the expression of the MR gene at a high concentration of 200 μg/ml, but HRP did not. In stimulation experiments, neither mannan nor HRP induced nitric oxide (NO) production or stimulated cytokine (IL-1β, IL-6, and IL-10) gene expression. A concentration of 200 μg/ml mannan induced a significant increase in Toll-like receptor 2 (TLR2) gene expression. The gene expression of TLR1 and 3 was significantly up-regulated by different HRP concentrations. In contrast to TLR1 and 3, TLR4 exhibited a significant decrease in gene expression in a dose-dependent manner. These results may suggest that MR ligands affect the innate immune response of TLRs in chicken monocytes.

Key words: chicken, mannose receptor, monocytes, Toll-like receptor

Introduction

Phagocytosis is an actin-mediated process that involves interactions with foreign material. Macrophages are specialized phagocytes that play an important role in clearance of microorganism. Macrophages express pattern recognition molecules to recognize foreign ligands during early of the immune response. Microorganisms typically express repeating patterns of molecular structures on their surface. These structures are generally known as pathogen-associated molecular patterns (PAMPs), and the receptors that recognize them are pattern recognition receptors (PRRs) (Murphy K et al., 2008). Many receptors have been implicated in pattern recognition, including the mannose receptor (MR), NOD-like receptors, Dectin-1, DC-SIGN and Toll-like receptors (TLRs) (Dostert et al., 2008; Ip et al., 2009; Reid et al., 2009 Jungi et al., 2011; Kumagai et al., 2008; Mathews et al., 2008; Meylan et al., 2006).

The mannose receptor (MR) is a cell-bound C-type lectin that recognizes a wide range of both endogenous and exogenous ligands found on the surface of many bacteria and viruses (Gazi and Martinez-Pomares, 2009; Miller et al., 2008). MR is considered a ‘non-canonical’ PRR that mediates physiological clearance in homeostatic processes (Le Cabec et al., 2005; Lee et al., 2002; Su et al., 2005) and acts as a professional phagocytic receptor in antigen processing and presentation (Burgdorf et al., 2006, 2007, 2008; Segura et al., 2009). Although lack of signaling motifs in the cytoplasmic tail (Jordens et al., 1999), MR remains to be a solo receptor that directly leads to cytokine production (Rizzetto et al., 2010; Van de Veerdonk et al., 2009). In some cases, this pathway appears to cooperate with other receptors to trigger immune cell activation or amplification by other PPR signals (Van de Veerdonk et al., 2009). The best studied candidates for cooperation with MR are the Toll-like receptors (TLRs), which are a family of evolutionarily conserved PRRs that play a critical role in initiating innate immune responses and activating antigen-specific adaptive immunity. Ten TLR family members have been identified in chickens, and each recognizes and responds to different microbial components (He et al., 2006a; 2006b; Jungi et al., 2011).

To date, most MR research has focused on mammals, mainly mice and humans. Although chicken MR was identified on macrophages in the early 1980s (Rossi and Himmele, 1985a, 1985b), the function of MR in chickens is not as well understood as that of MR in mice and humans. Nitric oxide (NO) is an important physiological messenger and effector molecule of the immune system. Acemannan, an extract isolated from tropical cactus plant Aloe vera...
Peripheral blood samples were diluted with Ca$^{2+}$- and Mg$^{2+}$-free Hank’s balanced salt solution (1:1, vol/vol), carefully layered onto a Histopaque gradient (Histopaque-1077, Sigma, St Louis, MO, USA) in 15-ml conical centrifuge tubes, and centrifuged at 400 $\times$ g for 30 min. The PBMC layer was collected, washed 3 times in Hank’s solution, and spun at 250 $\times$ g for 10 min. The PBMCs were resuspended in RPMI 1640 medium (HyClone, ThermoScientific, Waltham, MA, USA) supplemented with 10% chicken serum (Gibco BRL, Gaithersburg, MD, USA) and antibiotics (penicillin and streptomycin, HyClone).

**Culture and Stimulation of Monocytes**

Aliquots of 1 ml of PBMC (2 $\times$ 10$^7$ cells/ml) were dispensed into a round-bottom 12-well cell culture plate (Corning, NY, USA) and incubated at 37°C in a 5% CO$_2$ incubator for 3 h. Non-adherent cells were removed by washing twice with fresh culture medium. After washing, the adherent cells were cultured for 2 d. The culture medium was then refreshed, and monocytes were stimulated with the MR ligands mannan from *Saccharomyces cerevisiae* (Sigma) and HRP (Sigma) at different concentrations for 48 h.

**Nitrite Assay**

Aliquots of 200 $\mu$l of PBMC (2 $\times$ 10$^7$ cells/ml) were dispensed into a round-bottom 96-well microtiter plate (Corning). Monocytes were obtained using the adherent method. Monocytes NO production was determined by the Griess Reagent System (Promega, Madison, WI, USA). After stimulation for 48 h, 50 $\mu$l of each experimental sample was added to the wells in triplicate. Then, an equal volume of sulfanilamide solution was dispensed into each of the experimental samples. After a 10 min incubation at room temperature, 50 $\mu$l of NED solution was added to each of the wells. Following another incubation at room temperature for 10 min, the plate was read at 570 nm using an automated ELISA plate reader (Bio-Rad 550, Hercules, CA, USA).

**Real-time PCR Analysis of Gene Expression**

Quantitative real-time PCR amplification was performed in 15 $\mu$l of LightCycler® 480 SYBR Green I Master Mix (Roche, Rotkreuz, Switzerland) with the LightCycler® 480 Real-time PCR System (Roche). The individual primers used are shown in Table 1. The parameters were as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min; one cycle of 95°C for 15 s, 60°C for 15 s and 95°C for 15 s; and 40°C for 30 s. We performed standard curve experiments for all genes examined as previously described (Liu et al., 2010). The expression levels of the genes of interest were calculated relative to the expression of the reference gene GAPDH. Increases or decreases relative to the unstimulated sample were expressed as fold changes.

**Data Analysis**

Statistical analyses were performed with SPSS version 11.5 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA and multiple comparisons were used to test for significant differences between different groups. Differences were regarded as significant at $P<0.05$ and $P<0.01$.

**Results**

**Differential Induction of NO Production in Chicken Monoocytes**

In our study, different concentrations of LPS, mannan and HRP were used to stimulate chicken monocytes for 48 h. Compared with the other groups, NO production was significantly increased in the LPS-stimulated groups ($P<0.01$). This is consistent with a previous study in which LPS-induced NO production and cytokine gene activity were described in chicken macrophages (Dil and Qureshi, 2003). However, the nitrite levels in the mannan and HRP groups were quite low. There were no differences compared to control groups (Fig. 1). Thus, mannan and HRP have little effect on NO production in chicken monocytes.

**MR Ligands Stimulate MR and Cytokine Expression**

To determine if mannan and HRP could stimulate MR gene expression, we stimulated monocytes with mannan and HRP at various concentrations for 48 h and then detected MR gene expression. We found that 200 $\mu$g/ml mannan caused more than a six-fold increase in MR gene expression, which was significantly different from the change induced by other mannan concentrations (Fig. 2A). Although we observed a
Table 1. Primer sequences used in real-time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Direction</th>
<th>Sequence</th>
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<th>Accession no. in Gene Bank</th>
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<td>GAPDH&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Forward</td>
<td>TGCCATCACGACGCAACACGAAAG</td>
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<tr>
<td>IL-1β</td>
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<tr>
<td>IL-6&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> Primers sequence from reference (Heidari <i>et al.</i>, 2008); <sup>b</sup> primer sequence from reference (Liu <i>et al.</i>, 2010).

Fig. 1. Differential induction of NO production in chicken monocytes. Monocytes were stimulated with LPS, mannan and HRP for 48 h at 37°C in a 5% CO₂ and 95% humidity incubator. The nitrite content in the cell culture medium was determined. Each bar represents the mean of the nitrite levels ± SEM in the culture supernatant from samples established from 6–7 chicks per group (n = 6–7). The differences in nitrite levels between experimental treatments and the control were assessed by one-way ANOVA and multiple comparisons. P < 0.05 (*), P < 0.01 (**).
greater than two-fold change in MR gene expression in the 200 μg/ml HRP stimulation group, the multiple comparison results demonstrated that there were no significant differences between the HRP groups and the control group (Fig. 2B). These results demonstrate that, compared with HRP, mannan is a ligand that can induce a high level of MR gene expression in chicken monocytes.

Although we did not detect NO production in cells stimulated with HRP or mannan, we wanted to evaluate whether these two ligands could induce cytokine production by monocytes. Therefore, IL-1β, IL-6, and IL-10 gene expression were measured in chicken monocytes after cellular activation with mannan and HRP. We found that IL-1β gene expression increased by a small amount in the experimental groups as the concentration of the stimulus increased. Statistical analysis revealed that there were no significant differences between the treatment groups (data not shown). The effect of 200 μg/ml mannan was of marginal statistical significance (P=0.065). Conversely, IL-6 gene expression was no significant different and IL-10 gene expression was so low that it could not be detected in either the control groups or the experimental groups (data not shown).

Expression of TLRs in Chick Monocytes

We quantified the relative expression of several cytokine genes at 48 hours post-treatment by real-time RT-PCR. Furthermore, TLR gene expression was measured after activation with MR ligands at different concentrations. Following stimulation of monocytes with mannan, we observed a subtle (1.39-fold) change in TLR2 gene expression in the presence of a high mannan concentration (200 μg/ml). Statistical analysis confirmed the significantly up-regulated gene expression of TLR2 (P=0.024) (Fig. 3C), although we did not detect significant changes in the gene expression of other TLRs (Fig. 3A, E, G, TLR5, 7 data not shown). When stimulated with various concentrations of HRP, the gene expression of TLR1, TLR3 and TLR4 exhibited significant changes. Compared with the unstimulated control, TLR1 gene expression increased 2.56-fold in the 200 μg/ml HRP-stimulated group (P=0.031) (Fig. 3B). We also observed significant increases in TLR3 gene expression (Fig. 3F). As the stimulus concentration increased, the level of TLR3 gene expression increased (each group P<0.01). Moreover, with increasing HRP concentration, TLR4 gene expression was down-regulated (10 μg/ml HRP, 0.732 ± 0.100, P=0.047; 50 μg/ml HRP, 0.518 ± 0.107, P<0.01; 100 μg/ml HRP, 0.431 ± 0.110, P<0.01; 200 μg/ml HRP, 0.365 ± 0.085, P<0.01) (Fig. 3H). There were no significant changes in the gene expression of TLR2, TLR5, or TLR7 expression upon HRP stimulation (Fig. 3D, TLR5, 7 data not shown).

Discussion

There has been a rapid increase in the number of recognized PAMP and PRR interactions that mediate the activation of innate immunity. Several classes of PRRs recognize various PAMPs, of which C-type lectin receptors and TLRs are likely very important (Ip et al., 2009; Jungi et al., 2011). NO is an important pro-inflammatory mediator that is involved in macrophage activities against intracellular pathogenic microorganisms and viral proliferation. Chicken primary monocytes produce large quantities of NO when exposed to bacteria or viruses (Crippen et al., 2003; Guillermo and DaMatta, 2004; Lavric et al., 2007; Xu et al., 2005). The differential induction of individual NO and cytokine production has been observed in a chicken macrophage cell line (HD11) (Karaca et al., 1995) and in monocytes when cells were stimulated by various PRR ligands (He et al., 2006a; 2006b; 2007). However, MR ligands and their
Fig. 3. Changes in TLRs gene expression were quantified by real-time PCR. (A) TLR1 gene expression after stimulation with mannan. (B) TLR1 gene expression after stimulation with HRP. (C) TLR2 gene expression after stimulation with mannan. (D) TLR2 gene expression after stimulation with HRP. (E) TLR3 gene expression after stimulation with mannan. (F) TLR3 gene expression after stimulation with HRP. (G) TLR4 gene expression after stimulation with mannan. (H) TLR4 gene expression after stimulation with HRP. Each bar represents the mean±SEM of three replicate samples established from 6 chicks per group ($n=6$). The differences in TLR gene expression between experimental treatments and the control were assessed by one-way ANOVA and multiple comparisons. $P<0.05$ (*), $P<0.01$ (**).
effect in chicken immune cells are unclear. The only clue is that cultures of normal chicken spleen cells and HD11 cells have been reported to produce NO in response to acemannan, inferring that this effect is mediated by the macrophage mannose receptor (Karaca et al., 1995). To determine if MR ligands can induce NO production in chicken monocytes, we stimulated chicken monocytes with mannan, HRP and LPS (as a control) at different concentrations. The results showed that mannan and HRP failed to induce NO in chicken monocytes. Thus, it appears that these two ligands have no stimulatory effect on the NO functional response in chicken monocytes. *S. cerevisiae* mannan and acemannan are composed of polymannoses in similar structures, but the responses of NO production are different. *S. cerevisiae* yeast had suppressive mechanisms which could avoid potentially harmful inflammatory process. Mannan is the main component of *S. cerevisiae* cell wall mediating interaction between yeast and immune cells (Rizzetto et al., 2010). Acemannan, 1-(1,4)-linked acetylated mannan, has diverse immunomodulatory properties. Acemannan has acetylated terminal mannose residues (Karaca et al., 1995). Receptor specificity for acetylated mannose activated macrophage cytotoxicity against tumor cells (Zhu et al., 1993). Thus, the source of the polymannoses extracts and their chemical structures may be the important reasons for the different NO production.

To determine if mannan and HRP are strong ligands for MR production, we examined MR gene expression by quantitative RT-PCR. In chicken monocytes, we observed a dose-dependent up-regulation of MR at a high concentration of mannan (200 μg/ml) but no response to HRP. Thus, compared to HRP, mannan could be a more potent ligand for MR gene expression. These results suggested that mannan could also be a general ligand for MR functional studies in both mammal and chicken (Rizzetto et al., 2010). Although HRP was inferred to be a competitive ligand for MR in chickens, its effect on MR gene expression was not obvious in our study. It is unclear why mannan up-regulated MR gene expression but HRP did not in this study. In human PBMCs, *Candida albicans* mannan induces strong IL-17 production, whereas *S. cerevisiae* mannan does not, suggesting that MR could be differentially engaged by *C. albicans* mannan and *S. cerevisiae* mannan (Van de Veerendonk et al., 2009). While *S. cerevisiae* mannan mainly contains short linear chains of mannose polymers, a large portion of HRP glycoproteins carry eight trimannosyl N-glycan structures (Wührer et al., 2005). Based on current research, this differential recognition and stimulation by *S. cerevisiae* mannan and HRP is most likely related to the different branching of their structures.

When stimulated with external materials, macrophages secrete an array of cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6). MR has been shown to be involved in the production of both pro-inflammatory and anti-inflammatory cytokines. It has been implicated in the stimulation of cytokine production by fungal mannan in chicken and mammals (Karaca et al., 1995; Rizzetto et al., 2010). In our study, neither mannan nor HRP induced significant changes in cytokine gene expression in chicken monocytes. Previous studies suggested that freshly isolated monocytes are negative for MR, but in vitro monocyte derived macrophages are strongly positive. MR levels are up-regulated by anti-inflammatory molecule IL-10, but down-regulated by pro-inflammatory stimulus LPS (Allavena et al., 2004; Gordon, 2003). In our study, MR expression was significantly increased in high mannan concentration, but cytokine expression including IL-10 was not detected. In human PBMCs, MR can directly lead to cytokine production, and this pathway could be amplified by dectin-1/TLR2 signaling (Van de Veerendonk et al., 2009). In addition, some studies have suggested that MR requires the assistance of other receptors to trigger signaling cascades (Gazi and Martinez-Pomares, 2009; Xaplanteri et al., 2009). When binding with a pathogen such as *Pneumocystis*, MR alone was not sufficient to promote cytokine release but required co-expression of TLR. MR may form a functional complex with TLR on the cell surface and facilitate signal transduction (Tachado et al., 2007). Besides, a single chicken TLR member alone may have weak ability to induce immune response. The chicken TLR1 and TLR2 recognize bacterial lipoproteins and peptidoglycan in distinct combinations. Either type of chicken TLR2 alone exhibits minimal ability of NF-κB activation (Hiquet et al., 2008). The lack of significant cytokine production in response to MR ligands in chicken monocytes may therefore be due to the weak responses of the signal pathway. Combination of MR and other TLRs stimulation may effectively initiate and strengthen the immune responses to pathogens in chicken monocytes.

This is the first report to investigate the different immune responses resulting from stimulation with MR ligands in chicken monocytes. Our data suggest that MR ligands play a role in the TLR expression and may affect the innate immune responses in chicken monocytes.

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