β-1,4-Mannobiose Stimulates Innate Immune Responses and Induces TLR4-Dependent Activation of Mouse Macrophages but Reduces Severity of Inflammation during Endotoxemia in Mice¹–³

Jennifer Kovacs-Nolan,⁴ Hiroyuki Kanatani,⁵ Akihiro Nakamura,⁵ Masahisa Ibuki,⁶ and Yoshinori Mine⁴*

¹Department of Food Science, University of Guelph, Guelph, Canada; ²Tsukuba R&D Centre, Fuji Oil Co. Ltd., Ibaraki Prefecture, Japan; and ³Soy Protein Processed Food Overseas Sales Department, Fuji Oil Co. Ltd., Tokyo, Japan

Abstract

β-1,4-Mannobiose (MNB) has been shown to exert prebiotic activity and modulate mucosal gene expression. In this study, the immune-modulating effect of MNB in healthy and endotoxemic mice and its role in Toll-like receptor (TLR) 2/4-mediated macrophage activation were investigated. Mice were supplemented daily with MNB (0, 5, 10, or 25 mg/kg) for 14 d. To examine the effect of MNB during endotoxemia, mice were supplemented with or without MNB (25 mg/kg) for 14 d, followed by challenge with intraperitoneal LPS or saline. MNB induced expression of both T helper (Th) 1- and Th2-type cytokines in the ileum (P < 0.05) and increased fecal IgA production and splenic NK cell activity (P < 0.05) in healthy mice. In endotoxemic mice, MNB reduced the expression of Tnfa, Il-6, iNos (P < 0.05), and Il-10 (P < 0.05), and reduced LPS-induced weight loss but increased Ifng, Il-12p40, Il-5, and Ifna expression (P < 0.05) and NK cell activity relative to positive control (LPS) mice. Treatment of RAW 264.7 macrophages with MNB induced TNF-α and IL-6 secretion (P < 0.05), and this effect was abrogated by inhibiting TLR4, but not TLR2, signaling. Pretreatment of RAW 264.7 cells with MNB induced tolerance to TLR2 and TLR4 agonists, reducing TNF-α production (P < 0.05) upon secondary stimulation with LPS or lipoteichoic acid. These results indicate that MNB can modulate intestinal and systemic immune responses in healthy and endotoxemic mice and prevent LPS-induced immune suppression, as well as directly stimulating innate immune mechanisms in vitro as a TLR4 agonist. J. Nutr. doi: 10.3945/jn.112.167866.

Introduction

The mucosal surface of the intestine plays a key role in control of the host immune response, both by acting as a barrier against microorganisms and food antigens and as the location of the gut-associated lymphoid tissues, the largest collection of lymphoid tissues in the body (1). There is increasing evidence that dietary components, including prebiotics, can modulate host immune functions and prevent disease (2,3).

Prebiotics are nondigestible oligosaccharides. Fermentation of prebiotics by bacteria in the colon produces SCFAs and decreases the pH, which favors the growth of commensal bacteria including Bifidobacteria and Lactobacilli, which in turn modulate intestinal and systemic immune responses (1,4).

Oligosaccharides such as inulin (IN)7 and fructooligosaccharides (FOS) have well-established prebiotic properties and have been shown in a number of human studies to alter the composition of intestinal microflora by increasing fecal Bifidobacteria levels (3). More recently, galactooligosaccharides (GOS) have also been described with strong clinical support for promoting digestive and immune health (5).

β-1,4-Mannobiose (MNB), composed of D-mannose units joined by a β-1,4 linkage, is a nondigestible disaccharide obtained from food materials, including coconut flour (6) and has shown promise as a potential prebiotic and immune-modulator. In vitro, mannooligosaccharides, including MNB, are fermented by Bifidobacterium adolescentis, Lactobacillus acidophilus, and Lactobacillus gasseri to produce SCFAs, including acetic, propionic, and butyric acids (7). In vivo, a mixture of

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mannooligosaccharides containing mainly MNB and manno-
triose was found to significantly increase fecal *Bifidobacteria*
levels in human subjects (8). Moreover, we have shown in
previous studies that dietary supplementation with MNB could
modulate local intestinal gene expression and upregulate genes
involved in host defense and immunity, increase IgA produc-
tion, and prevent *Salmonella* Enteritidis infection in chickens (9,10).

In addition to their effect on commensal bacteria in the
intestine, there is increasing evidence that prebiotic carbohydrates
may also exert direct effects on the immune system, by interact-
ing with receptors on immune cells (11). We have recently shown
that MNB can increase the phagocytic activity and *Salmonella*
-killing activity of chicken macrophages in vitro, as well as in-
creasing hydrogen peroxide and NO production, and expression
of genes critical for host defense and antimicrobial activity (12).
However, the mechanism by which MNB exerts this activity is
not known.

The recognition of pathogen-associated molecular patterns
by Toll-like receptors (TLRs) initiates signaling pathways that
stimulate host defenses through the induction of reactive
oxygen and nitrogen intermediates and proinflammatory cyto-
kines necessary for the development of effective immunity.
Nine conserved TLRs have been identified in both humans and
mice, including TLR2, which is involved in the recognition of
peptidoglycan and lipoteichoic acid (LTA) from Gram-positive
bacteria, and TLR4, which recognizes bacterial LPS (13). There
is increasing evidence that LPS is not the only ligand for TLR4,
and endogenous molecules such as hyaluronic acid, heparin
sulfate, and fibrinogen have also been shown to signal through
the TLR4 complex (14). The branched carbohydrate struc-
ture of MNB would suggest potential similarity to bacte-
rial cell components and may act as a TLR agonist. Indeed,
other naturally occurring polysaccharides have been shown to
stimulate innate immunity via TLR2 and TLR4, including
β-D-mannuronate oligomers, which were found to in-
duce TLR2/4-mediated production of inflammatory cytokines
TNF-α and IL-6 by mouse macrophage cells (15,16). More
recently, Jiang et al. (17) have described the synthesis of β-1,4-D-
mannobiose derivatives as potential TLR2/4 ligands for vaccine
adjuvants.

In the present study we evaluated the immune-modulating
effects of MNB in both healthy mice and in an LPS-induced
model of systemic inflammation (endotoxemia) and examined
the role of TLR2/4 in the immune-stimulating activity of MNB.

**Materials and Methods**

**Reagents**

MNB (99% pure) was prepared from coconut flour as previously
described (12). The structure of pure MNB was confirmed by using
13C-NMR and 1H-NMR (Supplemental Figs. 1–4). Endotoxin levels
were <1 endotoxin U/mg as measured by the ToxinSensor Chromogenic
LAL Endotoxin Assay Kit (GenScript). LPS from *Escherichia coli* O111:
B4 and LTA from *Bacillus subtilis* were purchased from Sigma-Aldrich
Co. Monoclonal anti-mouse TLR2 antibody (T2.5) and the TLR4
inhibitor CL-095 (18) were purchased from InvivoGen and were
prepared and used according to the manufacturer’s instructions.
Recombinant mouse TNF-α and IL-6, anti-mouse/rat TNF-α (TN3-
19.12), biotin-conjugated anti-mouse/rat TNF-α, anti-mouse IL-6
(MP5-20F3), biotin-conjugated anti-mouse IL-6, purified mouse IgA
(M18-254), anti-mouse IgA (C10-3), biotin-conjugated anti-mouse IgA
(C10-1), and avidin-conjugated HRP were purchased from BD Biosci-
ences. Unless otherwise specified, all cell culture reagents were purchased from Life Technologies.

**Animals and experimental design**

Six- to 8-wk-old female BALB/c mice (n = 6/group) (Charles River),
weighing 20–25 g, were housed 6 per cage in a temperature-controlled
environment (23–25°C) on a 12-h light-dark cycle. Mice were fed a
nonpurified diet (2014 Teklad Global Diet; Harlan Teklad) and provided
water ad libitum. All animal experiments were approved by the University of
Guelph Animal Care Committee and carried out in accordance with
the Canadian Council on Animal Care Guide to the Care and Use of
Experimental Animals.

**Expt. 1.** To study the immune-modulating effects of MNB in healthy
mice and determine optimal MNB dose, animals were randomly divided
into groups and given MNB (5, 10, or 25 mg/kg body weight per mouse
in 0.2 mL water) or vehicle (water) daily for 14 d by oral gavage. Fecal
samples were collected on d 0, 7, and 14. On d 14, mice were killed by
CO2 asphyxiation, and the spleen and sections of the ileum (~2 cm) were
collected from each mouse to measure NK cell activity and gene
expression, respectively.

**Expt. 2.** To compare the effect of MNB administration in healthy and
endotoxemic mice, animals were given MNB (25 mg/kg in 0.2 mL water)
or vehicle (water) daily for 14 d. Body weights were recorded on d 0, 5,
10, and 14. On d 14, mice were injected intraperitoneally with LPS (1.25
mg/kg in 0.1 mL sterile saline) to induce endotoxemia. Control animals
received intraperitoneal saline only. After 24 h, mice were weighed and
then killed by CO2 asphyxiation, and the spleen and sections of the ileum
(~2 cm) were collected from each mouse to measure NK cell activity and
gene expression, respectively.

**Isolation of splenocytes**

Splenocytes were aseptically removed from all mice and placed into cold
RPMI (Roswell Park Memorial Institute) 1640 medium containing
0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 10
mmol/L HEPES, 10 U/mL penicillin-streptomycin, and 50 mmol/L 2-
mercaptoethanol (Sigma-Aldrich). Splenocytes were passed through a 100
μm nylon mesh screen (Thermo Fisher) to obtain a single cell suspension,
and erythrocytes were lysed using RBC Lysis Buffer (Sigma-Aldrich).
Cells were washed twice and re-suspended in the above medium
containing 10% FBS at the desired concentration.

**Splenic NK cell cytotoxicity assay.** YAC-1 mouse tumor cells
(RIKEN) were grown in RPMI 1640 medium containing 10 mmol/L
HEPES, 1 mmol/L sodium pyruvate, and 10% FBS (HyClone). NK cell
cytotoxic activity of splenocytes against the NK-sensitive YAC-1 cells
was measured by fluorescent concentration release assay as described
elsewhere (19) by using an effector (splenocyte) to target (YAC-1) cell
country of 50:1. Results are expressed as percentage lysis of YAC-1 cells
and were calculated as follows: (Fmed − Fexp)/Fmed × 100, where
F represents the fluorescence of the solubilized cells after the supernatant
has been removed, Fmed is fluorescence from target incubated with
medium alone, and Fexp is fluorescence from target cells incubated with
effector cells.

**Measurement of fecal IgA**

Pooled samples of fecal pellets were collected from each individual
cage and resuspended in PBS according to Tress et al. (20). Total IgA was
measured by ELISA according to the manufacturer’s instructions (BD
Biosciences), and results are expressed as micrograms of IgA per milligram
of fecal dry matter.

**Treatment of RAW 264.7 cells and splenocytes with TLR
agonists and inhibitors**

RAW 264.7 mouse macrophage cells (American Type Culture
Collection) grown in DMEM containing 1 mmol/L sodium pyruvate, 10 U/mL
penicillin-streptomycin, and 10% FBS were seeded into 48-well tissue
culture plates (Corning) at a density of 5 × 105 cells/well and allowed to
adhere overnight before treatment.

**Expt. 3.** To examine dose-response in vitro, RAW 264.7 cells were
treated with MNB (at indicated concentrations) for 6 h (TNF-α) or 24 h


(IL-6), and cytokine concentrations in culture supernatants were measured by ELISA.

**Expt. 4.** For TLR2/4 inhibition, RAW 264.7 cells were pretreated with anti-TLR2 antibody (100 µg/L) for 0.5 h or CLI-095 (1 mg/L) for 6 h before addition of MNB (50 mg/L), LTA (1 mg/L), or LPS (10 µg/L). To measure TL4 inhibition in splenocytes, cells were isolated from the spleens of 6 untreated mice as described above. Splenocytes (5 × 10⁵ cells/well) were pretreated for 6 h with CLI-095 followed by stimulation with MNB (50 mg/L) or LPS (10 µg/L) for 24 h, and TNF-α concentrations in culture supernatants were measured by ELISA.

**Expt. 5.** To test for MyD88-dependent and -independent gene expression, RAW 264.7 cells were treated with MNB (50 mg/L) or LPS (10 µg/L) for 6 h, and relative mRNA expression was measured by real-time RT-PCR.

**Expt. 6.** To measure endotoxin tolerance, RAW 264.7 cells were pretreated with MNB (50 mg/L), LPS (10 µg/L), or LTA (1 mg/L) for 24 h, and then washed twice and reincubated (challenged) with LPS or LTA for an additional 6 h. TNF-α concentrations in culture supernatants were measured by ELISA.

**TNF-α and IL-6 ELISA**

TNF-α and IL-6 concentrations in cell culture supernatants were measured by ELISA according to the manufacturer’s instructions (BD Biosciences).

**RNA isolation and measurement of gene expression by real-time RT-PCR**

Total RNA was extracted from ileum tissue or RAW 264.7 cells by using the Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer’s instructions. The quantity and quality of the RNA were verified by spectrophotometry (NanoDrop 8000; Thermo Fisher) and gel electrophoresis. cDNA was synthesized from 1 µg RNA by using a qScript cDNA Synthesis Kit (Quanta Biosciences), and real-time PCR was carried out as previously described (12) using the primers listed in Supplemental Table 1. Relative gene expression was calculated by using the 2⁻ΔΔCt method (21) using Gapdh as the reference gene.

**Statistical analysis.** Values are expressed as mean ± SEM. Data analysis was carried out by using GraphPad Prism (version 5.0; GraphPad Software, Inc.). When variances were not homogeneous, the data were log-transformed before analysis. One-way ANOVA followed by Dunnett’s post hoc test was used for comparisons of MNB treatment with a control group (gene expression and NK cell activity in Expt. 1, Expt. 3, Expt. 5, Expt. 6). A linear trend post test was used to compare cytokine secretion to MNB dose in vitro (Expt. 3). Two-way ANOVA was used to determine the effects of MNB supplementation (vehicle vs. MNB) in healthy and endotoxemic mice (Expt. 2), and Tukey’s post hoc test was performed when a significant effect was observed (gene expression, NK cell activity). Two-way repeated-measures ANOVA was used for body weights from d 0 to d 14, before LPS administration. To examine the effect of MNB on weight loss after LPS administration, changes in body weight over 24 h were analyzed by 2-way ANOVA, followed by post hoc testing to detect changes in group means, which differed significantly from a hypothetical mean of zero. In Expt. 4, the effect of TLR2/4 inhibitors on MNB- vs. LPS- or LTA-treated cells was examined by using 2-way ANOVA, and differences between cells treated with or without inhibitor were analyzed by post hoc t test by using the difference between means divided by the corresponding SE of difference. Differences were considered significant when P < 0.05.

**Results**

**MNB modulates local and systemic immune responses in healthy mice (Expt. 1).** MNB supplementation increased the d 14 expression of all cytokine genes measured (Table 1). Expression of Tnfa, Ifng, Il-10, and Il-4 was significantly higher (P < 0.05) in mice that received 25 mg/kg MNB when compared with the control (0 mg/kg) group. Total fecal IgA increased in all groups, but on d 14 was highest in mice supplemented with 25 mg/kg MNB when compared with control mice or mice treated with 5 or 10 mg/kg MNB (Table 1). MNB treatment also increased splenic NK cell activity, and mice given 10 or 25 mg/kg MNB had significantly higher NK cell activity on d 14 than control mice (P < 0.05) (Table 1). On the basis of these preliminary experiments, a dose of 25 mg/kg was chosen for further in vivo studies on endotoxemic mice.

**MNB reduces severity of inflammation and modulates immune responses in endotoxemic mice (Expt. 2).** Positive control mice (LPS) displayed significant weight loss (P < 0.05) after induction of endotoxemia (24 h postchallenge) (Table 2). Although

**TABLE 1** Effect of MNB dose on ileum cytokine gene expression, fecal total IgA, and splenic NK cell activity in healthy mice supplemented with 0, 5, 10, or 25 mg/kg MNB for 14 d (Expt. 1)¹

<table>
<thead>
<tr>
<th></th>
<th>MNB, mg/kg</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ileum gene expression², fold of 0 mg/kg</td>
<td></td>
</tr>
<tr>
<td>Tnfa</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Il-6</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Ifng</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Il-12p40</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Il-10</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Il-4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Fecal total IgA³, µg/mg</td>
<td></td>
</tr>
<tr>
<td>d 0</td>
<td>0.1</td>
</tr>
<tr>
<td>d 7</td>
<td>3.2</td>
</tr>
<tr>
<td>d 14</td>
<td>16.7</td>
</tr>
<tr>
<td>Splenic NK cell activity, % target cell lysis</td>
<td>18.3 ± 6.7</td>
</tr>
</tbody>
</table>

¹ Values are mean ± SEM unless otherwise indicated, n = 6 mice/group. Data were log transformed before analysis, but untransformed values are shown. *Different from control mice receiving 0 mg/kg MNB, P < 0.05 (1-way ANOVA and Dunnett’s post test). MNB, β-1,4mannobiose.

² Gene expression is reported as fold change relative to control mice given 0 mg/kg MNB, calculated by using the 2⁻ΔΔCt method. Values were normalized by using Gapdh as reference gene.

³ Values represent total IgA concentration of a single pooled fecal sample from each cage (6 mice) on d 0, 7, and 14; therefore, no statistical analysis was performed.
TABLE 2  Body weights of mice supplemented with 25 mg/kg MNB or vehicle orally for 14 d followed by challenge with intraperitoneal saline or LPS (Expt. 2) \(^1\)

<table>
<thead>
<tr>
<th>d 0</th>
<th>d 5</th>
<th>d 10</th>
<th>d 14</th>
<th>24-h Postchallenge</th>
<th>P value (2-way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MNB</td>
</tr>
<tr>
<td>Control</td>
<td>18.4 ± 0.2</td>
<td>19.1 ± 0.3</td>
<td>19.2 ± 0.3</td>
<td>19.5 ± 0.2</td>
<td>19.9 ± 0.3</td>
</tr>
<tr>
<td>MNB</td>
<td>18.2 ± 0.3</td>
<td>18.5 ± 0.3</td>
<td>18.4 ± 0.4</td>
<td>18.8 ± 0.3</td>
<td>19.0 ± 0.3</td>
</tr>
<tr>
<td>LPS</td>
<td>17.8 ± 0.4</td>
<td>18.2 ± 0.2</td>
<td>18.5 ± 0.3</td>
<td>18.9 ± 0.4</td>
<td>18.7 ± 0.3*</td>
</tr>
<tr>
<td>MNB+LPS</td>
<td>18.4 ± 0.2</td>
<td>19.1 ± 0.4</td>
<td>19.2 ± 0.4</td>
<td>19.3 ± 0.2</td>
<td>18.1 ± 0.4*</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 6 mice/group. Control, vehicle, and saline; MNB, 25 mg/kg MNB and saline; LPS, vehicle and challenge with 1.25 mg/kg LPS; MNB+LPS, 25 mg/kg MNB and challenge with 1.25 mg/kg LPS. *Significant change in body weight 24 h after LPS challenge, \(P < 0.05\) (post hoc test as described in Materials and Methods). Data were log transformed before analysis, but untransformed values are shown. MNB, \(\beta\)-1,4-mannobiose.

weight loss was also observed in mice treated with MNB+LPS (\(P < 0.05\)), an overall effect of MNB treatment (\(P = 0.048\)) was detected by 2-way ANOVA. As expected, no weight loss was observed in control mice (control) or mice given MNB alone (MNB). No effect of MNB supplementation on body weight was observed up to d 14, before LPS challenge (2-way ANOVA, \(P = 0.85\)).

Similar to Expt. 1, when compared with control mice MNB increased the gene expression of Tnfa (\(P < 0.05\)) (Fig. 1A), Il-6 (\(P < 0.05\)) (Fig. 1B), Ifng (\(P < 0.05\)) (Fig. 1C), Ifna (\(P < 0.05\)) (Fig. 1D), Il-12p40 (\(P < 0.05\)) (Fig. 1E), Ifna (\(P < 0.05\)) (Fig. 1F), and Il-10 (\(P < 0.05\)) (Fig. 1G) in the ileum of healthy mice not treated with LPS. In contrast, treatment with MNB+LPS reduced the LPS-induced expression of Tnfa, Il-6, Il-10 (\(P < 0.05\), and iNos (inducible NO synthase) (\(P < 0.05\)) (Fig. 1H) when compared with positive control LPS mice 24 h postchallenge, whereas expression of Ifng, Il-12p40, Il-5, and Ifna (\(P < 0.05\)) was elevated relative to the LPS group.

Splenic NK cell activity was greater in healthy mice treated with MNB (47.8 ± 4.7%) when compared with control mice (26.0 ± 3.5%). Similarly, NK cell activity was increased in endotoxemic mice treated with MNB (30.5 ± 2.9%) when compared with the positive control LPS group (13.7 ± 5.4%) 24 h after challenge with LPS.

**MNB dose-dependently increases TNF-\(\alpha\) and IL-6 production in vitro in mouse macrophage cells (Expt. 3).** MNB at doses of 50, 100, and 500 mg/L significantly increased secretion of TNF-\(\alpha\) (\(P < 0.05\)) and IL-6 (\(P < 0.05\)) in RAW 264.7 cells when compared with untreated cells (0 mg/L) (Fig. 2), and this effect was dose dependent (\(P\)-trend < 0.0001) for both cytokines. On the basis of these results, a dose of 50 mg/L, the lowest dose that resulted in a significant increase in cytokine secretion, was chosen for further in vitro studies.

**MNB activates immune cells in vitro via MyD88-dependent TLR4 signaling (Expt. 4 and Expt. 5).** Pretreatment of RAW 264.7 cells with the TLR4 inhibitor CLI-095 significantly reduced MNB-induced TNF-\(\alpha\) secretion (\(P < 0.05\)),...
as well as that of the TLR4 agonist LPS (P < 0.05), when compared with control cells not exposed to inhibitor (Table 3). Pretreatment with anti-TLR2 antibody did not affect TNF-α secretion by MNB but significantly reduced LTA-induced TNF-α secretion (P < 0.05) when compared with the no-inhibitor control, suggesting that the activation of RAW 264.7 cells by MNB occurred through TLR4 but not TLR2. Likewise, CLI-095 pretreatment reduced both MNB- and LPS-induced TNF-α secretion (P < 0.05) from mouse splenocytes when compared with control (Supplemental Table 2).

Expression of the MyD88-dependent cytokines II-6 and II-1b was upregulated (P < 0.05) in RAW 264.7 cells treated with either MNB or LPS when compared with untreated control cells (Table 4). However, only LPS upregulated expression (P < 0.05) of the MyD88-independent cytokines Ip-10 (IFN-γ-inducible protein), Rantes (regulated upon activation normal T cell expressed and secreted), and Ifnbs, suggesting that in contrast to LPS, MNB may signal exclusively through a MyD88-dependent pathway.

MNB induces endotoxin tolerance in vitro in mouse macrophages (Expt. 6). Pretreatment of RAW 264.7 cells with MNB significantly reduced TNF-α secretion upon restimulation (challenge) with LPS (P < 0.05), resulting in an ~50% reduction in TNF-α production when compared with untreated control cells challenged with LPS (Fig. 3). MNB pretreatment also significantly reduced cytokine secretion in response to challenge with LTA (P < 0.05) when compared with the corresponding control.

**TABLE 3** Effect of TLR2/4 inhibitors on TNF-α production in RAW 264.7 cells treated for 6 h with MNB, LPS, or LTA (Expt. 4) 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>MNB</th>
<th>LPS</th>
<th>LTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1520 ± 138</td>
<td>2330 ± 124</td>
<td>—</td>
</tr>
<tr>
<td>CLI-095</td>
<td>51 ± 22*</td>
<td>124 ± 51*</td>
<td>—</td>
</tr>
<tr>
<td>TLR2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1110 ± 120</td>
<td>787 ± 88</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Anti-TLR2</td>
<td>1090 ± 122</td>
<td>107 ± 15*</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 3, representative of at least 3 independent experiments. Data were log transformed before analysis, but untransformed values are shown. Cells were pretreated with TLR4 inhibitor (CLI-095), TLR2 inhibitor (anti-TLR2), or no inhibitor (control), followed by 50 mg/L MNB, 10 μg/L LPS, or 1 mg/L LTA. *Different from corresponding control, P < 0.05 (post hoc test as described in Materials and Methods). MNB, β-1,4-mannobiase; LTA, lipoteichoic acid; TLR, Toll-like receptor.

**FIGURE 2** TNF-α and IL-6 secretion from RAW 264.7 cells treated with 0, 10, 50, 100, or 500 mg/L MNB for 6 h (TNF-α) or 24 h (IL-6) (Expt. 3). Values are mean ± SEM, n = 3. *Different from 0 mg/L MNB for each cytokine, P < 0.05 (1-way ANOVA and Dunnett’s post hoc test). A linear trend post test was used to compare cytokine concentration to MNB dose. MNB, β-1,4-mannobiase.

**Discussion**

Prebiotics exert beneficial effects on the immune system both indirectly, by promoting the growth of probiotic bacteria and increasing IgA secretion and production of SCFAs, and directly, by competitively inhibiting pathogen adherence and signaling (1). There is increasing evidence that prebiotics may also exert direct immunostimulatory effects, independent of their effects on probiotics. In initial dose-finding experiments, supplementation with 25 mg/kg MNB induced a balanced T helper (Th) 1/Th2 response, increasing the expression of both Th1- (IL-12p40, Ifng, Tnfa) and Th2-type (IL-4, IL-6, IL-10) cytokine genes in healthy mice. These results are consistent with findings that dietary FOS could increase Th1 and Th2 cytokine secretion from restimulated mouse T cells (22). Similarly, an oligofructose (OF)-enriched IN prebiotic was found to simultaneously activate different T cell subpopulations in the intestines of rats, resulting in an increased production of IL-10 and IFN-γ (23). Both studies also reported that FOS and OF-enriched IN increased secretory IgA production in the intestine (22,23). Here, MNB increased fecal IgA concentrations when compared with untreated mice, which is in line with our previous findings that dietary MNB could increase IgA production in chickens, and upregulates the expression of interferon-related genes in the ileum (9,10). Prebiotics have also been shown to exert systemic immunomodulatory effects (11). In mice, supplementation with OF or IN increased splenic NK cell activity (24), and GOS were shown to increase phagocytosis and NK cell activity in humans (25). Moreover, increased resistance to systemic Salmonella and Listeria infections was observed in mice fed OF or IN (26). In the present study, MNB administration increased splenic NK cell activity in mice, indicating that MNB may likewise exert systemic immune-modulating effects. Fermentation of prebiotics, as well as MNB, produces SCFAs, including butyrate, which has been shown to enhance NK cytotoxic activity in the spleen (24).

Evidence suggests that prebiotics may also be effective for the prevention of inflammatory disorders, including sepsis (27,28), and in the present study the immune-modulating effects of MNB were evaluated by using an LPS-induced model of nonlethal endotoxemia in mice. During sepsis, expression of both Th1 and Th2 cytokines are altered; however, there are mixed reports as to the role of specific cytokines and disease outcome. The exaggerated inflammatory response typically leads to sustained systemic inflammation, which contributes to failure to clear pathogens by causing defective innate and adaptive immune responses.
TABLE 4 Expression of cytokine/chemokine genes involved in MyD88-dependent and -independent signaling in untreated RAW 264.7 cells or cells treated for 6 h with MNB or LPS (Expt. 5)1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>MNB</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relative gene expression, fold of control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Il-6</td>
<td>1.0 ± 0.3</td>
<td>3.3 ± 0.4*</td>
<td>4.4 ± 0.4*</td>
</tr>
<tr>
<td>Il-1b</td>
<td>1.0 ± 0.1</td>
<td>5.7 ± 1.8*</td>
<td>9.6 ± 2.0*</td>
</tr>
<tr>
<td>Ip-10</td>
<td>1.0 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>4.0 ± 0.3*</td>
</tr>
<tr>
<td>Rantes</td>
<td>1.0 ± 0.03</td>
<td>1.2 ± 0.2</td>
<td>3.2 ± 0.4*</td>
</tr>
<tr>
<td>Ifnb</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.3</td>
<td>5.0 ± 1.2*</td>
</tr>
</tbody>
</table>

1 Values are mean ± SEM, n = 3, representative of at least 3 independent experiments. Data were log transformed before analysis, but untransformed values are shown. Cells were treated with medium alone (Control), 50 mg/L MNB, or 10 μg/L LPS. Gene expression is reported as fold change relative to control, calculated using the 

Values were normalized by using Gapdh as reference gene.

*Different from control, P < 0.05 (1-way ANOVA and Dunnett’s post test). MNB, β-1,4-mannobiose; MyD88, myeloid differentiation primary response gene 88.

FIGURE 3 TNF-α production by RAW 264.7 cells pretreated for 24 h with medium (Control), MNB (50 mg/L), LPS (10 μg/L), or LTA (1 mg/L) and restimulated (challenged) with LPS or LTA for 6 h (Expt. 6). Results are expressed as percentage of TNF-α relative to control. Values are mean ± SEM, n = 3. *Different from corresponding control, P < 0.05 (1-way ANOVA and Dunnett’s post test). LTA, lipoteichoic acid; MNB, β-1,4-mannobiose.
mediated mainly by improved effector functions of the innate immune system (47). In conclusion, we have shown that MNB can exert prebiotic effects and modulate local intestinal and systemic immune responses and increase IgA secretion in healthy mice, as well as reduce inflammation and preventing immune suppression during LPS-induced endotoxemia. Moreover, we have shown that MNB can exert direct immune-stimulating effects in vitro on immune cells by acting as a novel TLR4 agonist, a previously unreported finding. MNB may be a multifunctional immune-modulator of innate immune responses, capable of maintaining immune homeostasis.

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Literature Cited

Immune-modulating activity of β1,4-mannobiose


