Megalo-type α -1,6-glucosaccharides induce production of tumor necrosis factor α in primary macrophages via toll-like receptor 4 signaling

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ABSTRACT

The term “megalo-saccharide” is used for saccharides with ten or more saccharide units, whereas the term “oligo-saccharide” is used for saccharides containing fewer than ten monosaccharide units. Megalo-type α -1,6-glucosaccharide (M-IM) is a non-digestible saccharide and not utilized by intestinal bacteria, suggesting that ingested M-IM may encounter ileum Peyer’s patches that contains immune cells such as macrophages. Macrophages are responsible for antigen incorporation and presentation during the initial step of immune responses. We investigated whether M-IMs modulate macrophage functions such as cytokine production, nitric oxide production, cell viability, and phagocytosis. Primary macrophages collected from the rats were cultured with the existence of M-IM or lipopolysaccharides (LPS). M-IM and LPS induced the production of tumor necrosis factor α (TNF α), interleukin 6 (IL6), and nitric oxide in the primary macrophages. The gene expression profile of inflammatory factors including TNF α , IL6, and IL1 β in M-IM-stimulated cells was similar to that of LPS-stimulated cells. The M-IM did not affect phagocytosis in the primary macrophages. The M-IM-induced TNF α production was suppressed in the cells treated with a toll-like receptor 4 (TLR4) inhibitor called TAK-242. In conclusion, the M-IM modulates cytokine expression via TLR4 signaling and may play a role in the modulation of immune responses.

Non-digestible saccharides are used by bacteria in the large intestine, leading to the growth of beneficial bacteria in the colon, which contributes to health promotion via the digestive system (15, 17). For example, they are involved in the regulation of blood glucose in diabetics, the suppression of pathogen penetration through the immune system, the promotion of mineral absorption, the inhibition of oxidant-induced apoptosis, the modulation of lipid metabolism in hyperlipidemias, the reduced risk of colon cancer, and the modulation of microbiota (19). Because all the physiological effects mentioned above are not necessarily induced by every non-digestible saccha-

ride, these physiological influences are thought to depend on the structures of the saccharides such as their side-chain branching, linkage types, and degree of polymerization (DP), etc.

The DP in carbohydrates is defined as the number of monosaccharide units in a polysaccharide. In literature, the “megalosaccharides” consist of 10 to 100 monosaccharide units (26), whereas “oligosaccharides” represents a polysaccharide less than 10 monosaccharide units (DP < 10). Isomalto-glucosaccharides (IMs) are one of the non-digestible saccharides, and they primarily consist of α -1,6-glycoside linkages. Ingestion of oligo-type IM increases fecal *Bifidobacteria* population in humans (8). However, it is unknown whether megalo-type IM has beneficial influence on health maintenance and promotion. Megalo-type IM is not distributed commercially. Therefore, we have enzymatically synthesized two fractions of oligo-type α -1,6-glucosaccharides such as short-

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sized isomaltooligosaccharides (S-IMO) (average DP = 3.3) and long-sized isomaltooligosaccharides (L-IMO) (average DP = 8.6) (6). The latter contains megalosaccharides. The ingestion of L-IMO including a megalosaccharide fraction does not promote organic acid production in the cecal contents of rats, although the ingestion of S-IMO increases the butyric acid concentration in the rat cecum. This result suggests that relatively long IMs remain in the gastrointestinal tract without degradation or utilization by intestinal bacteria.

In a separate experiment, we used megalotype IM (M-IMs) to investigate whether M-IM influences flavonoid solubility and its intestinal absorption (25). Quercetin, one of the flavonoids that observed in onion, green tea, and apple, has beneficial functions via radical scavenging and anti-inflammation (1). An increased concentration of quercetin was observed in the portal blood of rats that received quercetin-3-glucosides with M-IM and isomaltooligosaccharides (O-IMs), via the promotion of quercetin solubilization in the small intestinal contents. This influence is prominent in the case of M-IM rather than O-IM. Taken together, these findings indicate that ingested M-IM remains in the gastrointestinal tract and has a chance to encounter the innate immune system especially at Peyer's patches (13).

Macrophages are members of the innate immune cell group and are involved in non-specific defense as well as the initiation of adaptive immunity, and they are specialized phagocytic cells that incorporate exogenous substances, cellular debris, microbes as well as cancer cells (12, 16). These cells play important roles in the development of diseases such as inflammatory bowel disease, diabetes, as well as cancer in its various aspects (9, 12).

Lipopolysaccharide (LPS), a cell-wall component in gram-negative bacteria, induces a variety of inflammatory reactions via inflammatory cytokines, such as tumor necrosis factor α (TNF α), interleukin 6 (IL6), and IL1 β . Some pro-inflammatory mediators including nitric oxide (NO) that are produced from activated macrophages have a strong influence on regional and systemic immune responses. In fact, LPS treatment induces sepsis-like symptoms that are accompanied by the inflammatory mediators from activated macrophages (22). In particular, LPS activates macrophages, producing inflammatory cytokines at the early stage of the innate immune response (3, 22). Accordingly, the activation of macrophages with LPS is widely used as a model of the innate immune response. Reportedly, the activation of macrophages with LPS requires a receptor called toll-like

receptor 4 (TLR4) (3, 10). The TLR4 signaling pathway is critical for the expression of pro-inflammatory cytokines in innate immune cells (7) and the development of inflammatory disease (2).

At this moment, there is almost no information on the regulation of immune responses to IMs, especially for those that depend on the DP of non-digestible polysaccharides. We expected that the innate immune system might have the chance to encounter IMs that had been ingested, and the responses presumably depend on the DP. In the present study, we investigated whether the DP of non-digestible polysaccharides affects the responses in rat primary macrophages.

MATERIALS AND METHODS

Materials. IMs were prepared from maltodextrin through the transglycosylation activity of dextran dextrinase (EC 2.4.1.2) (6). This enzyme catalyzes the successive transfer of a glucosyl group from a terminal position in a dextrin molecule to a non-reducing terminal position in another molecule to make an α -1,6-glucosidic linkage (4). The average DPs of the M-IM and the O-IM used in this study were 11.0 and 3.6, respectively.

Animals. These experiments were approved by the Institutional Animal Care and Use Committee of the National University Corporation of Hokkaido University, and the rats were maintained in accordance with the National University Corporation of Hokkaido University Regulations on Animal Experimentation (permission number: 08-0131, 14-0026). The rats were housed in individual stainless steel cages with wire-mesh bottoms. The cages were placed in a room with controlled temperature ($22 \pm 2^\circ\text{C}$), relative humidity (40–60%), and lighting (lights on 8 : 00–20 : 00) throughout the experiment. Male WKAH/Hkm Slc rats (5–6 weeks old; Japan SLC, Hamamatsu, Japan) were used to isolate peritoneal exudate cells.

Isolation of rat peritoneal exudate cells. Sodium periodate (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 0.9% physiological saline and sterilized with a sterile filter (0.2 μm). The final concentration of sodium periodate solution was 5 mM. Male WKAH rats were intraperitoneally given 3–5 mL of the sodium periodate solution (23). At 72 h after administration, the peritoneal exudate cells were collected. The cell suspension was centrifuged, and the cell number in the cell pellet was counted by trypan blue dye exclusion. We used

Table 1 The primer sequences for quantitative reverse transcription-polymerase chain reaction

Name	Forward primer (5'→3')	Reverse primer (3'→5')	Taqman assay ID	NCBI reference Sequence
<i>Tnfa</i>	CAGAACTCCAGGCGGTGTC	CTTGGTGGTTTGCTACGACG	Rn01525859_g1	NM_012675.3
<i>Il1β</i>	GCTACCTATGTCTTGCCCGT	TGCTGATGTACCAGTTGGGG	Rn00580432_m1	NM_031512.2
<i>Il6</i>	AGAGACTTCCAGCCAGTTGC	AGAGCATTGGAAGTTGGGGT	Rn01410330_m1	NM_012589.1
<i>Nos2</i>	GGTTCCTCAGGCTTGGGTCT	GGCTTGTCTCTGGGTCCTCT	Rn00561646_m1	NM_012611.3
<i>Cox2</i>	GACTGTACCCGGACTGGATTC	GGTCCTCGCTTCTGATCTGT	Rn01483828_m1	NM_017232.3
<i>Nlrp3</i>	TAGGGGATCCAGGCATGAGG	CAGGTGTGTGAGGTTCTGGT	Rn04244625_m1	NM_001191642.1
<i>Ccl2</i>	CCCAGAAACCAGCCAACTCTC	GTGCTGAAGTCCTTAGGGTTGA	Rn00580555_m1	NM_031530.1
<i>Icam1</i>	GTCCTCCAATGGCTTCAACC	CAGGATGAGGTTCTTGCCCA	Rn00564227_m1	NM_012967.1
<i>Vcam1</i>	AGCAGACAGCTAAAGAACGGG	ATGGCGGGTATTACCAAGGAG	Rn00563627_m1	NM_012889.1
<i>Tlr2</i>	CCACAAAACGTGTTCGTGCT	GTTACAGTTCCTGTCACAAAGAC	Rn02133647_s1	NM_198769.2
<i>Rplp0</i>	GGCAAGAACACCATGATGCG	GTGATGCCCAAAGCTTGAA	Rn03302271_gH	NM_022402.2

CD45, F4/80, and CD163 as macrophage markers (14, 21). The percentage of CD45⁺ and F4/80⁺ was 80.5 in the peritoneal exudate cells as analyzed by FACScalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Almost all the cells expressed CD163 (99.8%) in the peritoneal exudate cells that expressed CD45⁺ and F4/80⁺.

Measurements of cytokine concentrations. Peritoneal exudate cells (1×10^5 cells/well) were seeded in a 24-well plate. The cells were maintained in RPMI 1640 (Gibco; Cat No. 31800-22) that was supplemented with 10% FBS and incubated at 37°C for 1 h to adhere. The wells were washed with pre-warmed PBS to remove non-adherent cells. The adherent peritoneal exudate cells used as primary macrophages were serum-starved with RPMI 1640 medium supplemented with 0.1% FBS for 24 h. They were then stimulated with O-IM or M-IM (10 mg/mL) at 37°C for 48 h. LPS from *Escherichia coli* 0111 : B4 (Sigma Chemicals, St. Louis, MO, USA) was used as a positive control (5 µg/mL). The culture supernatants were collected, centrifuged, and kept at -80°C until analysis. The TNFα and IL6 production in the culture medium was measured with ELISA kits (Biolegend, San Diego, CA, USA).

We used TAK-242 as a TLR4 inhibitor (Chemscience LLC, Monmouth Junction, NJ, USA). TAK-242 was dissolved in dimethyl sulfoxide (DMSO) and diluted with the medium. The final concentration of DMSO was 0.1% in the culture medium. The starved primary macrophages were pretreated with TAK-242 (100 µM) for 20 h, and then stimulated with M-IM for another 48 h. The culture supernatants were kept at -80°C until analysis. The TNFα and IL6 production in the culture medium was measured with ELISA kits (Biolegend).

NO production and cell viability. Peritoneal exudate cells (2×10^5 cells/well) were seeded in a 96-well plate and starved for 24 h. They were cultured with 200 µL of DMEM (Gibco) containing M-IM (10 mg/mL) or LPS (1 µg/mL) for 24 h. The culture supernatants (100 µL) were mixed with 50 µL of Griess reagent and left to stand for 15 min at room temperature. NaNO₂ was used as the standard. The optical density was measured at 540 nm. For cell viability, 10 µL of the cell counting kit-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to the remaining medium (100 µL) in the wells containing primary macrophages and kept at 37°C for 1 h. The optical density was measured at 450 nm.

Quantitative real-time PCR. The peritoneal exudate cells (1×10^7) were seeded in a 6-cm dish and starved for 24 h. The primary macrophages were exposed to M-IM or LPS for 3 h, and cytokine mRNA expression was measured by using real-time PCR with a Taqman probe. RNA was extracted from the cells with an RNeasy mini kit (Qiagen, Hilden, Germany). The RNA concentration was measured by spectrophotometry (SmartSpec™ Plus spectrophotometry; Bio-Rad, Hercules, CA, USA). RNA (1 µg) was used for reverse transcription with ReverTra Ace® qPCR RT master mix with gDNA remover according to the manufacturer's instructions. The sequences of the primers used for quantitative RT-PCR are listed in Table 1. The qPCR reaction was performed using an Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) with Taqman gene expression assays for each target gene as follows: *Tnfa*; *Il1β*; *Il6*; nitric oxide synthase 2 (*Nos2*); cyclooxygenase2 (*Cox2*); NLR family, pyrin domain containing 3 (*Nlrp3*); chemokine (C-C mo-

Fig. 1 TNF α production by primary macrophages in response to IMs. **(A)** Difference of TNF α production between O-IM and M-IM (10 mg/mL). **(B)** TNF α and **(C)** IL6 productions at 48 h after treatment with LPS (5 μ g/mL) or M-IM (10 mg/mL). The TNF α concentration in the supernatant was measured with ELISA kits. Means not sharing a common letter differ significantly ($n = 3$, $P < 0.05$, by Tukey-Kramer's test).

tif) ligand 2 (*Ccl2*); intercellular adhesion molecule 1 (*Icam1*), vascular cell adhesion molecule 1 (*Vcam1*); and toll-like receptor 2 (*Tlr2*). Ribosomal protein, large, p0 (*Rplp0*) was used as a control. A serial dilution of the cDNA solution for each target gene was used as a standard to confirm the ranges of the PCR reactions. For the PCR array, RNA was extracted from the primary macrophages or rat small intestinal mucosa using an RNeasy mini kit. The pooled mRNA solution in the group was used to check the expression profile by using a PCR Array and RT2 SYBR green master mix (Qiagen). Complementary DNA was synthesized with an RT2 first strand kit (Qiagen). One μ g of total RNA was used for the RT reaction. The qPCR reaction was performed with an Mx3000P real-time PCR system with a custom PCR array. The data were analyzed by using web-based software according to the manufacturer's instructions (Qiagen).

Phagocytosis assay. Peritoneal exudate cells (1×10^5 cells/well) were seeded in a 96-well plate and starved for 20 h. Phagocytosis was determined using a CytoSelect 96-well phagocytosis assay kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's guidance. The cells were cultured with RPMI 1640 containing M-IM (10 mg/mL), LPS (1 μ g/mL), or cytochalasin D (2 μ M as a phagocytosis inhibitor) for 4 h. The cells were added with the labeled *E. coli* suspension and incubated for 6 h. After washing, the incorporated *E. coli* in the macrophages was measured and relative abundance of the *E. coli* was determined.

Statistical analysis. Statistical analysis was per-

formed with JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). All the values are presented as the average and standard error of the mean. The analysis was performed with a Tukey-Kramer's multiple comparison test. A two-way ANOVA was used to evaluate differences in TNF α and the IL6 production on the TLR4 inhibitor (TAK-242 and M-IM). Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Cytokine production in response to M-IM in rat primary macrophages

M-IM (10 mg/mL) significantly increased the TNF α production in the primary macrophages (Fig. 1A). By contrast, there was no significant influence by O-IM (10 mg/mL) on TNF α production. Because the fundamental structure was almost the same between M-IM and O-IM, these results indicated that the recognition of IMs by macrophages requires a certain size. We used 1 mg/mL of M-IM as a stimulant in a separate experiment, but there was no induction of TNF α production in the macrophages (data not shown). We compared M-IM with LPS as positive control for the production of pro inflammatory cytokines, TNF α and IL6, in the primary macrophages (Fig. 1B and 1C). The TNF α and IL6 production significantly increased in response to 10 mg/mL of M-IM as well as 5 μ g/mL of LPS in the macrophages.

NO production and cell viability in response to M-IM

The primary macrophages produced NO in response to M-IM and LPS (Fig. 2A). The NO level in the

Fig. 2 NO production and cell viability of primary macrophages in response to M-IM or LPS. **(A)** NO production and **(B)** cell viability of the cells treated with LPS (1 µg/mL) or M-IM (10 mg/mL) for 24 h. The NO production was measured with Griss reagent and the cell viability was determined with the cell counting kit. Means not sharing a common letter differ significantly ($n = 10$, $P < 0.05$, by Tukey-Kramer's test).

M-IM-stimulated macrophages was significantly lower than that in LPS-stimulated macrophages, but both of them were significantly higher than that of the control. Treating with LPS or M-IM promoted cell viability (Fig. 2B), but no difference in cell viabilities was found between LPS and M-IM. NO production was not necessarily consistent with cell viability.

Gene expression profiles in primary macrophages treated with M-IM

We examined the time course for TNF α expression in the primary macrophages in response to LPS in a preliminary experiment. The highest expression was observed at 3 h after the stimulation (data not shown), and a variety of cytokine expressions were measured at this time point. The induction of inflammation-related factors can be detected by using a PCR array in response to M-IM as well as LPS. The patterns were nearly the same between M-IM and LPS (Fig. 3). We selected some of the factors to confirm the quantitative difference. As a result, treating with LPS or M-IM significantly increased the expression of *Tnfa*, *Il6*, *Il1b*, *Cox2*, *Nlrp3*, *Nos2*, *Ccl2*, *Icam1*, and *Tlr2* in the primary macrophages (Fig. 4). In the case of *Vcam1*, more than 33 cycles were required to detect expression, whereas approximately 25 cycles were needed for the other genes, suggesting that the *Vcam1* expression level was extremely low even in the LPS-treated macrophages. The overall gene expression pattern in response to M-IM was quite similar to that of LPS.

Phagocytic ability of macrophages by M-IM

To investigate whether the existence of M-IM influ-

Fig. 3 Expression profile of cytokines and other inflammatory factors in primary macrophages using the PCR array. The cells were cultured for 3 h with stimulants such as M-IM (10 mg/mL) or LPS (5 µg/mL). Pooled cDNA was used for the PCR array.

ences the phagocytosis, we measured antigen incorporation activity of the primary macrophages by using labeled *E. coli* particle. Cytochalasin D, an inhibitor of actin polymerization, inhibited phagocytosis of the macrophages in response to *E. coli*. No difference was observed of phagocytic ability in the macrophages among control, LPS, and M-IM (Fig. 5).

The role of TLR4 in cytokine production by M-IM

We investigated whether a TLR4 signal inhibitor (TAK-242) influences the cytokine production induced by M-IM. TAK-242 suppressed the TNF α production induced by M-IM in the primary macrophages (Fig. 6A). The finding raises the possibility that TLR4 is responsible for TNF α production by M-IM. There was no statistical significance in IL6 production from the TAK-242 treatment, although the IL6 production was attenuated (Fig. 6B) and resembled those found with TNF α production.

DISCUSSION

In a preliminary experiment, we tested whether the DP of IM was a response determinant in leukocytes that were isolated from rat mesenteric lymph nodes

as well as in macrophage cell line Raw 264.7. As a result, an increase in TNF α production against M-IM was possibly dependent on the DP (data not shown). The TNF α production induced by M-IM was much higher in Raw 264.7 cells compared with the production in isolated leukocytes. These results suggested that macrophages recognize M-IM. Because Raw 264.7 cells are an established cell line, the responsiveness is not necessarily consistent with the *in vivo* responses of macrophages. Thus, we isolated primary macrophages to investigate their response to M-IM. As expected, the primary macrophages produced TNF α in response to M-IM. Likewise, O-IM did not influence TNF α production in the primary macrophages, indicating that the DP of IMs is a determinant in the recognition performed by macrophages.

LPS receptors such as TLR4 and CD14 may be the candidate molecules that recognize M-IM, because the cytokine expression profile in response to M-IM was similar to the profile that occurred in response to LPS. Recently, the role of TLR4 in recognizing non-digestible saccharides was reported in intestinal epithelial cell lines (18). *Tlr4* expression in IEC-18 cells is 40-fold higher than that in Caco-

2/TC7 cells, resulting in the determination of the difference in responsiveness by CCL2 production against non-digestible saccharides such as fructooligosaccharides, galactooligosaccharides, inulin, and goat milk oligosaccharides. Induced CCL2 secretion by non-digestible saccharides was suppressed in colonic explants of TLR4 knockout mice as well as TLR4 or myeloid differentiation primary response gene 88 knockdowns in IEC-18 cells (18). Additionally, the inhibitors of TLR4 signaling-related molecules such as ERK1/2, JNK, p38, MAPK, and NF- κ B suppressed CCL2 production in IEC-18 cells (18), suggesting that the activation of the TLR4 signaling pathway is involved in the response induced by non-digestible saccharides. Based on these studies, we examined the role of TLR4 in the responses to M-IM by using TLR4 inhibitor TAK-242, which suppresses the cytokine and NO production induced by LPS stimulation in innate immune cells (5) and *in vivo* (24). TAK-242 reportedly binds with TLR4 and inhibits the association of TLR4 with its two adaptor molecules, toll/inteleukin-1 receptor domain-containing adaptor protein (TIRAP) and the TIRAP-inducing interferon- β -related adaptor molecule, accompanied by the suppression of NF- κ B activation (11). We

Fig. 4 Gene expressions of pro-inflammatory factors in primary macrophages in response to M-IM or LPS. The cells were cultured for 3 h with stimulants such as LPS (5 μ g/mL) or M-IM (10 mg/mL). Gene expressions of factors involved in inflammation were measured by using RT-qPCR. Means not sharing a common letter differ significantly ($n = 6$, $P < 0.05$, by Tukey-Kramer's test).

demonstrated in the present study that TAK-242 suppressed TNF α production in the M-IM-treated primary macrophages. These data suggested a link between TLR4 signaling and M-IM-induced cytokine production.

In the expression profile, *Nos2*, the NO-inducing

Fig. 5 Phagocytosis of primary macrophages in response to cytochalasin D, LPS, or M-IM. The cells were pretreated with stimulants such as cytochalasin D (2 μ M), LPS (1 μ g/mL), or M-IM (10 mg/mL) for 4 h. *E. coli* suspension was added to each well and cultured for 6 h. Relative incorporation of *E. coli* was evaluated in response to LPS or M-IM. Means not sharing a common letter differ significantly ($n = 6$, $P < 0.05$, by Tukey-Kramer's test).

enzyme, significantly increased when the cells were exposed to M-IM or LPS. The NO induced by *Nos2* mediates many biological functions such as vasodilation and inflammation (3). NO production (Fig. 2A) was consistent with *Nos2* expression (Fig. 4). In general, the production of bioactive factors depends on the cell viability. The compound in the cell-counting kit solution is a highly water-soluble tetrazolium salt that is similar to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is known as MTT. LPS stimulation is reported to enhance cell viability (20). In the present study, the cell viability significantly increased in response to LPS and M-IM (Fig. 2B). The results showed no signs of apoptosis in the cells that were exposed to LPS or M-IM.

Phagocytosis is the first step of innate immune responses against exogenous antigens. The result indicates that M-IM does not disturb the activation of phagocytosis by macrophages. Luminal antigens in intestine are incorporated by antigen-presenting cells at Peyer's patches, and presented to T cells at Peyer's patches or mesenteric lymph nodes (13). In combination with the cytokine productions, M-IM might contribute to activation of T cells via antigen-presentation by macrophages at these sites. Ten mg/mL of M-IM was required to detect the induction of TNF α production. The concentration of M-IM was 2,000 times higher than that of LPS. Thus, the induction by M-IM was very weak compared with that of LPS. Our previous study showed that the in-

Fig. 6 TLR4 signaling inhibitor effects on TNF α and IL6 production in primary macrophages in response to M-IM. (A) TNF α and (B) IL6 productions. The cells were pretreated with or without TAK-242 (100 μ M) for 20 h, and stimulated with M-IM (10 mg/mL) for 48 h. The TNF α and IL6 concentrations in the supernatant were measured with ELISA kits. Two-way ANOVA P -values are inserted at the panel. Means not sharing a common letter differ significantly ($n = 4$, $P < 0.05$, by Tukey-Kramer's test).

gestion of an M-IM-containing diet does not exacerbate an experimental colitis induced by dextran sulfate sodium (6). These results indicate that the M-IM does not induce *in vivo* inflammation although M-IM induces the production of inflammatory cytokines in the culture experiments as shown in the present study. The ingestion of IMs itself is not considered to evoke unnecessary inflammatory responses. These observations indicate that M-IM plays a role in an initial step of the inflammation process and the prevention of infection via modulation of macrophage functions.

In conclusion, IMs modulate macrophage functions such as cytokine and NO production depending on their DP. It is possible that M-IM plays a role in the modulation of macrophage polarization via TLR4 signaling.

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