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Modulation of inflammatory responses by megalo-type isomaltosaccharides

イソマルトメガロ糖による炎症反応の調節作用

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LIST OF ABBREVIATIONS

Arg1	Arginase 1
ASC	Apoptosis-associated speck-like protein containing CARD
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CD14	Cluster of differentiation 14
COX2	Cyclooxygenase 2 (known as prostaglandin-endoperoxide synthase)
CRP	C-reactive protein
GaLN	D-Galactosamine
ICAM1	Intercellular adhesion molecule 1
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL10	Interleukin 10
IL18	Interleukin 18
IL1 β	Interleukin 1 β
IL6	Interleukin 6
IM	Isomaltosaccharide
KLH	Keyhole limpet hemocyanin
LPS	Lipopolysaccharide
M-IM	Megalo-type isomaltosaccharide
NAFLD	Non-alcoholic fatty liver disease

NCD	Non-communicable disease
NDS	Non digestible saccharide
NF- κ B	Nuclear factor-kappa B
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NO	Nitric oxide
Nos2	Nitric oxide synthase 2
O-IM	Oligo-type isomaltosaccharide
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF α	Tumor necrosis factor alpha
VCAM1	Vascular cell adhesion protein 1 (known as vascular cell adhesion molecule 1)
MAPK	Mitogen-activated protein kinase

Abstract

Non-digestible saccharides modulate various immune responses depending on their structures, which may contribute to host health condition. Megalo-type isomaltosaccharides (M-IMs) are composed of 10 to 50 glucose units with α -1,6-glucosidic linkages. M-IMs display delayed absorption and may stay relatively long time in the gastrointestinal tract, which enables them to encounter host immune cells. It is possible that M-IMs regulate not only innate immunity, but also adaptive immunity. To evaluate this possibility, we investigated the influence of M-IMs on immune system by using primary macrophages and experimental animal models.

1. M-IMs induce tumor necrosis factor α production in primary macrophages via toll-like receptor 4 signaling

Intestinal bacteria utilize M-IMs in a slow rate, suggesting that ingested M-IMs may encounter ileal Peyer's patches, which contain innate immune cells such as macrophages or dendritic cells. Macrophages are responsible for incorporation and presentation of antigens during the initial step of immune responses. We investigated whether M-IMs modulate macrophage functions such as cytokine productions, nitric oxide production, and phagocytosis. Primary macrophages collected from male WKAH/HkmSlc rats were cultured with the existence of M-IMs or lipopolysaccharides (LPS). M-IMs and LPS induced the production of tumor necrosis factor α (TNF α), interleukin 6 (IL6), and nitric oxide (NO) in the primary macrophages. The gene expression profile of inflammatory factors in the M-IM-stimulated cells was similar to those in the LPS-stimulated cells. Stimulation with the M-IMs 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, TAK-242. In conclusion, the M-IMs modulate cytokine expression via TLR4 signaling and may play a role in immune responses.

2. Ingestion of M-IMs ameliorates LPS-induced acute liver injury in rats

Interaction of innate and adaptive immune cells involved in elimination of pathogen and contributes to maintenance of host homeostasis. Innate immune cells incorporate foreign substances and present them as antigens to T cells. In other words, innate immune cells can activate adaptive immune cells. In our previous experiment, ingestion of the M-IMs dose not induce acute inflammation in a 2-week-study. However, in the cellular experiment shown above, the primary macrophages produce inflammatory cytokines in response to the M-IM stimulation. Obviously, there is a variety of immune cell population depending on organs and collaboration of various types of immune cells could decide the way of response against M-IMs. In addition, duration of exposure to M-IMs might alter cellular responses in innate as well as adaptive immune cells. To clarify *in-vivo* responses to M-IMs, we investigated whether a long-term ingestion of the M-IMs influences T-cell-dependent antibody productions and LPS-induced liver injury.

Male F344/Jcl rats (5 weeks old) were fed diet supplemented with or without M-IMs (30 g/kg diet) for 5 weeks. Keyhole limpet hemocyanin (KLH) was administered subcutaneously (1 mg/rat) at week 2 as an exogenous antigen. We measured the production of KLH-specific IgM and IgG in the serum on day 7 and day 18 after the administration, respectively. At the end of the experimental period, the rats were administrated with 4 mg/kg of LPS to induce acute liver injury. At 6 hours after the LPS administration, the aorta plasma was collected to measure alanine transaminase (ALT) and aspartate transaminase (AST) activities as well as factors related with acute inflammation such as TNF α , IL6, caspase-1, and interleukin 1 β

(IL1 β). The liver was collected to analyze gene expressions associated with immune functions with RT-qPCR. There was no significant difference in serum concentration of KLH-specific antibodies and plasma TNF α between the groups. Also, there was no significant difference in the inflammation related cytokine expressions in the liver between the groups. However, plasma AST and ALT activities were attenuated accompanied by reduced plasma IL1 β in the rats fed M-IMs. IL6 tend to decrease in the rats fed the M-IM diet. In a separate experiment in rats without LPS injection, hepatic *Cd14* expression decreased significantly in the M-IM-fed rats. These results suggest that long-term ingestion of the M-IMs suppresses acute liver inflammation via alteration of gene expressions associated with induction of inflammation.

In conclusion, ingestion of M-IMs is considered to modulate innate immune responses such as macrophage functions rather than adaptive immune cell functions, which may contribute to prevention of acute inflammation induced by endotoxin.

Chapter 1

General Introduction

General Introduction

There is an emerging concern worldwide in “non-communicable disease (NCD)”, which is various types of chronic diseases including obesity, diabetes, and cardiovascular diseases, etc. (Beaglehole R *et al.*, 2011; WHO, 2014). The risk factors for NCDs are considered to be unhealthy diet, insufficient exercise, alcohol consumption, and smoking. WHO reported that the global death rate by NCDs reached 52% under the age 70 years (WHO, 2014). There are also socioeconomic impacts by NCD especially on socially disadvantage population having limited access to medical care. Thus, prevention of NCDs is another option for those people. Because dietary habit is closely involved in the development of NCDs, dietary intervention is expected to be available to prevent NCDs. It is a possible way to prevent NCDs is to control energy consumption and dietary composition in the dietary intervention. Food science is able to provide several options to struggle such problems through modulation of dietary composition. One of the promising ingredients is non-digestible saccharides (NDS).

NDSs are hardly digestible in small intestine and some of them are utilized by bacteria in large intestine. They are abundant in grains, fruits, and vegetables (Marlett JA *et al.*, 2008). Physicochemical property is diverse depending on the type of NDSs such as solubility, water-holding capacity, viscosity, and fermentability (Mudgi D and Barak S, 2013), and the properties are involved in beneficial physiological functions such as improvement of glucose and lipid metabolism, excretion of harmful substances, production of fatty acids, regulation of immune response, and so on. Also, it is reported that NDSs can prevent or ameliorate chronic diseases, such as diabetes, cardiovascular disease, and cancer

via physiological influences (Galisteo M *et al.*, 2008; King DE, 2005).

Some of the NDSs promote satiety (Burton-Freeman B, 2000), which induces subsequent weight loss (Slavin JL, 2005). NDSs can be classified with solubility and viscosity (James SL *et al.*, 2003). Viscous water-soluble NDS slows down the digestion and absorption of glucose (James SL *et al.*, 2003) and consequently suppresses elevation of blood glucose level after meal and secretion of insulin, which participate in treatment and prevention of diabetes. (Sierra M *et al.*, 2001). Also, water-soluble NDS holding a large amount of water has strong viscosity and interact with bile acids and cholesterol, which contributes to excretion of these molecules into feces (Theuwissen E *et al.*, 2007). As bile acids decrease under this condition, cholesterol is utilized to synthesize bile acids in the liver, resulting in a decrease in cholesterol in the blood (Theuwissen E *et al.*, 2007). Also, NDSs reduce serum cholesterol concentration accompanied by decrease in the low-density lipoprotein (Brown L *et al.*, 1999), suggesting that NDS prevent cardiovascular disease. Insoluble NDSs are possible to increase the amount of defecation. Also, insoluble NDSs promote intestinal peristaltic movement, thereby shortening transit time in the large intestine (Marlett JA *et al.*, 2002), which improves constipation and contribute to prevention of diverticular disease (Aldoori W and Ryan-Harshman M, 2002). Some of the NDSs can be utilized by intestinal bacteria (James SL *et al.*, 2003), leading to growth of beneficial bacteria in the colon and production of short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate (Tungland BD and Meyer D, 2002). The SCFAs contribute to health promotion such as gut barrier function, mineral bioavailability, and reduction of secondary bile acid (Tungland BD and Meyer D, 2002). For example, SCFAs produced from NDS suppresses colonic damage in a rat colitis model (Rodríguez-Cabezas ME *et al.*, 1999).

NDSs influence several functions of immune system in human studies. Ingestion of whole-wheat meal reduces postprandial serum IL18 concentrations both in diabetic and non-diabetic subjects (Esposito K *et al.*, 2003). The National Health and Nutrition Examination Survey Data in the United States demonstrated that NDS consumption is negatively associated with serum C-reactive protein (CRP) (Ajani UA *et al.*, 2004). Increase in serum CRP concentration is frequently found in acute and chronic inflammation (Pepys MB and Hirschfield GM, 2003; Visser M *et al.*, 1999), suggesting that ingestion of NDS attenuates development of acute and chronic inflammation.

Recently, mechanisms of the influence of NDS on immune responses have been partially clarified in experimental animals. Fructo-oligosaccharides promote IgA production in mice and isolated Peyer's patch cells (Hosono A *et al.*, 2014). Pro-inflammatory cytokines and NF- κ B expression in Caco-2 cells was diminished by α 3-sialyllactose or fructo-oligosaccharides (Zenhom M *et al.*, 2011), raising the possibility that the oligosaccharides retards inflammation by activation of PPAR α and peptidoglycan recognition protein 3. Another study (Bermudez-Brito M *et al.*, 2015) reported that cytokine productions from dendritic cells in response to NDS depend on the chain length and sugar composition. These NDSs also modulate polarization of T cells via crosstalk between intestinal epithelial cells and dendritic cells depending on their types (Bermudez-Brito M *et al.*, 2015). These studies suggest that NDS regulates various immune cells including B cells, dendritic cells, and T cells as well as epithelial cells, which modulates symptoms in immune-related diseases.

Interaction between innate and adaptive immunity influences health maintenance (Fig. 1-1). Macrophages and neutrophils in innate immunity are able to incorporate exogenous substances, and microbes (Nathan C, 2002; Medzhitov R, 2008). Also, they process such substances into small pieces and present them as antigens to the adaptive immune cells,

which classifies these cells as antigen present cells (APCs). The fundamental roles of innate immunity are to provide an immediate defense against infection and effective induction of adaptive immunity (Medzhitov R and Janeway CA Jr, 1997). On the other hands, adaptive immunity such as T cells and B cells participate in antigen-specific responses including antibody production and elimination of cancer cells or virus-infected cells (Saito S *et al.*, 2010). The cooperation between innate and adaptive immunity is required to maintain homeostasis and host defense form pathogens (Fig. 1-1).

Macrophages are divided into two phenotypes, and the balance of the phenotypes is involved in inflammation, disease development, maintaining homeostasis and tissue repair (Gordon S, 2003). M1 macrophages, the classically-activated types of macrophages stimulated by LPS and IFN γ , are fighting against microorganisms and produce pro-inflammatory cytokines such as TNF α , IL1, and IL6, which sometimes induces tissue damages. In contrast, M2 macrophages, the alternatively-activated types of macrophages, are involved in tissue remodeling, clearance of parasites, and produce anti-inflammatory cytokines such as IL4, IL10, and IL13. Balance of the M1 and M2 types determines direction of the fate, for example tissue injury or repair (Mantovani A *et al.*, 2002; Biswas SK *et al.*, 2010; Laskin DL, 2010).

“Megalosaccharides” are one of the categories in carbohydrates and indicates a saccharide containing 10 to 100 monosaccharide units (Thoma JA *et al.*, 1959). Isomaltoglucosaccharides (IMs) have α -1,6-glycosidic linkages in the chain of the glucosaccharides (Figure 1-2). Previously, we investigated whether megalo-type isomaltoglucosaccharides (M-IMs) influences solubility and intestinal absorption of quercetin-3-glucoside (Shinoki A *et al.*, 2013) and found that M-IMs enhance absorption of quercetin in a small intestine via the promotion of the solubilization in the small intestinal contents.

In this study, we investigated whether M-IMs influence cytokine productions in primary macrophages isolated from rats and the ingestion of M-IMs affects antigen-specific antibody productions and acute hepatitis in an animal model. The dissertation is divided into two main parts, one is in chapter 2 showing that M-IMs induce TNF α production in primary macrophages via toll-like receptor 4 signaling. Another one is shown in chapter 3, which describes that ingestion of M-IMs ameliorates LPS-induced acute liver injury in rats.

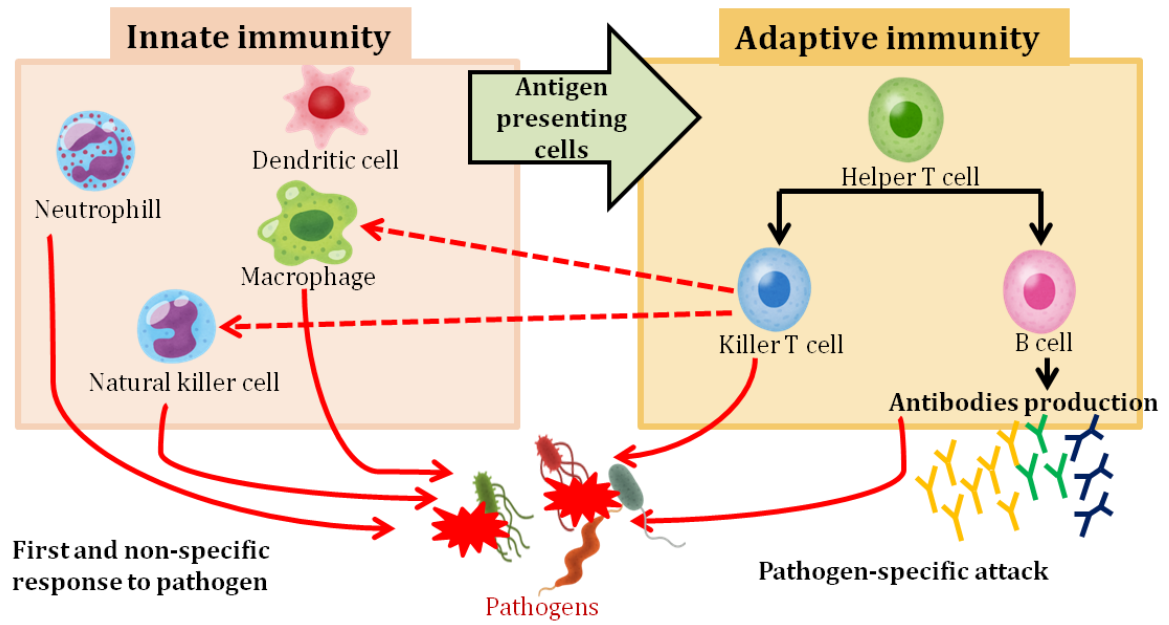


Figure 1-1. Interaction between innate and adaptive immunity

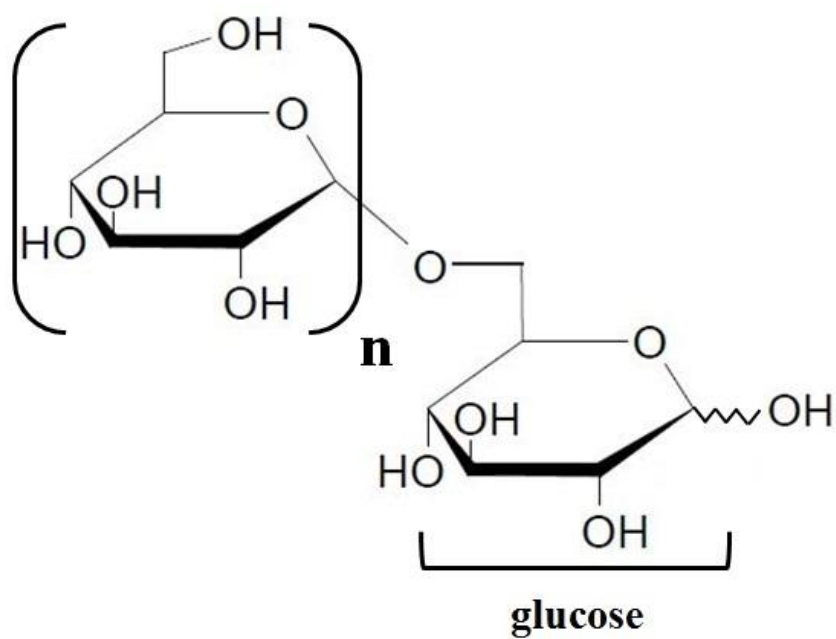


Figure 1-2. Structure of M-IMs

Isomaltosaccharides (IMs) have 10 to 50 degree of polymerization (DP) with α -1,6-glycosidic linkages.

Chapter 2

Megalo-type isomaltosaccharides induce tumor
necrosis factor α production in primary
macrophages via toll-like receptor 4 signaling

2-1. Introduction

NDSs 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 (Mussatto SI *et al.*, 2007, Olano-Martin E *et al.*, 2000). For example, they are involved in the regulation of blood glucose in diabetes, 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 hyperlipidemia, the reduced risk of colon cancer, and the modulation of microbiota (Patel S *et al.*, 2011). Because all the physiological effects mentioned above are not necessarily induced by every non-digestible saccharide, these physiological influences are thought to depend on the structures of the saccharides such as their side-chain branching, linkage types, and DP, etc.

The DP in carbohydrates is defined as the number of monosaccharide units in a sugar. In literature, the “megalosaccharides” consist of 10 to 100 monosaccharide units (Thoma JA *et al.*, 1959), whereas “oligosaccharides” represents a carbohydrate less than 10 monosaccharide units ($DP < 10$). Isomaltoglucosaccharides (IMs) are one of the slowly-digestible saccharides, and they primarily consist of α -1,6-glucosidic linkages. Ingestion of oligo-type IMs increases fecal *Bifidobacteria* population in humans (Kaneko T *et al.*, 1994). However, it is unknown whether megalo-type IMs 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-sized isomaltooligosaccharides (S-IMOs) (average $DP = 3.3$) and long-sized isomaltooligosaccharides (L-IMOs) (average $DP = 8.6$) (Iwaya H *et al.*, 2012). The latter

contains megalosaccharides. The ingestion of L-IMOs including a megalosaccharide fraction do not promote organic acid production in the cecal contents of rats, although the ingestion of S-IMOs increase the butyric acid concentration in the rat cecum. Because almost all parts of ingested M-IM possibly be digested slowly, but completely in the small intestine. 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 M-IMs to investigate whether M-IM influences flavonoid solubility and its intestinal absorption (Shinoki A *et al.*, 2013). Quercetin, one of the flavonoids that observed in onion, green tea, and apple, has beneficial functions via scavenging of reactive oxygen species (Boots AW *et al.*, 2008). An increased concentration of quercetin was observed in the portal blood of rats that received quercetin-3-glucosides with M-IMs and isomaltooligosaccharides (O-IMs), via the promotion of quercetin solubilization in the small intestinal contents. This influence is prominent in the case of M-IMs rather than O-IMs. Taken together, these findings indicate that ingested M-IMs remain in the gastrointestinal tract. It is possible for M-IMs a chance to encounter the innate immune system especially at Peyer's patches (Mowat AM, 2003).

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 (Medzhitov R, 2008; Nathan C, 2002). These cells play important roles in the development of diseases such as inflammatory bowel disease, diabetes, as well as cancer in its various aspects (Laskin DL *et al.*, 1995; Medzhitov R, 2008).

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 influence regional and systemic immune responses. In fact, LPS-treatment induces sepsis-like symptoms that are accompanied by the inflammatory mediators from activated macrophages (Russell JA, 2006). In particular, LPS activates macrophages, producing inflammatory cytokines at the early stage of the innate immune response (Gordon S, 2003; Russell JA, 2006). Accordingly, the stimulation 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) (Gordon S, 2003; Lu Y-C *et al.*, 2008). The TLR4 signaling pathway is critical for the expression of pro-inflammatory cytokines in innate immune cells (Janeway CA Jr *et al.*, 2002) and the development of inflammatory disease (Janeway CA Jr *et al.*, 2012).

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.

2-2. Materials and methods

2-2-1. Materials

IMs were prepared from maltodextrin through the transglycosylation activity of dextran dextrinase (EC 2.4.1.2) (Iwaya H *et al.*, 2012). 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 (Ii M *et al.*, 2006). The average DPs of the M-IMs and the O-IMs used in this study were 11.0 and 3.6, respectively.

2-2-2. 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%), lighting (lights on 8:00-20:00), and diet (Table 2-1, Table 2-2 and Table 2-3) throughout the experiment. Male WKAH/Hkm Slc rats (5-6 weeks old: Japan SLC, Hamamatsu, Japan) were used to isolate peritoneal exudate cells.

2-2-3. Isolation and culture of rat primary macrophages

Sodium periodate (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in 0.9% physiological saline and sterilized with a sterile filter (0.2 μ m). The final concentration of sodium periodate solution was 5 mM. Male WKAH rats were intraperitoneally given 3-5 mL of the sodium periodate solution (Schleicher U and Bogdan C, 2009). At 72 hours 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 CD45, F4/80, and CD163 as macrophage markers (Murray PJ *et al.*, 2011). 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⁺.

2-2-4. 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-IMs or M-IMs (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 μ g/mL). The culture supernatants were collected, centrifuged, and kept at -80°C until analysis. The TNF α 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 (Chemscence 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 μ M) for 20 h, and then stimulated with M-IMs for another 48 h. The culture supernatants were kept at -80°C until analysis. The TNF α and IL6 production in the culture medium was measured with ELISA kits (Biolegend).

2-2-5. Nitric oxide (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 μ L of DMEM (Gibco) containing M-IM (10 mg/mL) or LPS (1 μ g/mL) for 24 h. The culture supernatants (100 μ L) were mixed with 50 μ L 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 μ L of the cell counting kit-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to the remaining medium (100 μ L) in the wells containing primary macrophages and kept at 37°C for 1 h. The optical density was measured at 450 nm.

2-2-6. 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 (SmartSpecTM Plus spectrophotometry; Bio-Rad, Hercules, CA, USA). RNA (1 μ g) 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 2-4. 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 motif) 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). Complimentary DNA was synthesized with an RT2 first strand kit (Qiagen). The qPCR reaction was performed using an Mx3000P real-time PCR system (Agilent Technologies). One µg 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).

2-2-7. 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-IMs (10 mg/mL), LPS (1 µg/mL), or cytochalasin D (2 µM 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.

2-2-8. Statistical analysis

Statistical analysis was performed with JMP software (version 11.0; SAS Institute, Inc., Tokyo, Japan). All the values are presented as the averages and standard error of the means. The analysis was performed with Tukey-Kramer's multiple comparison test and Student's *t*-test for the comparison of two groups. A two-way ANOVA was used to evaluate differences in TNF α and the IL6 production on the TLR4 inhibitor (TAK-242 and M-IMs). Differences were considered to be significant at $P < 0.05$.

Table 2-1. The diet for primary macrophage isolation

Ingredient	g/kg
Acid casein ¹	250
Sucrose ²	602.5
Soybean oil ³	50
Crystalline cellulose ⁴	50
AIN-93G mineral mixture ⁵	35
AIN-93 vitamin mixture ⁶	10
Choline bitartrate	2.5

¹ NZMP Acid Casein (Fonterra. Ltd., Auckland, New Zealand).

² Nippon Beet Sugar Manufacturing Co., Ltd., Japan).

³ (J-Oil Mills Tokyo, Japan).

⁴ Ceolus PH102 (Asahi Chemical Industry, Tokyo, Japan).

^{5, 6} Mineral mixtures were prepared according to the AIN93-G formulation and vitamin mixtures were prepared according to the AIN93 formulation.

Table 2-2. AIN-93 vitamin mixture composition.

Ingredient		g/kg
Nicotinic acid	Niachin	3.0
Ca Pantothenate		1.6
Pyridoxine hydrochloride	V. B6	0.7
Thiamin hydrochloride	V. B1	0.6
Riboflavin	V. B2	0.6
Folic acid		0.2
D-Biotin	V. H	0.02
Cyanocobalamin Powder ¹	V. B12	2.50
Vitamin E Juvela Granule ²	V. E	75.0
Vitamin –A –palmitate ³	V. A	0.229
Cholecalciferol	V. D3	0.25
KATIV – N ⁴	V. K1	7.5
Powdered sucrose		907.801

¹ cyanocobalamin 10 mg in 9.99 g of sucrose

² all-*rac*-alpha-tocopheryl acetate 200 mg/g granule

³ retinyl palmitate 1,750 Unit/mg powder

⁴ phylloquinone (phytonadion) 10 mg/g powder

Table 2-3. AIN-93G mineral mixture composition.

Ingredient	g/kg
Essential mineral element	
Calcium carbonate, anhydrous	375
Potassium phosphate, monobasic	196
Potassium citrate, tri-potassium, monohydrate	70.78
Sodium chloride	74
Potassium sulfate	46.6
Magnesium oxide	24
Ferric citrate	6.06
Manganous carbonate	1.65
Zinc carbonate	0.63
Cupric carbonate	0.3
Potassium iodate	0.01
Sodium selenate, anhydrous	0.01025
Ammonium paramolybdate, 4 hydrate	0.00795
Potentially beneficial mineral element	
Sodium meta-silicate, 9 hydrate	1.45
Chromium Potassium sulfate, 12 hydrate	0.275
Lithium chloride	0.0174
Boric acid	0.0815
Sodium fluoride	0.0635
Nickel carbonate	0.0318
Ammonium vanadate	0.0066
Powdered sucrose	221.026

Table 2-4. The primer sequences for quantitative reverse transcription-polymerase chain reaction

<i>Name</i>	<i>Forward primer (5'→3')</i>	<i>Reverse primer (3'→5')</i>	<i>Taqman assay ID</i>	NCBI Reference sequence
TNF-a	CAGAACTCCAGGCGGTGTC	CTTGGTGGTTTGCTACGACG	Rn01525859_g1	NM_012675.3
IL-1b	GCTACCTATGTCTTGCCCCGT	TGCTGATGTACCAAGTTGGGG	Rn00580432_m1	NM_031512.2
IL-6	AGAGACTTCCAGCCAGTTGC	AGAGCATTGGAAGTTGGGGT	Rn01410330_m1	NM_012589.1
Nos2	GGTTCCTCAGGCTTGGGTCT	GGCTTGCTCTCTGGGTCCTCT	Rn00561646_m1	NM_012611.3
Ptgs/cox2	GACTGTACCCGGACTGGATTG	GGTCCTCGCTTCTGTATCTGT	Rn01483828_m1	NM_017232.3
Nlrp1a	GTGTGAGGGGCTCAGGAATC	TCAAACAGAGGTCAGCCAC	Rn01467483_m1	NM_001145755.1
Nlrp3	TAGGGGATCCAGGCATGAGG	CAGGTGTGTGAGGTTCTGCTGGT	Rn04244625_m1	NM_001191642.1
CCL2	CCCAGAAACCAGCCAACTCTC	GTGCTGAAGTCCTTAGGGTTGA	Rn00580555_m1	NM_031530.1
Icam1	GTCCTCCAATGGCTTCAACC	CAGGATGAGGTTCTTGCCCA	Rn00564227_m1	NM_012967.1
Vcam1	AGCAGACAGCTAAAGAACGGG	ATGGCGGGTATTACCAAGGAG	Rn00563627_m1	NM_012889.1
TLR2	CCACAAAACTGTGTTGCGTGCT	GTTACAGTTCCTGTACACAAAGAC	Rn02133647_s1	NM_198769.2

2-3. Results

2-3-1. Cytokine production in response to M-IMs in rat primary macrophages

M-IMs (10 mg/mL) significantly increased the TNF α production in the primary macrophages (Fig. 2-1). By contrast, there was no significant influence by O-IMs (10 mg/mL) on TNF α production. Because the fundamental structure was almost the same between M-IMs and O-IMs, these results indicated that the recognition of IMs by macrophages requires a certain size. We used 1 mg/mL of M-IMs as a stimulant in a separate experiment, but there was no induction of TNF α production in the macrophages (data not shown). We confirmed that the production of proinflammatory cytokines, including the IL6 in the primary macrophages that were exposed to M-IMs, was compared with LPS as a positive control (Fig. 2-2A and B). The TNF α and IL6 production significantly increased in response to 10 mg/mL M-IMs as well as 5 μ g/mL LPS in the macrophages.

2-3-2. NO production and cell viability in response to M-IMs

The primary macrophages produced NO in response to M-IMs and LPS (Fig. 2-3A). The NO level in the 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-IMs promoted cell viability (Fig 2-3B), but no difference in cell viabilities was found between LPS and M-IMs. NO production was not necessarily consistent with cell viability.

2-3-3. Gene expression profiles in primary macrophages treated with M-IMs

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 (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-IMs as well as LPS. The patterns were nearly the same between M-IMs and LPS (Fig 2-4). We selected some of the factors to confirm the quantitative difference. As a result, treating with LPS or M-IMs significantly increased the expression of *Tnf*, *Il6*, *Il1 β* , *Cox2*, *Nlrp3*, *Nos2*, *Ccl2*, *Icam1*, and *Tlr2* in the primary macrophages (Fig 2-5). 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-IMs was quite similar to that of LPS.

2-3-4. Phagocytic ability of macrophages by M-IMs

To investigate whether the existence of M-IMs influences 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-IMs (Fig. 2-6).

2-3-5. The role of TLR4 in cytokine production by M-IMs

We investigated whether a TLR4 signal inhibitor (TAK-242) influences the cytokine production induced by M-IMs. TAK-242 suppressed the TNF α production induced by M-IM in the primary macrophages (Fig 2-7A). These findings raise the

possibility that TLR4 is responsible for TNF α production by M-IMs. There was no statistical significance in IL6 production from the TAK-242 treatment, although the IL6 production was attenuated (Fig 2-7B) and resembled those found with TNF α production.

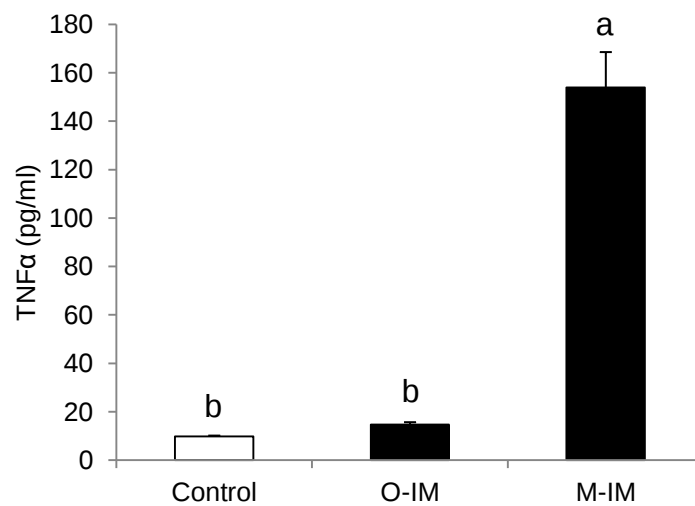


Figure 2-1. TNFα production by primary macrophages in response to IMs

The cells were cultured for 48 h with stimulants such as LPS (5 µg/mL), O-IMs, or M-IMs (10 mg/mL). The TNFα 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).

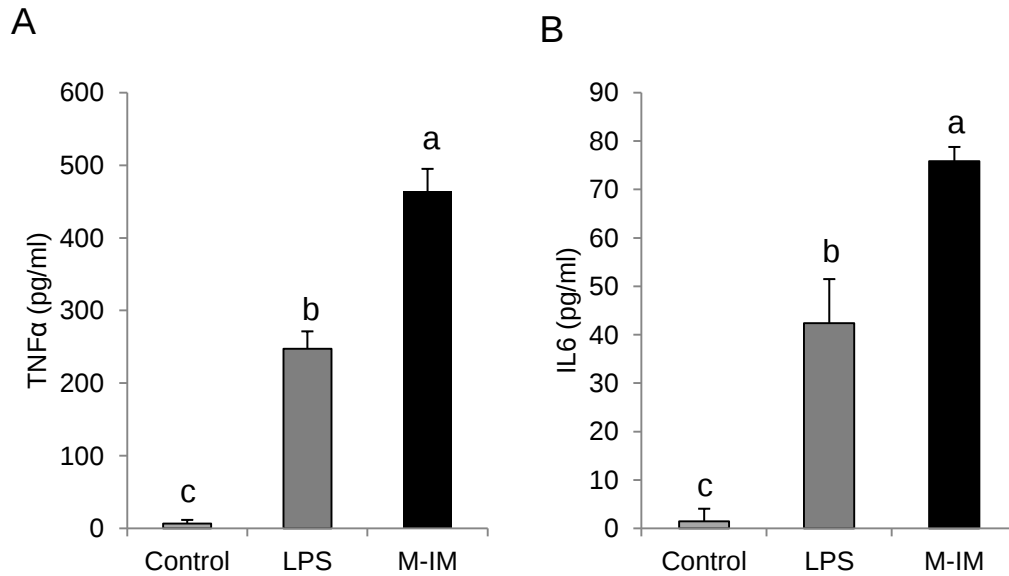


Figure 2-2. TNFα and IL6 production by primary macrophages in response to M-IMs

The cells were cultured for 48 h with stimulants such as LPS (5 µg/mL) or M-IMs (10 mg/mL). The (A) TNFα and (B) IL6 productions in the supernatant were measured with ELISA kits. Means not sharing a common letter differ significantly ($n = 3$, $P < 0.05$, by Tukey-Kramer's test).

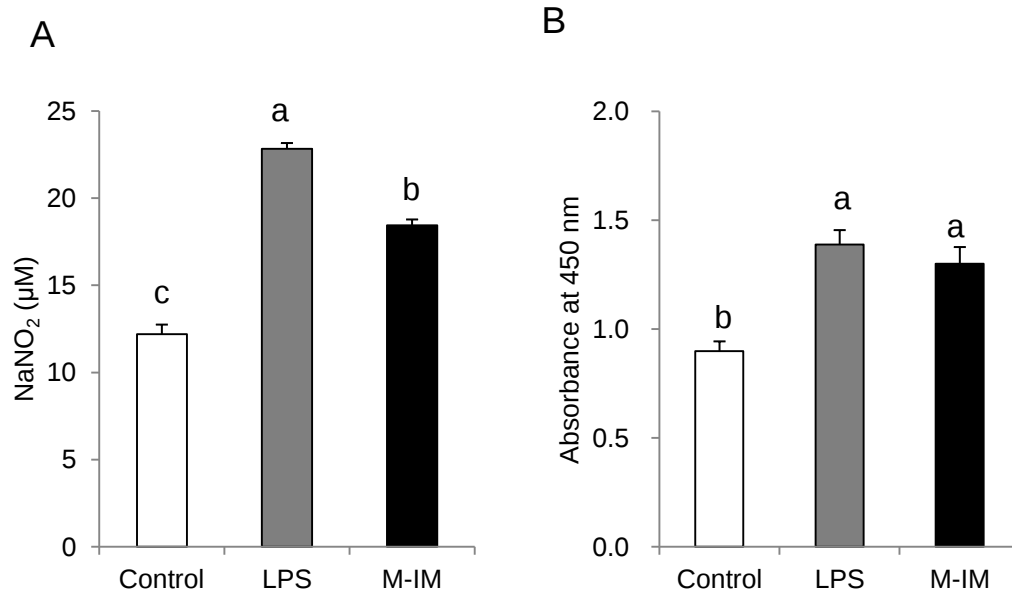


Figure 2-3. NO production and cell viability of primary macrophages in response to M-IMs

The cells were cultured for 24 h with stimulants such as LPS (5 μg/mL) or M-IMs (10 mg/mL). The (A) NO production and (B) cell viability were determined. Means not sharing a common letter differ significantly ($n = 10$, $P < 0.05$, by Tukey-Kramer's test).

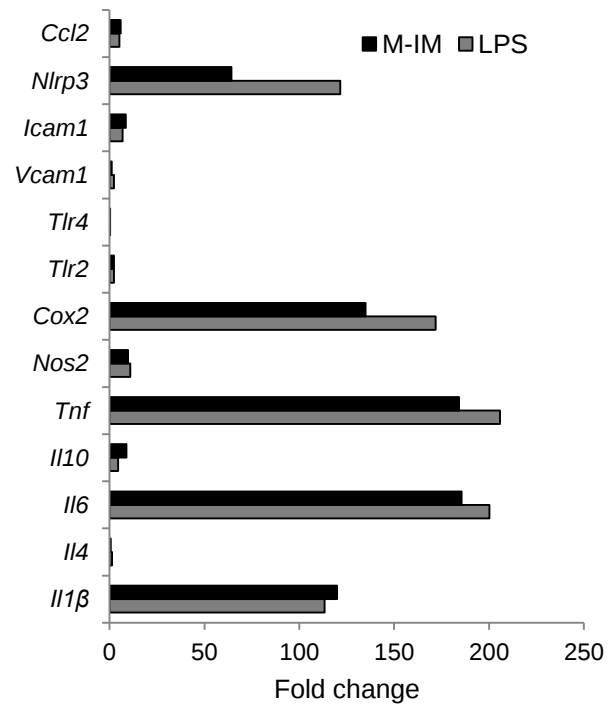


Figure 2-4. Expression profile of cytokines and other inflammatory factors using the PCR array

Macrophages were cultured for 3 h with stimulants such as M-IMs (10 mg/mL) or LPS (5 µg/mL). Pooled cDNA was used for the PCR array.

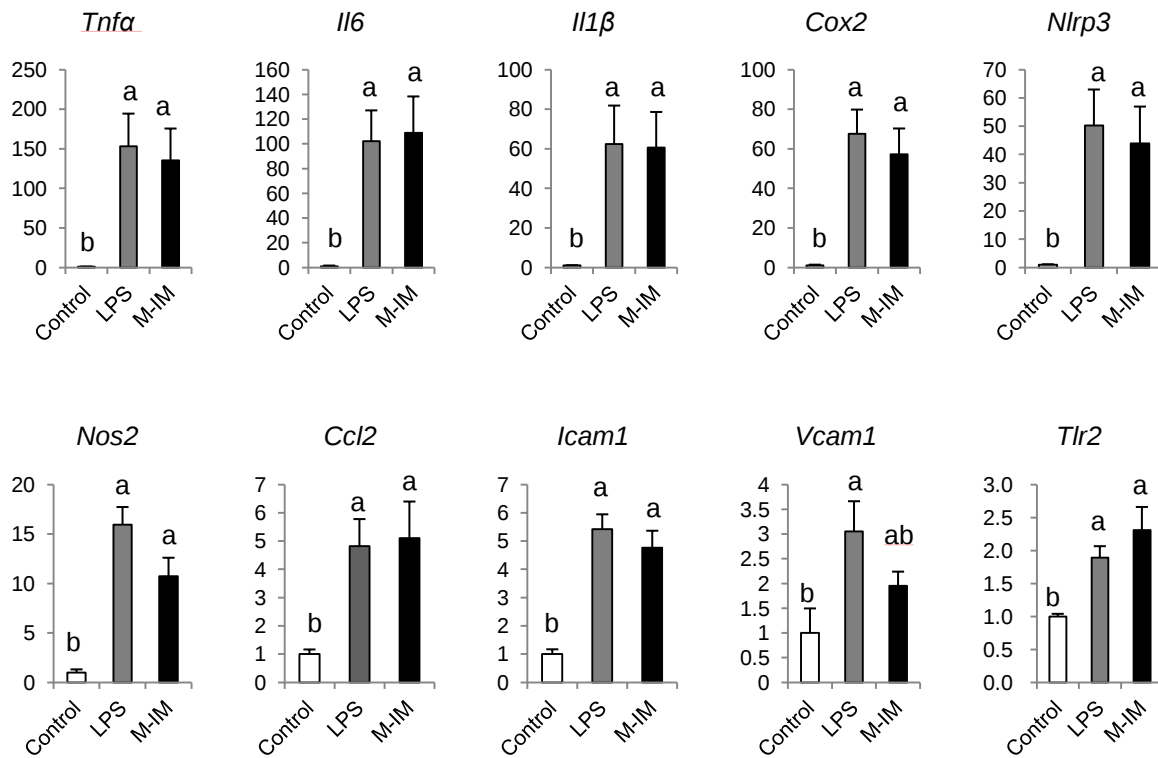


Figure 2-5. Gene expressions of pro-inflammatory factors in primary macrophages in response to M-IMs

The cells were cultured for 3 h with stimulants such as LPS (5 µg/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).

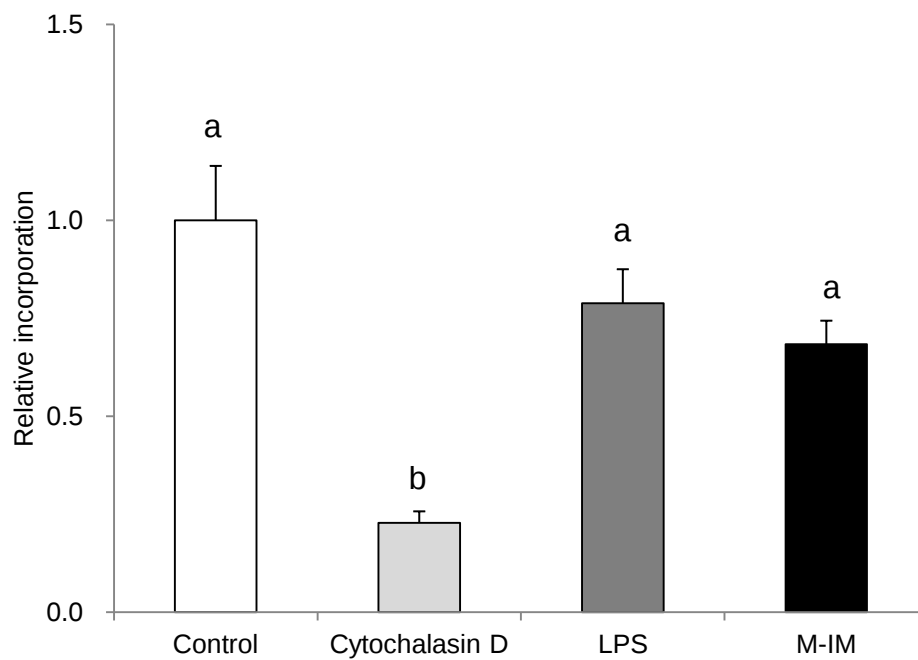


Figure 2-6. Phagocytosis of primary macrophages in response to cytochalasin D, LPS, or M-IMs

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

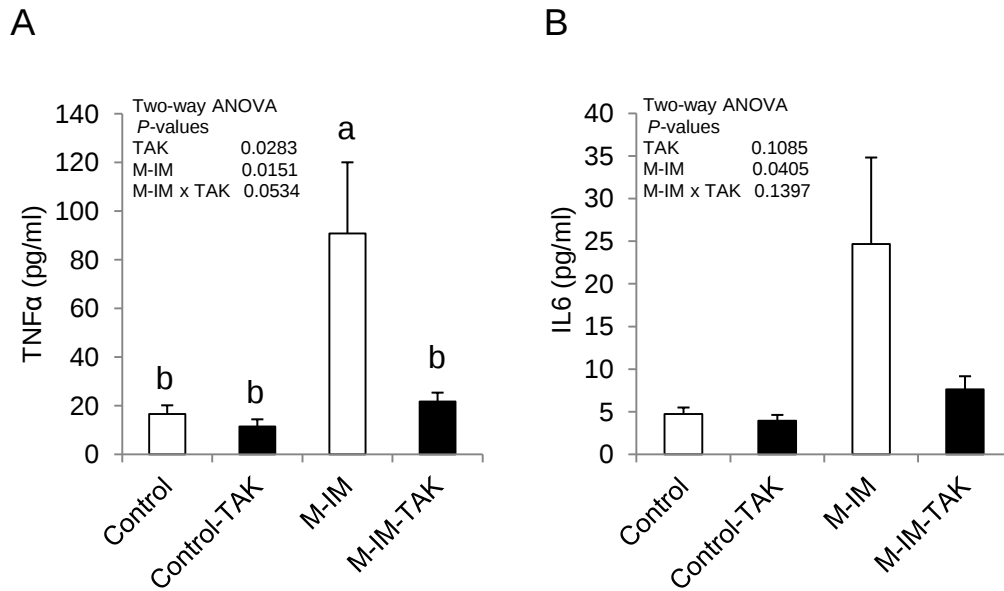


Figure 2-7. Effects of TLR4 signaling inhibitor on TNF α and IL6 production in primary macrophages in response to M-IMs

(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-IMs (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).

2-4. Discussion

In a preliminary experiment, we tested whether the DP of IMs 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-IMs was possibly dependent on the DP (data not shown). The TNF α production induced by M-IMs was much higher in Raw 264.7 cells compared with the production in isolated leukocytes. These results suggested that macrophages recognize M-IMs. 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-IMs. As expected, the primary macrophages produced TNF α in response to M-IMs. Likewise, O-IMs 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-IMs, because the cytokine expression profile in response to M-IMs were similar to the profile that occurred in response to LPS. TLR4 in an intestinal epithelia cell line IEC-18 is responsible for CCL2 secretion induced by NDSs such as fructooligosaccharides, galactooligosaccharides, inulin, and goat milk oligosaccharides (Ortega-González M *et al.*, 2014). Induced CCL2 secretion by NDS 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 (Ortega-González M *et al.*, 2014). 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 (Ortega-González M *et al.*, 2014), suggesting

that the activation of the TLR4 signaling pathway is involved in the response induced by NDS. Based on these studies, we examined the role of TLR4 in the responses to M-IMs by using TLR4 inhibitor TAK-242, which suppresses the cytokine and NO production induced by LPS stimulation in innate immune cells (Ii M *et al.*, 2006) and *in vivo* (Sha T *et al.*, 2005). 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- γ -related adaptor molecule, accompanied by the suppression of NF- κ B activation (Matsunaga N *et al.*, 2011). We 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 enzyme, significantly increased when the cells were exposed to M-IMs or LPS. The NO induced by *Nos2* mediates many biological functions such as vasodilation and inflammation (Gordon S, 2003). NO production (Fig. 2-3A) was consistent with *Nos2* expression (Fig. 2-5). 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 (Pozzolini M *et al.*, 2003). In the present study, the cell viability significantly increased in response to LPS and M-IMs (Fig. 2-3B). The results showed no signs of apoptosis in the cells that were exposed to LPS or M-IMs.

Phagocytosis is the first step of innate immune responses against exogenous antigens. The result indicates that M-IMs do 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 (Mowat AM, 2003). In combination with the cytokine productions, M-IMs 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-IMs was 2,000 times higher than that of LPS. Thus, the induction by M-IMs was very weak compared with that of LPS. Our previous study showed that the ingestion of an M-IM-containing diet does not exacerbate an experimental colitis induced by dextran sulfate sodium (Iwaya H *et al.*, 2012). These results indicate that the M-IMs do not induce *in vivo* inflammation although M-IMs induce 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-IMs play 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-IMs play a role in the modulation of macrophage polarization via TLR4 signaling.

Chapter 3

Ingestion of megalo-type isomaltosaccharides
ameliorates LPS-induced acute liver injury in rats

3-1. Introduction

Liver is involved in metabolism of nutrients, such as carbohydrate, protein, and fat, stores nutrients and supplies them to other organs when necessary (Rui L, 2014). In addition, liver plays roles in metabolism of alcohol and toxic substances to detoxify these substances (Liska DJ, 1998). Bile acids produced by liver as a detergent contribute to digestion and absorption of dietary lipids (Mitra V and Metcalf J, 2009). As shown above, liver is the center of metabolism in the body not only for nutrients, but also for toxic substances. These indicate that liver failure provokes massive impact in enormous aspects of metabolism, resulting in development of several disorders. Excess consumption of alcohol induces alcoholic liver diseases (ALD), whereas excess energy consumption associated with obesity and diabetes triggers development of non-alcoholic fatty liver disease (NAFLD) even without alcohol consumption (Pagano G *et al.*, 2002). Lipid accumulation was observed both in ALD and NAFLD, which leads to inflammation via oxidative stress, resulting in hepatic fibrosis. Subsequently, several types of liver disorders develop, for example, cirrhosis and liver cancer.

Kupffer cells regulate inflammatory responses in liver and are involved in the prevention or pathogenesis of liver diseases. Damage of hepatocytes induces production of inflammatory cytokines from Kupffer cells that are involved in development of insulin resistance (Shoelson SE *et al.*, 2006). Also, these cytokines produced lead to hepatocytes apoptosis in turn, indicating that Kupffer cells play pivotal roles in development of liver disease. There are several models of hepatitis to identify preventive effect of drugs and food ingredients on liver disease. For example, deficiency of choline with or without methionine is

frequently used to induce steatohepatitis with fibrosis (Sahai A *et al.*, 2004). High-fat diets induce steatohepatitis (Anstee QM and Goldin RD, 2006) and promotes the increase in serum ALT activity in response to LPS (Li Z *et al.*, 2005), but relatively long-term (4 to 12 weeks) experiment is required for development of the hepatitis. Carbon tetrachloride induces much severe hepatitis with high serum TNF α concentration and ALT activity (Lee C-H *et al.*, 2007). Other drugs such as D-galactosamine (GalN), thiocetamide, concanavalin A, and LPS are also used as inducers of acute hepatic failure (Rahman TM and Hodgson HJ, 2000).

In these inducers, LPS, one of the cell-wall components in Gram-negative bacteria (Schumann RR, 1992), is naturally found in luminal contents of the gut. Once disruption of gut barrier occur, LPS is possible to penetrate host tissues. Enhancement of gut permeability is observed in genetically-modified obese mice (*ob/ob* and *db/db* mice) and high-fat diet-fed mice, where blood LPS concentration increases (Brun P *et al.*, 2007; Cani PD *et al.*, 2007). Hepatocytes produce LPS-binding protein (LBP) and LPS-LBP complex binds to CD14 on the membrane of Kupffer cells, resulting in activation of TLR4 signaling (Su GL, 2002). The stimulation of TLR4 signaling by LPS leads to liver inflammation by producing cytokines such as TNF α and IL1 β .

In the previous chapter, the stimulation with M-IMs enhances inflammatory cytokine productions via TLR4 signaling in primary macrophages in a culture experiment, suggesting modulation of inflammatory responses in *in vivo*. We have already investigated whether the ingestion of M-IMs modulates in-vivo inflammation by using DSS-induced colitis rats (Iwaya H *et al.*, 2012). However, there is no apparent induction in inflammatory response with or without the M-IM ingestion, suggesting that the M-IMs do not directly induce mucosal inflammation. Recently, it is reported that the ingestion of NDS improve symptom of liver injury. For example, monosaccharides of corn bran hemicellulose partially normalize increased blood levels of ALT and AST activities in GalN-induced hepatitis rats (Geng X *et*

al., 2005). Dietary fiber in edible seaweeds diminished the ALT and AST activities in GalN-injected rats (Kawano N *et al.*, 2007).

The immune system is classified as innate and adaptive immunity. Macrophages are one of the innate immune cells to present antigens to T-cells, adaptive immune cells, resulting in production of antigen-specific antibodies. It is possible that M-IMs regulate not only innate immunity, but also adaptive immunity. T-cell-dependent antibody response (TDAR) is used as an immuno-toxicology test to identify alteration of antigen-specific antibody production (Plitnick LM and Herzyk DJ, 2010). Keyhole limpet hemocyanin (KLH) is widely used as an antigen in TDAR. Thus, we investigated influence of M-IMs on adaptive immunity using the TDAR to KLH. In this study, we investigated influence on M-IMs in both in-vivo innate and adaptive responses at the same time. Because it is required for 3 weeks at least to determine TDAR in the standard protocol, we consider that it is necessary for the rats to get accustomed to the M-IM diet beforehand to facilitate TDAR response in this condition.

The aim of this study is to investigate whether the M-IM ingestion modulates TDAR and LPS-induced liver injury in rats.

3-2. Materials and methods

3-2-1. Animals and diets

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: 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 ± 2 °C), relative humidity (40-60%), and lighting (lights on 8:00-20:00) throughout the experiment. The food intake and body weight during the experimental period were recorded every two days at 10:00 a.m.

To determine the dose of LPS, male WKAH/HkmSlc rats (5-6 weeks old: Japan SLC, Hamamatsu, Japan) were used and fed a modified American Institute of Nutrition (AIN)-93G rodent diet (Reeves et al., 1993) containing sucrose as the carbohydrate source for 1 week (control diet, Table 3-1). LPS was intraperitoneally administered at 2, 4, or 8 mg/kg in 0.9% physiological saline (*E. coli*, serotype 055:B5; Sigma Chemicals). At 6 h after the LPS administration, the rats were euthanized with sodium pentobarbital (50 mg/kg) and the blood plasma was collected from aortic vessel with heparin (50 U/mL blood) and aprotinin (500 KIU/mL blood).

To determine whether M-IM ingestion modulates KLH-specific antibody production and LPS-induced liver injury (Fig 3-1), male F344/Jcl rats (5 weeks old; Japan) were fed control diet (Table 3-1) for 4 days. After acclimation, the rats were divided into two groups and fed diets with or without the M-IM supplementation (at 30 g/kg) for 35

days (Table 3-1). The rats were administrated with KLH at day 14. At the end of the experimental period (day 35), the rats were subcutaneously administrated with 4 mg/kg of LPS to induce acute liver injury. At 6 h after the LPS administration, the rats were euthanized with sodium pentobarbital and the blood plasma was collected from aortic vessel with heparin and aprotinin.

In a separate experiment (Fig 3-2), male F344/Jcl rats (5 weeks old; Japan) were fed control diet (Table 3-1) for 5 days. Then, the rats were divided into two groups and fed diets supplemented with or without M-IMs (at 30 g/kg) for 37 days. At the end of the experimental period, the rats were euthanized with sodium pentobarbital and the blood plasma was collected from aortic vessel with heparin and aprotinin.

In all experiment, the plasma was separated after centrifugation and stored at -80°C. The liver was weighed and collected and stored at -80°C.

3-2-2. T-cell-dependent antibody production

KLH (Sigma Chemicals, St. Louis, MO, USA) was dissolved in PBS (2 mg/ml). The final dose of KLH was 1 mg/animal. Male F344 rats were subcutaneously given 500 µL of KLH-containing solution. The bloods were collected from tail vein, centrifuged 2,500 g for 15 min at room temperature, and kept at -80°C until analysis. KLH-specific IgM and IgG production in serum were analyzed by ELISA kits (Shibayagi, Shibukawa Gunma, Japan) at day 21 (IgM) and day 32 (IgG), respectively (Fig 3-1).

3-2-3. Plasma parameters

The aortic plasma was analyzed using a transaminase C II test Wako kit (Wako Pure Chemical Industries, Ltd.) to measure liver injury markers alanine transaminase (ALT) and aspartate transaminase (AST) activity. The concentration of creatinine, kidney injury

marker, was determined by creatinine assay Wako kit (Wako Pure Chemical Industries, Ltd.). Also, the concentrations of TNF α (Biolegend, San Diego, CA, USA), IL6 (Biolegend), Caspase-1 (CUSABIO, College Park, MD, USA), and IL1 β (Thermo, Waltham, MA, USA) were measured with ELISA kits.

3-2-4. Quantitative real-time PCR

At 6 h after the LPS administration, the liver were collected and stored at -80°C. Inflammatory 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 (NanoDrop Lite; Thermo Scientific, Waltham, MA, USA). RNA (1 μ g) was used for reverse transcription with ReverTra Ace® qPCR RT master mix with gDNA remover according to the manufacturer's instructions. The qPCR reaction was performed using an Mx3000P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA) with Taqman gene expression assays for *Tnfa*, *Il6*, *Il1 β* , *Il10*, *Arg1*, and *Cd14*. *Rplp0* was used as a control (Table 3-2). A serial dilution of the cDNA solution for each target gene was used as a standard to confirm the ranges of the PCR reactions.

3-2-5. Statistical analysis

Statistical analysis was performed with JMP software (version 12.0; 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 Student's *t*-test for the comparison of two groups. Differences were considered to be significant at $P < 0.05$.

Table 3-1. Diet composition for feeding study of M-IMs

Ingredient	g/kg	
	Control	M-IM
Casein ¹	200	200
Soybean oil ²	70	70
Crystalline cellulose ³	50	50
AIN-93G mineral mixture ⁴	35	35
AIN-93 vitamin mixture ⁵	10	10
Choline bitartrate	2.5	2.5
L-Cystine	3	3
M-IM		30
Sucrose ⁶	To make 1 kg	

¹ NZMP Acid Casein (Fonterra. Ltd., Auckland, New Zealand).

² (J-Oil Mills Tokyo, Japan).

³ Ceolus PH102 (Asahi Chemical Industry, Tokyo, Japan).

^{4, 5} Mineral mixtures were prepared according to the AIN93-G formulation and vitamin mixtures were prepared according to the AIN93 formulation.

⁶ Nippon Beet Sugar Manufacturing Co., Ltd., Japan).

Table 3-2. The primer sequences for quantitative reverse transcription-polymerase chain reaction

Gene	Primer	Taqman Probe Assay ID	NCBI reference Sequence
	Forward (5'→3')		
	Reverse (3'→5')		
<i>Arg1</i>	CCCGCAGCATTAAGGAAAGC GCAAGCCGATGTACACGATG	Rn00691090_m1	NM_017134.3
<i>Cd14</i>	GCCAGAGAACGCTGCTGTAA ACCGCACAGTAAGCCTCTTC	Rn00572656_g1	NM_021744.1
<i>Il1β</i>	GCTACCTATGTCTTGCCCGT TGCTGATGTACCAGTTGGGG	Rn00580432_m1	NM_031512.2
<i>Il6</i>	AGAGACTTCCAGCCAGTTGC AGAGCATTGGAAGTTGGGGT	Rn01410330_m1	NM_012589.1
<i>Il10</i>	GTAGATGCCGGGTGGTTCAA CCTCTGGATACAGCTGCGAC	Rn00563409	NM_012854.2
<i>Tnfa</i>	CAGAACTCCAGGCGGTGTC CTTGGTGGTTTGCTACGACG	Rn01525859_g1	NM_012675.3
<i>Tlr4</i>	CAGGAAGCTTGAATCCCTGC TTTTGTCTCCACAGCCACCA	Rn00569848_m1	NM_019178.1
<i>Rplp0</i>	GGCAAGAACACCATGATGCG GTGATGCCCAAAGCTTGGA	Rn03302271_gH	NM_022402.2

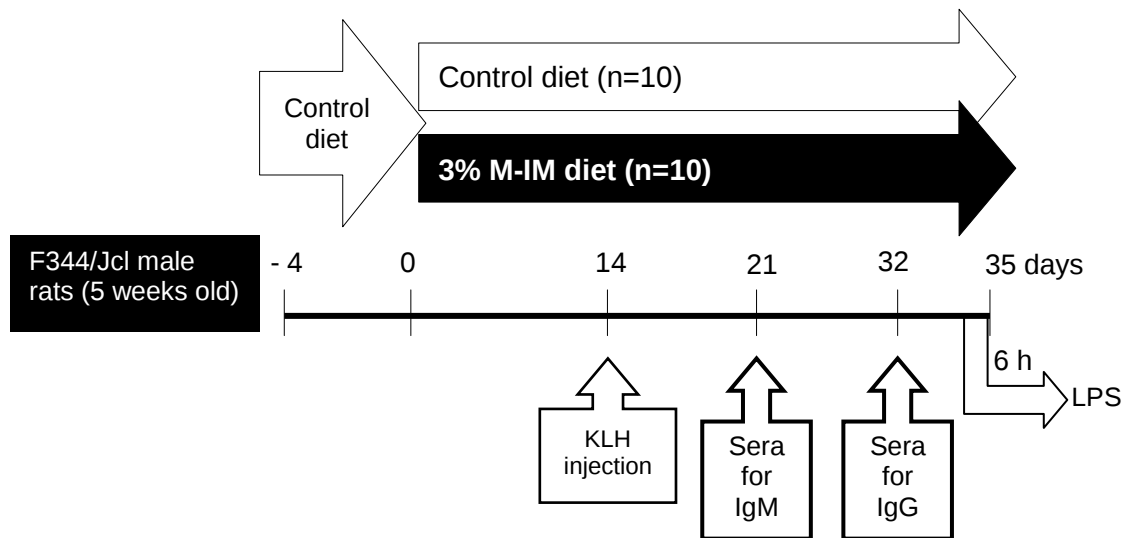


Figure 3-1. Experimental design with LPS treatment

Male F344/Jcl rats (5 weeks old) were fed diets supplemented with or without M-IMs (30 g/kg diet) for 35 days. KLH was administered subcutaneously (1 mg/rat) at day 14 as an exogenous antigen. KLH-specific antibody production in serum were analyzed by ELISA kits at day 21 (IgM) and day 32 (IgG), respectively. At the end of the experimental period, the rats were administrated with 4 mg/kg of LPS to induce acute liver injury. At 6 h after the LPS administration, the aorta plasma and liver were collected.

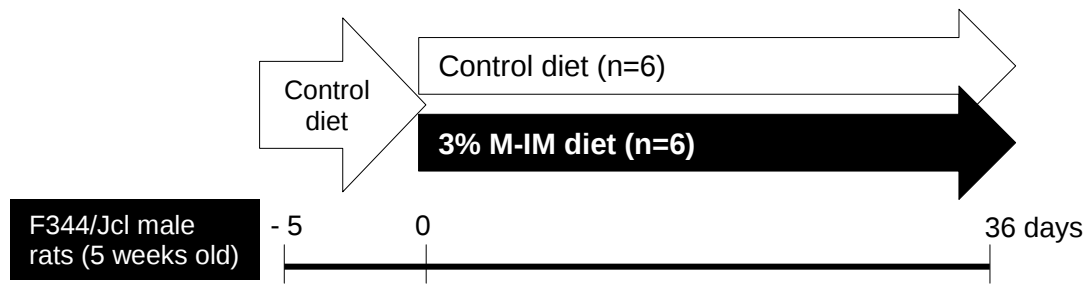


Figure 3-2. Experimental design without LPS treatment

The rats were fed the diet supplemented with or without 3% M-IMs for 36 days. The aorta plasma and liver were collected and stored at -80°C ($n = 6$).

3-3. Results

3-3-1. Partial suppression of increases in plasma AST, ALT and IL1 β in the rats fed M-IMs in response to LPS

In a preliminary experiment, plasma TNF- α concentration (Fig. 3-3A) and ALT activity (Fig. 3-3B) increased depending on LPS concentration. Apparent increases in TNF- α concentration and ALT activity were observed at 4 mg/kg of LPS. Thus, we assessed the influence of M-IMs on LPS-induced liver injury at 4 mg/kg.

Our previous study demonstrates that M-IMs were recognized by macrophage in primary culture (Chapter 2), indicating that the M-IMs may modulate not only innate immunity, but also adaptive immunity in vivo. Thus, we examined the impact of M-IMs on antigen-specific antibody production by using KLH. There was no significant difference in body weight and food intake by the ingestion of M-IMs (Fig. 3-4A and B), suggesting no effect of the ingestion of M-IMs on the growth. Although serum KLH-specific IgM (Fig. 3-5A) and production tended to increase, there was no significant difference in the KLH-specific IgG (Fig. 3-5B) at last.

No significant difference was found in the weight of liver, kidney, whole cecum, spleen, and epididymal adipose tissue (Fig. 3-6A to E) in the LPS-treated rats. Liver injury markers, plasma ALT (Fig. 3-7A) and AST (Fig. 3-7B) decreased significantly by the ingestion of M-IMs. On the other hand, there was no significant difference in the plasma creatinine concentration (Fig. 3-7C).

It is possible that suppression of the liver injury markers by ingestion of M-IMs likely to be associated with the inflammatory cytokines production. Therefore, we

confirmed production of the pro-inflammatory cytokines of the ingestion of M-IM in LPS-induced hepatitis models. As a result, the ingestion of M-IMs significantly suppressed IL1 β production (Fig. 3-8D). Also, both IL6 (Fig. 3-8B) and caspase-1 (Fig. 3-8C) productions tended to decrease in the rat fed the M-IM supplemented diet. However, there was no significant difference in the TNF α production (Fig. 3-8A).

Then, we measured the expression of inflammatory cytokine in the liver. No significant difference was observed in liver expression of *Tnfa*, *Il6*, *Il1 β* , *Arg1* and *Cd14* by the ingestion of M-IMs. (Fig. 3-9A to E). In contrast, the *Nlrp3* expression (Fig. 3-9F) was increased by the ingestion of M-IMs.

3-3-2. Down-regulation of liver *Cd14* expression in the M-IM-fed rats

To assess whether the M-IM-ingestion modulates factors associated with inflammation under untreated condition, we analyzed mRNA expressions and cytokine production in liver and plasma at the rats without LPS-treatment. No difference was observed in the growth and food intake as well as organ weights (Fig. 3-10 and Fig. 3-11). The plasma TNF α concentration in the rats fed control diet was below the range of the calibration curve (Fig. 3-12A). Also, in the M-IM-ingestion group, 3 out of 6 samples were below the range of the calibration curve.

There was no significant difference in plasma IL6 concentrations (Fig. 3-12B) as well as the gene expressions of *Arg1*, *Il1 β* , *Nlrp3* and *Tlr4* in the liver (Fig. 3-13A to E). The *Il6* expression tended to increase in liver of the M-IMs supplemented diet (Fig. 3-13E). Meanwhile, the *Cd14* expression (Fig. 3-13F) was significantly decreased in the liver of the rats fed the M-IM supplemented diet.

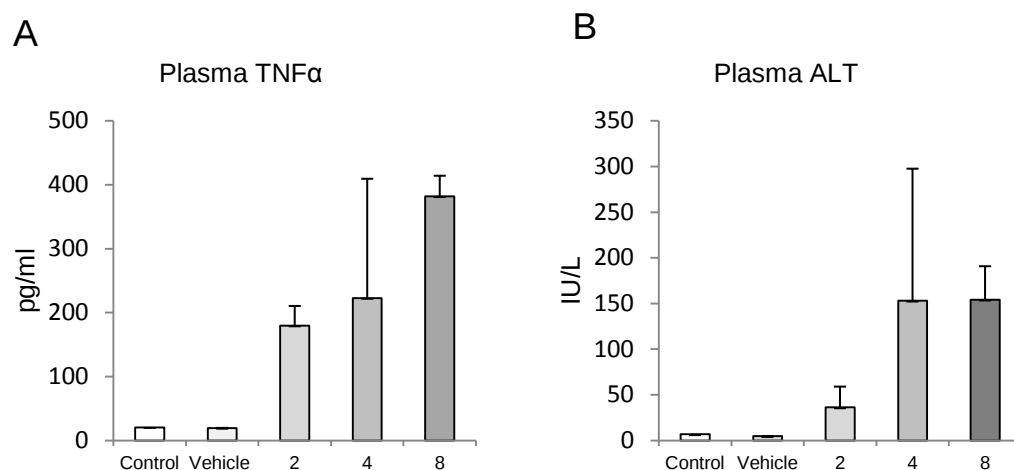


Figure 3-3. Changes in TNF α concentration and ALT activity by intraperitoneal administration of LPS

Male WKAH/Hkm Slc rats (5 weeks old) were fed AIN-93G sucrose based diets. To determine appropriate dose of LPS to induce liver injury, the rats intraperitoneally administered with various concentration of LPS. Saline was administered as vehicle. At 6 h after the administration, the aorta plasma was collected. (A) TNF α concentration in the aortic plasma and (B) ALT in the aortic plasma (n=1-2).

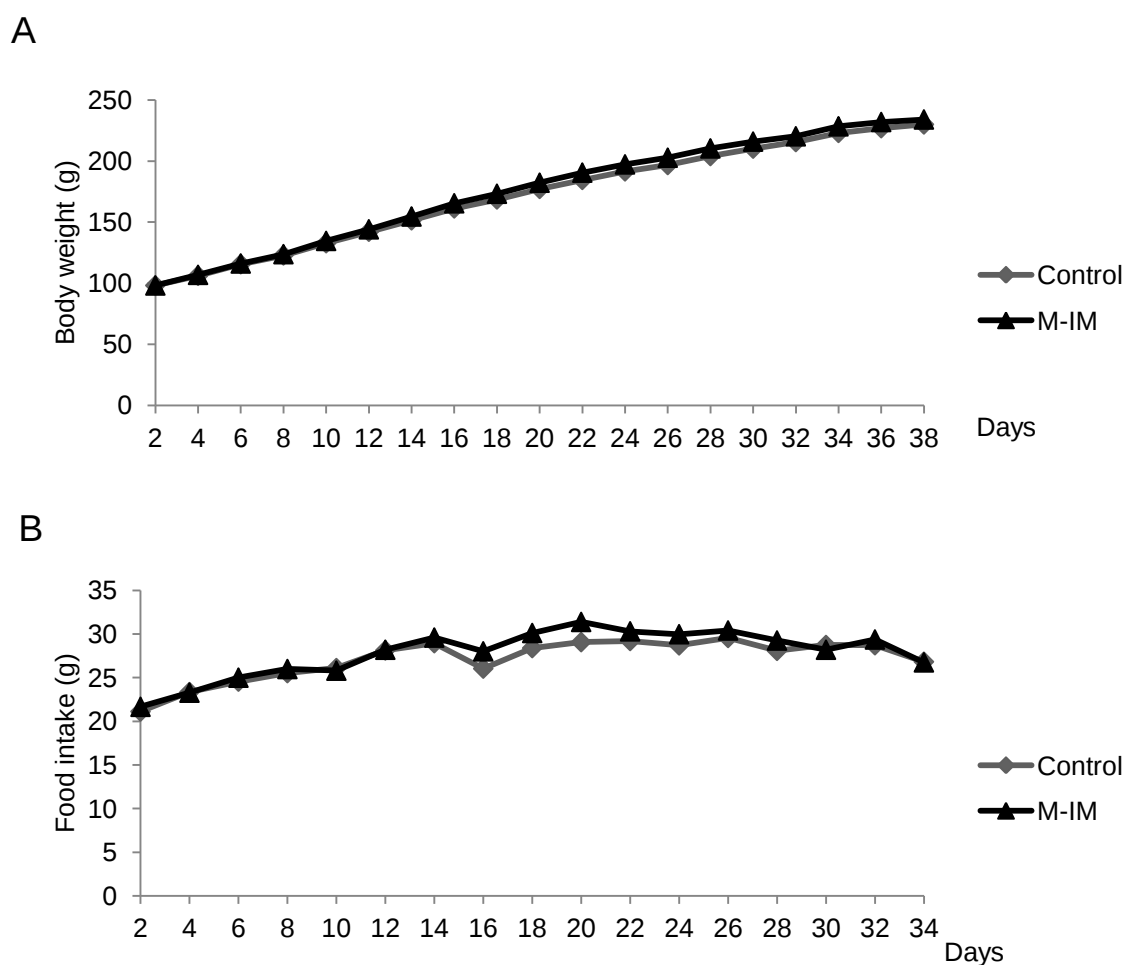


Figure 3-4. Changes in body weight and food intake in the rats fed the diet with or without 3% M-IMs

The rats were fed the diet containing M-IM (30 g/kg diet) for 35 days. (A) The body weight and (B) food intake were recorded every two days.

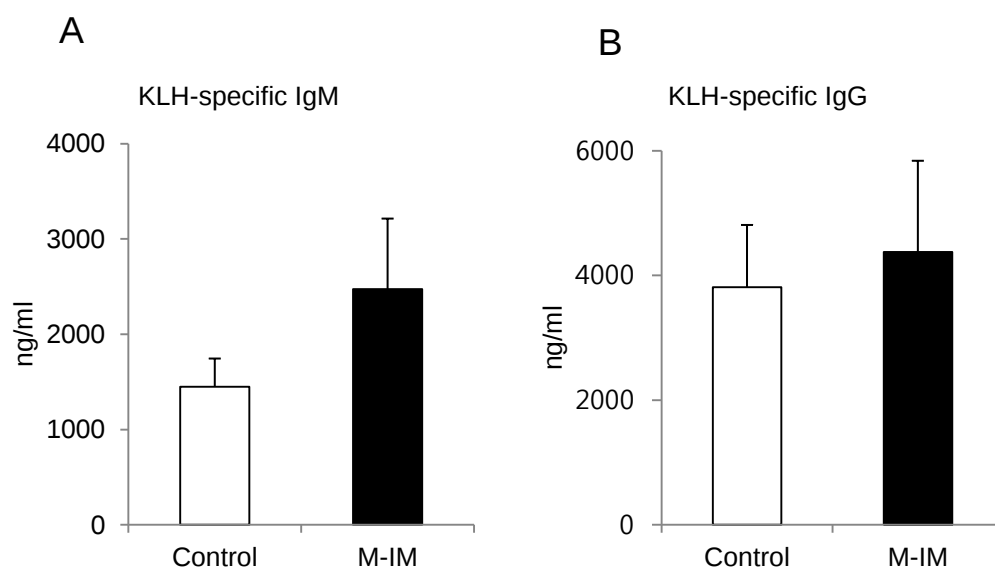


Figure 3-5. Antigen-specific antibody in sera of the rats fed the M-IM-supplemented diet

KLH was administered subcutaneously (1 mg/rat) at day 14 as an exogenous antigen. KLH-specific (A) IgM and (B) IgG in the serum were measured at day 7 and day 18 after the administration of KLH, respectively. The antibody production was measured with ELISA kits.

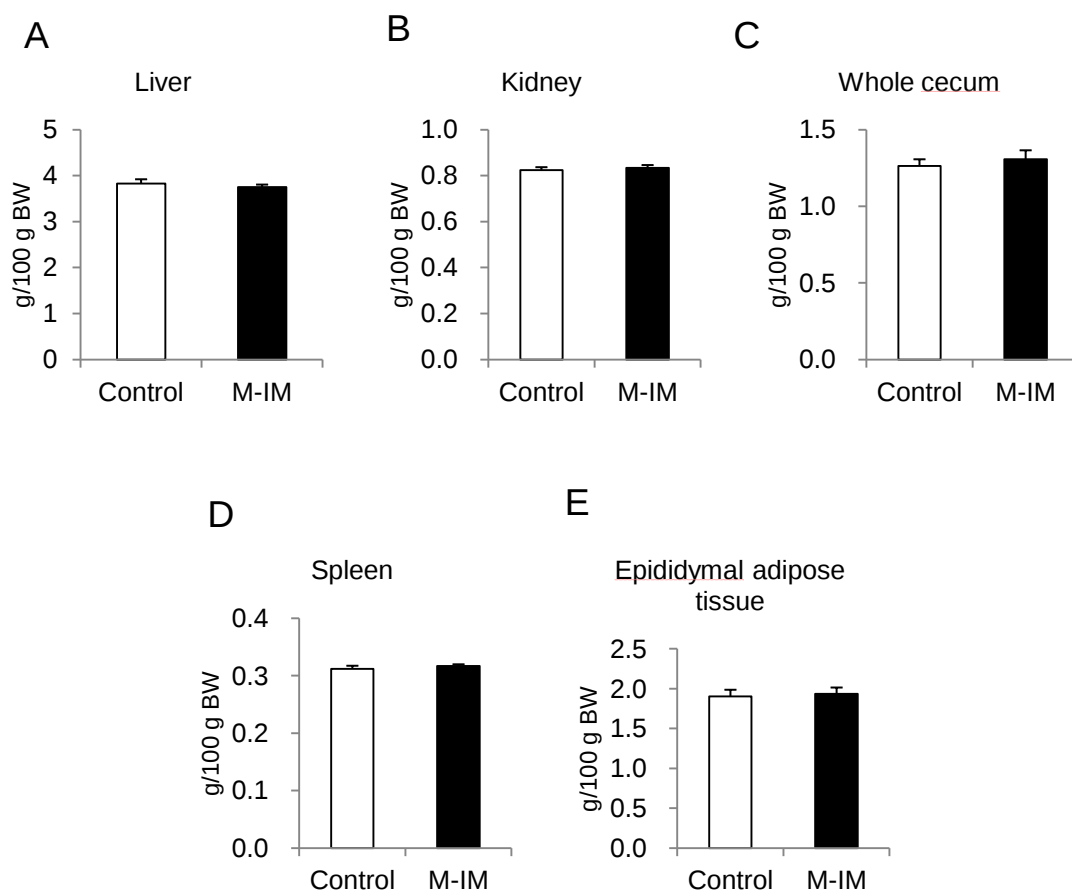


Figure 3-6. Tissue weights of the rats fed the diet with or without 3% M-IMs in LPS-treated condition

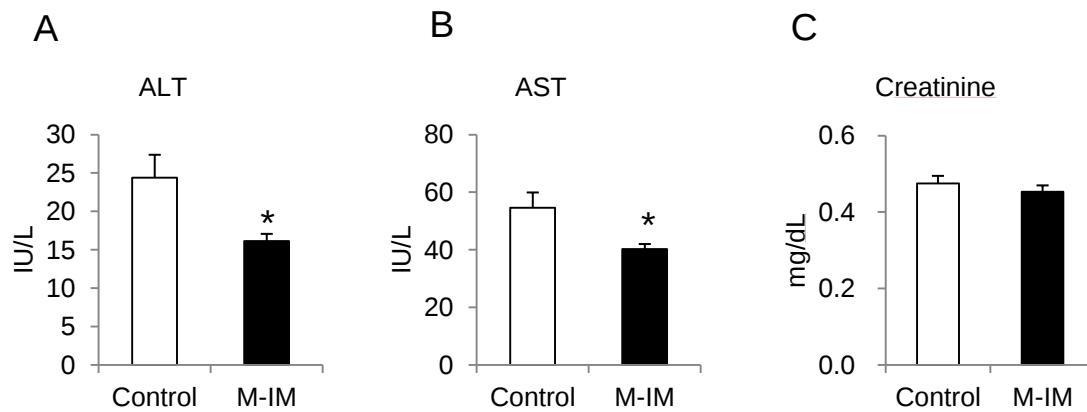


Figure 3-7. Plasma liver and kidney injury makers in the LPS-treated rats

At 6 h after the 4 mg/kg LPS subcutaneous administration, the aorta plasma was collected and stored at -80°C. (A) ALT and (B) AST in the aortic plasma were measured with wako transaminase C II test kits. (C) creatinine concentration in the aorta plasma was analyzed by using wako creatinine assay kits. Means not sharing a common letter differ significantly (n = 9-10, * $P < 0.05$, by Student's *t*-test).

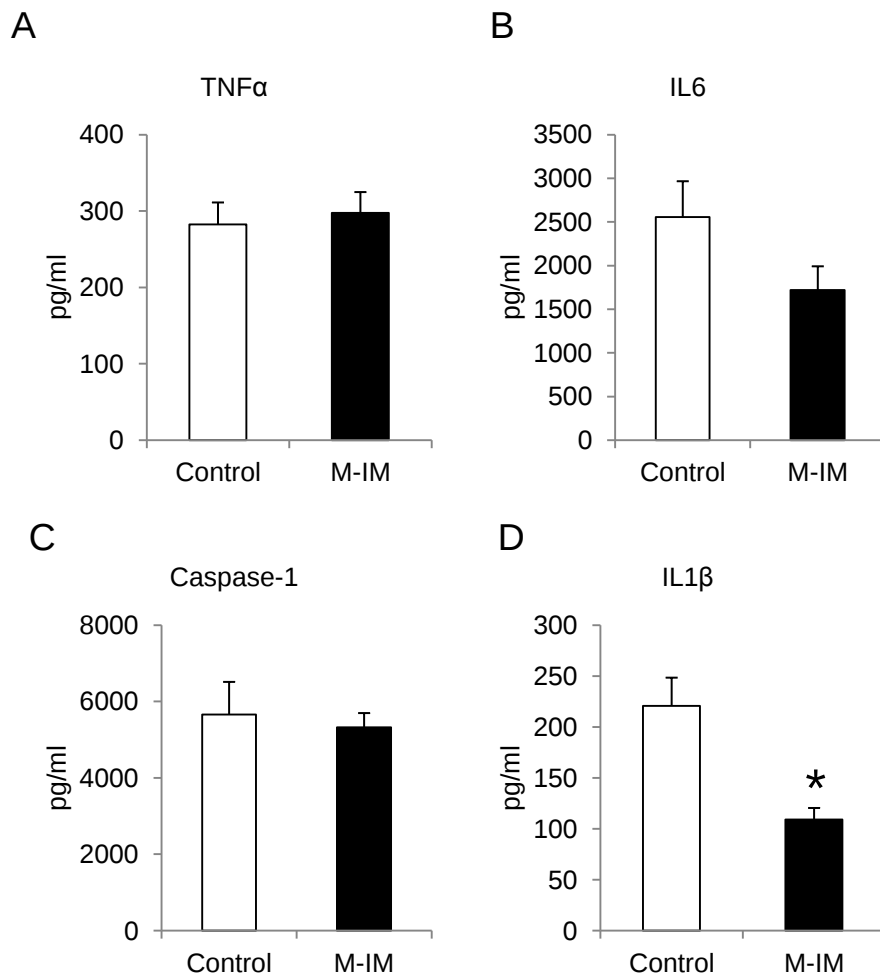


Figure 3-8. Inflammatory cytokine concentrations in aortic plasma of the LPS-treated rats

The rats were fed the diet supplemented with or without 3% M-IMs for 35 days. The rats were administrated with 4 mg/kg of LPS at day 35 to induce acute liver injury. At 6 h after the LPS administration, (A) TNF α , (B) IL6, (C) Caspase-1 and (D) IL1 β concentrations in the aorta plasma was measured with ELISA kits. Means not sharing a common letter differ significantly (n = 9-10, * $P < 0.05$, by Student's t -test).

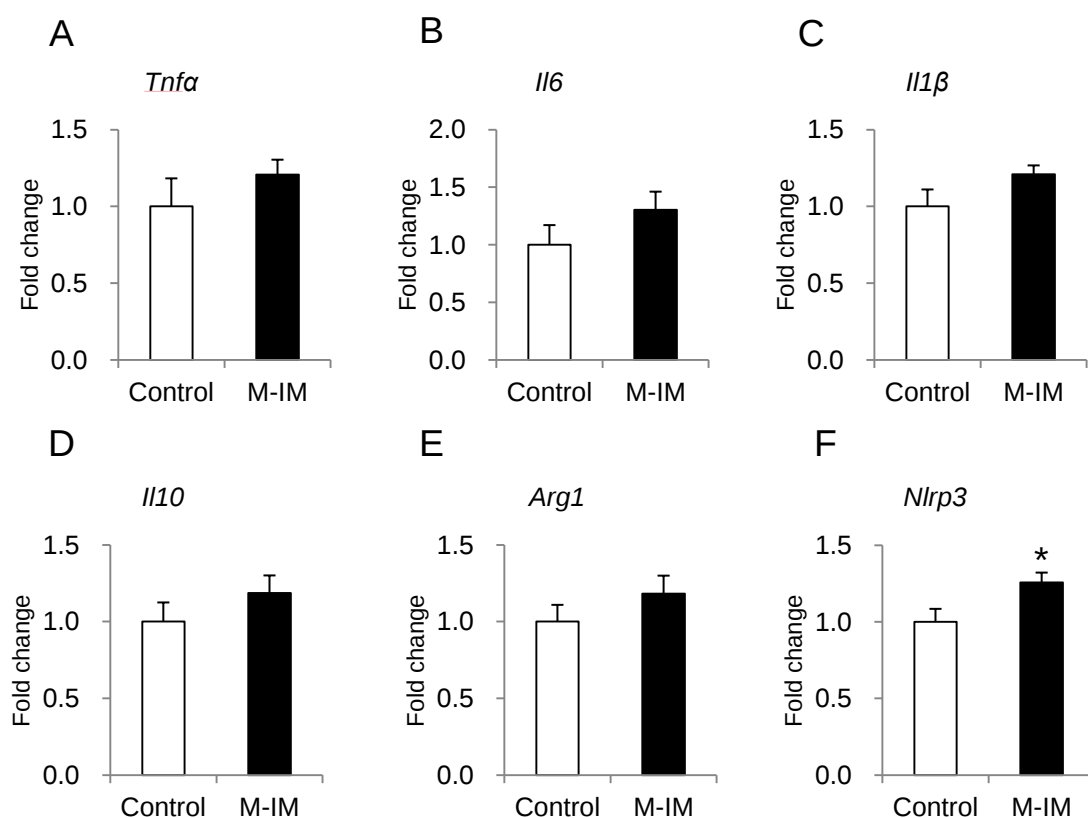


Figure 3-9. mRNA expressions in liver of the LPS-treated rats

The rats were fed the diet supplemented with or without 3% M-IMs for 35 days. The rats were administrated with 4 mg/kg of LPS at day 35 to induce acute liver injury. At 6 h after the LPS administration, the liver was collected and stored at -80°C. The RNA was isolated from liver, and the expressions of (A) *Tnfa*, (B) *Il6*, (C) *Il1β*, (D) *Il10*, (E) *Arg1*, (F) *Nlrp3* were measured by using RT-qPCR. Means not sharing a common letter differ significantly (n = 9-10, * $P < 0.05$, by Student's *t*-test).

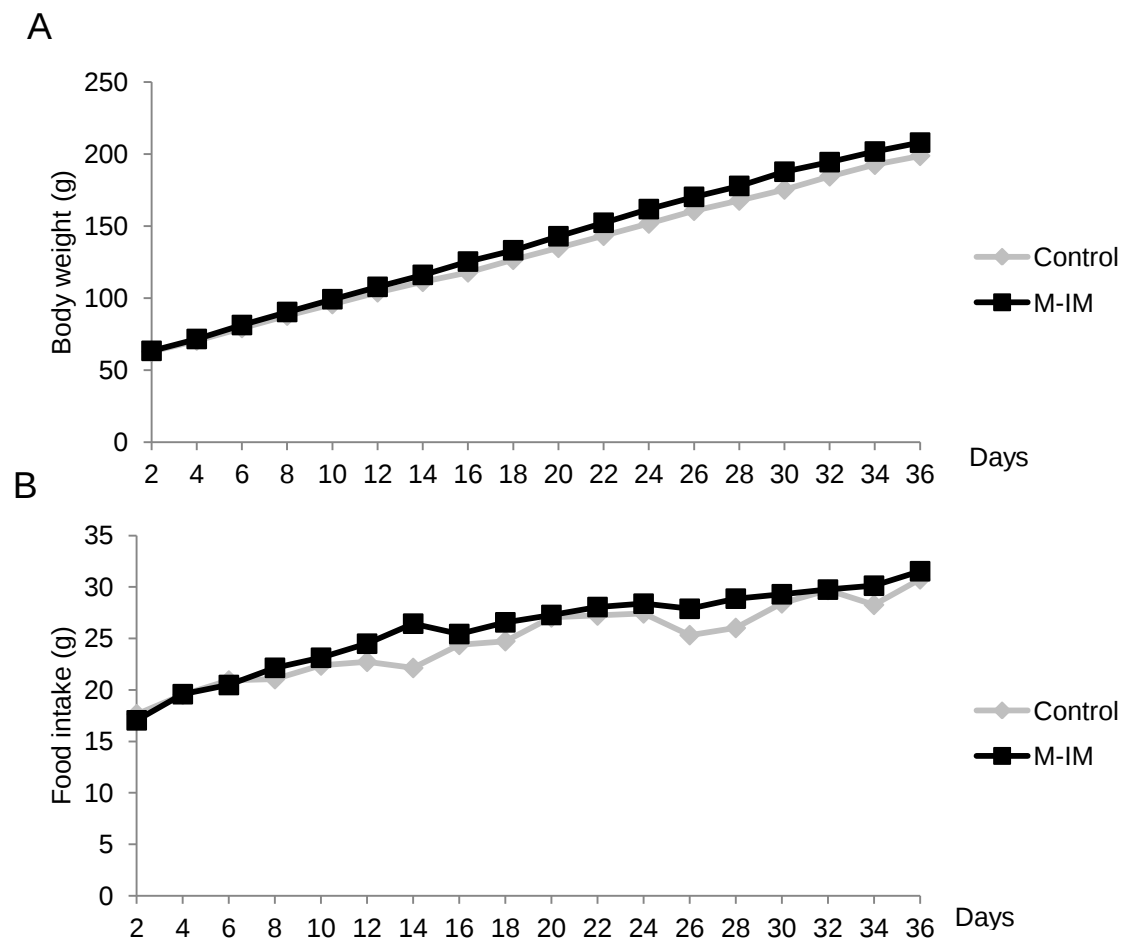


Figure 3-10. Changes in body weight and food intake in the rats fed the diet with or without 3% M-IMs

The rats were fed the diet containing M-IM (30 g/kg diet) for 36 days. (A) The body weight and (B) food intake were recorded every two days (n =6).

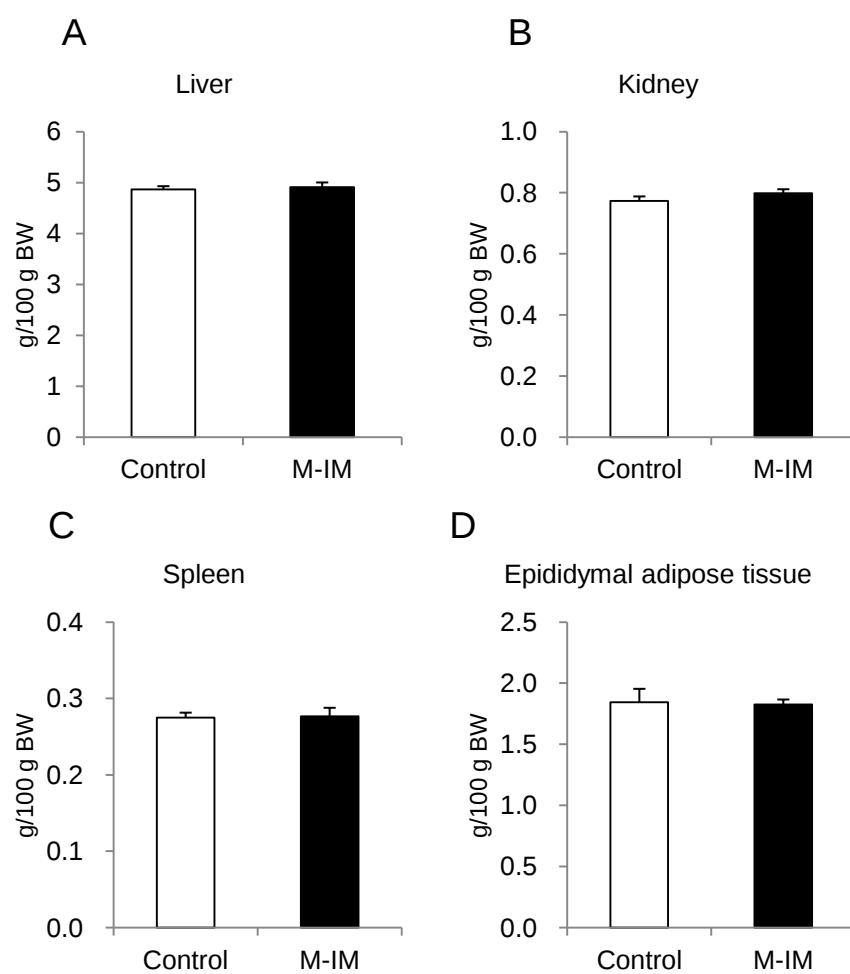


Figure 3-11. Tissue weights of the rats fed the diet with or without 3% M-IMs

(n =6)

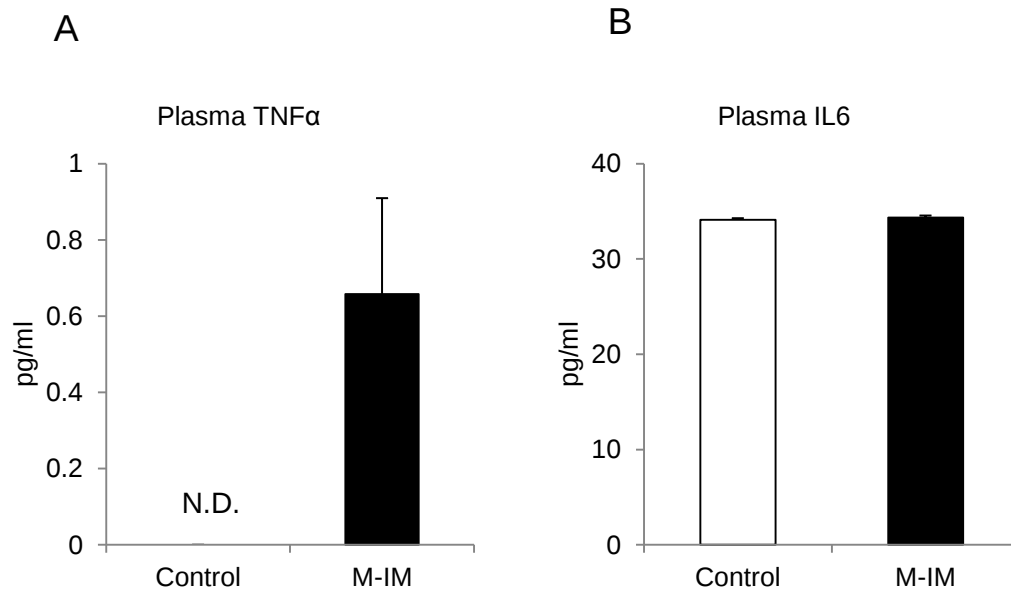


Figure 3-12. Inflammatory cytokines concentrations in aortic plasma

(A) TNF α (n =0-3). and (B) IL6 (n =6) concentrations in the aorta plasma were measured with ELISA kits. Means not sharing a common letter differ significantly. Means not sharing a common letter differ significantly.

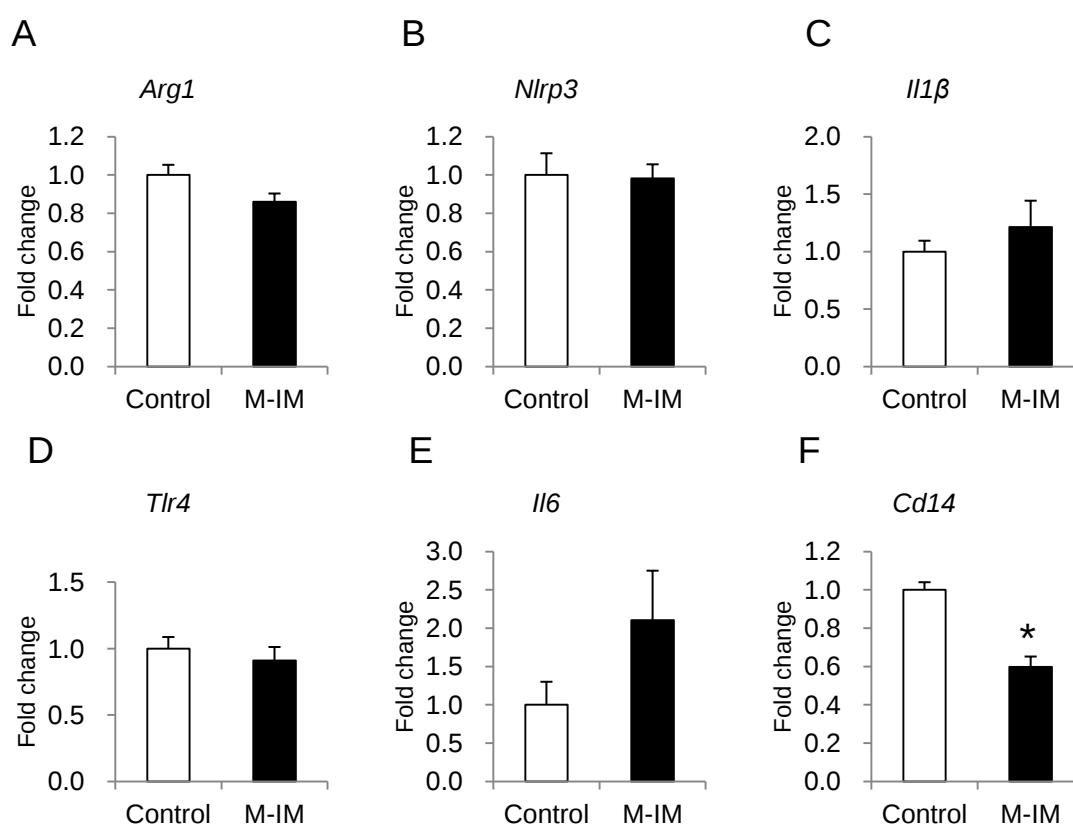


Figure 3-13. mRNA expressions in liver of the rats fed M-IM-supplemented diet

The RNA was isolated from liver, and (A) *Arg1*, (B) *Nlrp3*, (C) *Il1β*, (D) *Tlr4*, (E) *Il6*, and (F) *Cd14* expressions of factors involved in immune response were measured by using RT-qPCR. Means not sharing a common letter differ significantly (n =5-6, * $P < 0.05$, by Student's *t*-test).

3-4 Discussion

Increase in portal LPS concentration is reported in *ob/ob* and *db/db* mice (Brun P *et al.*, 2007) and a similar increase is observed in mice fed a high-fat diet (Cani PD *et al.*, 2007). Both genetically-modified mice and diet-induced obesity mice are representative models in metabolic syndrome that are accompanied by insufficient gut barrier functions. Such deterioration of gut barrier “leaky gut” would induce penetration of LPS from the gut. Interestingly, there is a postprandial increase in blood LPS even in mice fed a normal diet (Cani PD *et al.*, 2007), suggesting that such transient increase of blood LPS is involved in the onset of chronic inflammation. LPS is frequently used to induce liver failure in several studies and usually administered with other reagents such as GalN, carbon tetrachloride, or alcohols (Rahman TM *et al.*, 2000), but these treatments provoke quite severe inflammatory symptoms. On the other hand, a simple treatment with LPS can induce acute inflammation in brain (Hong CH *et al.*, 2004) and liver (Bohlinger I *et al.*, 1996). LPS treatment also increases plasma inflammatory cytokines (Kato R *et al.*, 2009). A single administration with LPS can induce inflammatory responses in many organs although the symptoms are relatively subtle. To evaluate prevention of liver failure by dietary factors, it is necessary to use models with early events rather than with severe symptoms. We then investigate to find appropriate experimental condition with single injection of LPS to induce liver damages. There is a report (Kato R *et al.*, 2009) showing that significant increases in the plasma concentrations are detected in rats at 3 and 6 h in TNF α and at 3, 6, and 9 h in IL1 β after peritoneal injection of LPS. In contrast, the increase in nitric oxide production was not observed at 3 h after LPS

administration in the same study. A similar LPS administration also enhances the activities of ALT and AST at 3 and 6 h post-administration in mice (Sato M *et al.*, 1995). Judged by alteration of these plasma parameters, we determined that appropriate endpoint was at 6 h after injection of LPS in the present study.

There is a report showing that a reduction of survival is observed when the rats received 5 mg/kg of LPS (Tavares E and Miñano FJ, 2010). Although massive fluctuation in the values was detected at 4 mg/kg of LPS, we predict that a significant difference can be secured stably if the number of individuals is increased per group. In general, it might be quite difficult to detect significant influence of dietary intervention in severe inflammation models. Also, we would like to determine preventive effects of dietary intervention in an early phase of disease models. According to such points of view, we tried to establish a mild liver inflammation model by using LPS administration. In order to ensure to induce liver injury, we selected 4 mg/kg of LPS in the present experiment. If LPS is injected intraperitoneally, abdominal organs including liver are exposed to enormous amount of LPS, which would induce unexpected responses against LPS in this site. To avoid this situation, we decided to inject LPS subcutaneously.

AST is present in liver, heart skeletal muscle, kidney, brain and red blood cell (Giannini EG *et al.*, 2005), whereas ALT intensively exists in the liver compared to other tissues such as kidney. Blood ALT activity is considered to increase specifically in liver injury (Giannini EG *et al.*, 2005). To determine whether the LPS treatment influences kidney functions, we measured creatinine concentration in aortic plasma. In the present study, no difference of the creatinine concentration with or without LPS administration accompanied by LPS-induced increase in liver inflammatory factors suggests that this model represents inflammation in liver rather than that in kidney. On the other hand, liver injury can be induced by GalN (Kawano N *et al.*, 2007), but there are extremely high levels of ALT and

AST activities in response to the treatment as compared to those in the present study, suggesting unexpected influences not only in liver but in the other organs such as kidney by administration with GalN. Therefore, the liver injury in the present experiment might be a reasonable model to represent an early stage of liver inflammation. As a result, we observed amelioration of the LPS-induced liver injury by the ingestion of M-IMs.

Attenuation of the transaminase activities in the M-IM-fed rats suggests modulation of inflammatory cytokines by M-IMs. Both TNF α and IL1 β are produced in initial step of liver inflammation (Su GL, 2002) and regulate IL6 production (aloisi F *et al.*, 1992; Shalaby MR *et al.*, 1989). Due to no difference of the TNF α production between the groups, the M-IM ingestion may suppress IL1 β - IL6 pathway in the LPS-induced liver injury. IL1 β production requires formation of inflammasome in innate immune cells (Guo H *et al.*, 2015). Inflammasome activates caspase-1, apoptosis-associated speck-like protein containing CARD, and NLRP3, which produces inflammatory cytokines such as IL1 β and IL18 in response to LPS. No apparent suppression of inflammasome by the M-IM ingestion in terms of gene expression in the present study. The M-IM ingestion may modulate some of these factors in protein levels. It is reported that serum IL1 β and IL18 concentrations increase in response to GalN/LPS in mice with a myeloid cell-specific knockout of autophagy protein 5 (Atg5) (Ilyas G *et al.*, 2016). Also, deficiency of Atg5 promotes serum ALT activity and liver injury grade in response to GalN/LPS, and the liver injury reduced by IL1 receptor antagonist (IL1Ra) (Ilyas G *et al.*, 2016). It is suggested that autophagy protects liver injury by inhibition of IL1 β . Also, the ALT activity was decreased by IL1Ra treatment in alcohol-induced liver inflammation mice (Petrasek J *et al.*, 2012), suggesting that IL1 β promotes liver injury. It is reported that lack of Atg5-dependent autophagy in macrophages promoted M1 polarization and enhanced inflammatory cytokines such as TNF α , CCL5, IL6, CCL2 and IL1 β (Liu K *et al.*, 2015), suggesting that such population of macrophages may affect inflammation process

in the liver after exposure to LPS. Considering that LPS-induced liver injury is exacerbated by lack of autophagy in mice fed a high-fat diet (Liu K *et al.*, 2015), it is possible that M-IM attenuates liver injury via modulation of autophagy.

LPS-induced inflammatory response occurs via interaction of proteins such as LBP, CD14, MD-2, TLR4 (Akira S *et al.*, 2006; Lu YC *et al.*, 2008). The TLR4 signaling induces activation of NF- κ B and MAPK, and produces inflammatory cytokines. In literature (Su GL, 2002), CD14 is an essential factor to exacerbate LPS-induced inflammation and liver injury. TNF α production in response to LPS decreases in Kupffer cells isolated from CD14 knockout mice (SU GL *et al.*, 2002). Also, in alcohol-induced liver injury mice, ALT activity is reduced in CD14-knockout mice (Yin M *et al.*, 2001). As a result, the ingestion of M-IMs reduced the expression of *Cd14* in liver, which may contribute to attenuation of liver injury in the LPS-treated rats.

In the cellular experiment, the M-IMs were recognized by primary macrophages and induced inflammatory cytokines but not O-IMs. It is possible that M-IMs modulate adaptive immunity via regulation of innate immunity such as macrophages functions. The M-IMs have no effect on body weight and food intake for experimental period. There were no significant differences between groups in KLH-specific antibody production, indicating that the M-IMs modulate innate immunity rather than adaptive immunity. Also, M-IMs specifically ameliorate inflammation with local immunity.

In conclusion, ingestion of M-IMs ameliorated LPS-induced acute liver injury in rats. Such reduced inflammation by the M-IMs may be associated with dysfunction of CD14 signaling (Fig. 3-14). Also, there is no influence of the M-IM ingestion on antibody production, indicating that M-IMs influence the innate immunity rather than the adaptive immunity, which may contribute to prevention of acute inflammation induced by endotoxin.

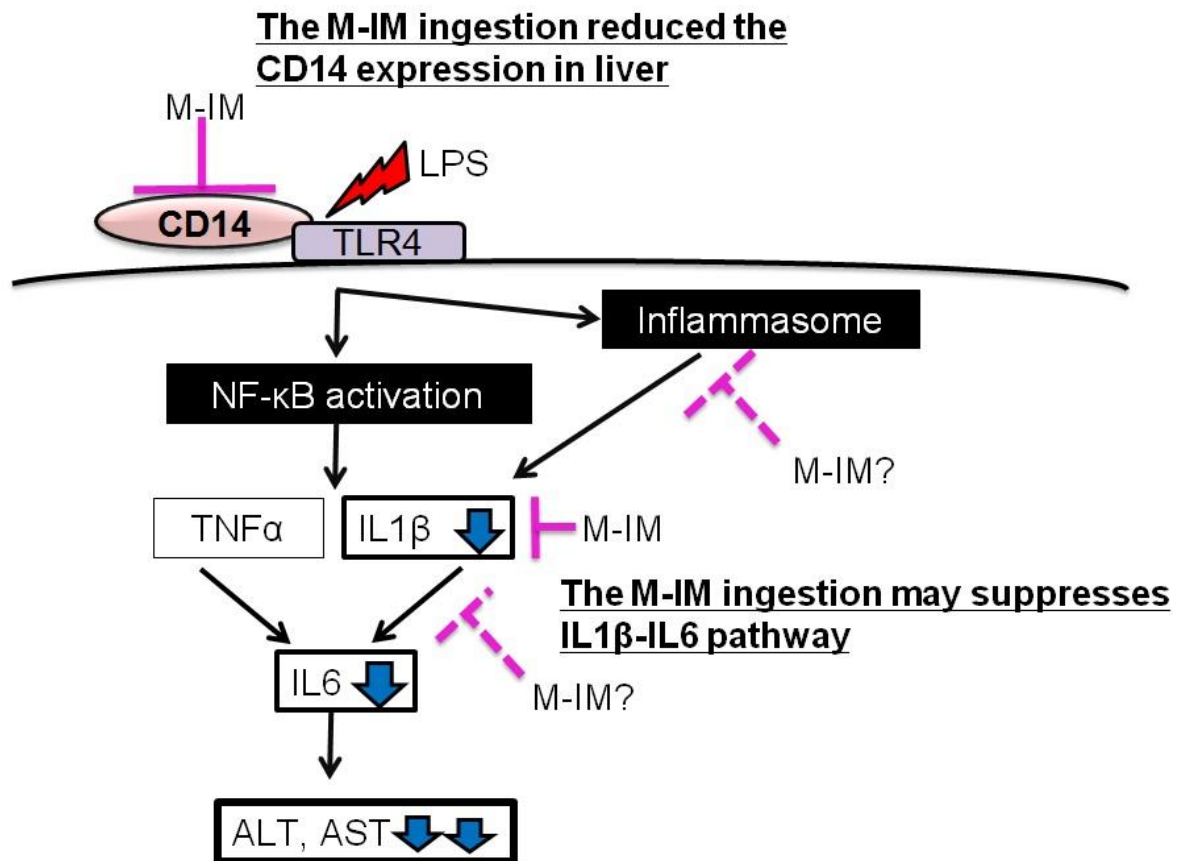


Figure 3-14. Presumable mechanisms of suppression of LPS-induced hepatitis by M-IMs

Chapter 4

General Discussion

General Discussion

Currently, a variety of hepatitis models in animal experiments are available such as GalN, LPS with GalN, and carbon tetrachloride etc. The symptoms of these models are basically quite severe although it depends on the dose of the reagents, which ensure a clear difference of parameters of interest including blood cytokines and transaminases, gene expressions as well as histological observations. Such severe symptoms might be appropriate to evaluate efficiency of pharmaceutical reagents because some clinical symptoms are as such. However, development of the disease established by the hepatitis inducers is not accordance with those of naturally-occurred hepatitis. Dietary habit can be one of the environmental factor in disease development, which is frequently involved in chronic inflammation. The influence of dietary intervention is not strong as compared to that of pharmaceutical reagents and cannot be detected in severe hepatitis models. In literature (Cani PD *et al.*, 2007), high-fat diet consumption induces steady increase in blood LPS concentration with leaky gut. As shown in chapter 3, a single administration of LPS can induce hepatitis, but the symptom is relatively weak than that induced by other treatments. However, the situation happened in a high-fat-fed condition. LPS-induced hepatitis in this experiment shows the symptoms in initial inflammation, which represents an early phase in chronic inflammation. We propose that the experimental model with single injection of LPS can be used to examine preventive effect of dietary intervention or food ingredients on hepatitis.

By using this hepatitis model, one of the possible targets, CD14 signaling, was identified in suppression of the LPS-induced hepatitis by the ingestion of M-IMs. Although We confirmed the suppressing effect of M-IMs in the liver inflammation, the precise

mechanisms are remains to be elucidated. For example, (1) How does the M-IM ingestion reduces gene expression of *Cd14* in the liver, (2) Does the M-IM modulates macrophage population (M1 and M2) in the liver, (3) What is the effective size in DP or the effective composition of sugars in M-IMs, (4) the M-IMs are able to be absorbed as the intact form, (5) Can luminal M-IMs be a signal to inducing responses in the cells in Peyer's patches, etc. These might be future working hypothesis to clarify the mechanism. One concern is the fate of LPS injected in the model. There might be significant problem if the LPS remains in the animals, which can induce another inflammation in the body. To elucidate this possibility, a long-term is necessary experiment in a similar treatment by using LPS. Obviously, appropriate dosage should be determined for the long-term experiment. If the LPS is successfully eliminated in the rats fed M-IMs, the M-IMs can be used as a functional ingredient in food to prevent chronic inflammation as well as NCDs in a clinical application.

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Publication and Academic Conferences

A) Publication

- ① **Joe GH**, Andoh M, Nomura M, Iwaya H, Lee JS, Shimizu H, Tsuji Y, Maseda H, Miyazaki H, Hara H and Ishizuka S (2014) Acyl-homoserine lactones suppress IEC-6 cell proliferation and increase permeability of isolated rat colon. *Biosci Biotechnol Biochem* 78, 462-5.
- ② **Joe GH**, Andoh M, Shinoki A, Lang W, Kumagai Y, Sadahiro J, Okuyama M, Kimura A, Shimizu H, Hara H and Ishizuka S (2016) Megalo-type α -1,6-glucosaccharides induce production of tumor necrosis factor α in primary macrophages via toll-like receptor 4 signaling. *Biomed Res* 37(3), 179-186.
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- ④ Shimizu H, Baba N, Nose T, Taguchi R, Tanaka S, **Joe GH**, Maseda H, Nomura N, Hagio M, Lee JY, Fukiya S, Yokota A, Ishizuka S and Miyazaki H (2015) Activity of ERK regulates mucin 3 expression and is involved in undifferentiated Caco-2 cell death induced by 3-oxo-C12-homoserine lactone. *Biosci Biotechnol Biochem* 79(6), 937-42.
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- ⑥ Taguchi R, Tanaka S, Joe GH, Maseda H, Nomura N, Ohnishi J, Ishizuka S, Shimizu H and Miyazaki H (2014) Mucin is involved in the apoptosis of intestinal epithelial cells via *N*-(3-oxododecanoyl)-L-homoserine lactone-induced suppression of Akt phosphorylation. *Am J Physiol Cell Physiol* 307, 162-8.

B) Academic conference

- ① Joe GH, Iizuka T, Fujimoto Y, Kimura A, Hara H and Ishizuka S. Ingestion of Megalo-type Isomaltoglucosaccharides Ameliorates Lipopolysaccharide-induced Acute Liver Injury in Rats. Experiment Biology 2017, Chicago, USA, April, 2017.
- ② Joe GH, Iizuka T, Fujimoto Y, Kimura A, Hara H and Ishizuka S. Modulation of inflammatory responses by megalo-type isomaltoglucosaccharides. The Japan Society for Bioscience, Biotechnology and Agrochemistry Hokkaido Branch, Sapporo, Japan, November, 2015.
- ③ Joe GH, Hori S, Shimizu H, Hara H and Ishizuka S. A reduction of myeloperoxidase and interleukin-10 expressions in mesenteric lymph nodes of the rats fed a cholic acid-supplemented diet. Experiment Biology 2016, San Diego, USA, April, 2016.
- ④ Joe GH, Kikuchi K, Tsuji M, Tada K, Shimizu H, Hara H and Ishizuka S. A diet supplemented with cholic acid induced hepatic steatosis without apparent inflammation and reduced myeloperoxidase and interleukin-10 expressions in the mesenteric lymph nodes in rats. The 21th Hindgut Club Japan, Tokyo, Japan. December, 2015.
- ⑤ Joe GH, Shinoki A, Kumagai Y, Kimura A, Funane K, Shimizu H, Hara H and Ishizuka S. Isomaltomegalosaccharides modulate rat macrophage functions in

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- ⑥ Joe GH, Shinoki A, Kumagai Y, Kimura A, Funane K, Shimizu H, Hara H and Ishizuka S. Modulation of rat macrophage functions by isomaltomegalosaccharides. 12th Asian Congress of Nutrition, Japan, Yokohama, May, 2015.
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