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LPS suppresses expression of asialoglycoprotein-binding protein through TLR4 in thioglycolate-elicited peritoneal macrophages

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Abstract Macrophages are known to express various types of endocytosis receptors that mediate the removal of foreign pathogens. Macrophage asialoglycoprotein-binding protein (M-ASGP-BP) is a Gal/GalNAc-specific lectin, which functions as an endocytosis receptor. We found here that LPS is able to down-regulate the mRNA expression of M-ASGP-BP in a time-dependent manner using thioglycolate-elicited rat and mouse peritoneal macrophages. However, LPS does not modulate the mRNA expression of M-ASGP-BP from macrophages of C3H/HeN mice, which have a point mutation of TLR4, the primary LPS receptor. Furthermore, an inhibitor of NF-κB was observed to efficiently block the suppressive effect of LPS on M-ASGP-BP as well as to inhibit the phosphorylated IκB. These results demonstrate that the mRNA expression of M-ASGP-BP is down-regulated by the LPS-mediated TLR4 pathway involving NF-κB activation, suggesting that engagement of M-ASGP-BP by LPS may yield a negative signal that interferes with the LPS-induced positive signals mediated by proinflammatory cytokines.

Keywords Macrophage asialoglycoprotein-binding protein · C-type lectin · LPS · TLR4 · NF-κB

Abbreviations

M-ASGP-BP	macrophage asialoglycoprotein-binding protein
MGL	macrophage C-type galactose/*N*-acetylgalactosamine-specific lectin
Mϕ	macrophage
LPS	lipopolysaccharide
TLR4	toll-like receptor 4
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated protein kinase
JNK	c-Jun amino-terminal kinase
NF-κB	nuclear factor kappa B
IκB	inhibitor of NF-κB

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Introduction

Macrophages (Mϕs) are phagocytic cells widely distributed throughout the body, and play essential roles in host defense by coordinating innate immunity and inflammatory responses. Resident Mϕs mediate the clearance of senescent or apoptotic cells, produce and secrete cytokines, are involved in hemopoiesis and bone resorption, transport and present antigens, and regulate neuroendocrine processes. Activated Mϕs are recruited to sites of infection or injury by immune or inflammatory stimuli, and play crucial roles in acute and chronic inflammation, tissue repair, immuno-

pathology and neoplasia [1]. The Mϕ possesses a sizeable repertoire of lectins (carbohydrate-binding proteins) that it utilizes to mediate an extensive range of functions, both immune and otherwise. On its surface, this multipotent cell is equipped with C-type (calcium-dependent), I-type (immunoglobulin-like), and R-type (ricin-like) lectins. Among them, the C-type lectins are the largest, most diverse and most prevalent in immune responses, and contribute to carbohydrate-mediated endocytosis [2, 3].

Lipopolysaccharides (LPS) are major integral components of the outer membrane of gram-negative bacteria. When released from bacteria, LPS elicit in higher organisms a broad spectrum of biological activities, especially activation of immune and inflammatory cells, including Mϕs, monocytes, and endothelial cells [4]. Vascular endothelial cells, like Mϕs, play a central role in a host's defense against bacterial infection and are a major cellular target for the LPS action. LPS has multiple effects on Mϕs, including the induction of secreted inflammatory mediators such as leukocyte adhesion molecules, soluble cytokines, and chemokines [5, 6]. Many diseases are characterized by inflammatory reactions involving both the innate and adaptive arms of the immune system. Thioglycolate medium injection into the peritoneal cavity has long been used as a stimulus for eliciting inflammatory Mϕs for study and for determining the importance of a particular mediator in inflammation.

Mϕ asialoglycoprotein-binding protein (M-ASGP-BP), also referred to as Mϕ C-type galactose/*N*-acetylgalactosamine-specific lectin (MGL), was first isolated from rat peritoneal elicited Mϕs and characterized as a Gal/GalNAc-specific lectin, and was shown to be structurally and functionally similar to rat liver ASGP-BP (or rat hepatic lectin; RHL) [7, 8]. M-ASGP-BP is a 42 kDa type II transmembrane glycoprotein that is responsible for carbohydrate-mediated endocytosis [7]. M-ASGP-BP mRNA is intensely expressed in thioglycolate-elicited peritoneal Mϕs but only slightly in resident peritoneal Mϕs [9]. It has been reported that the Mϕs accumulated at the sites of chronic rejection of rat heart transplants express M-ASGP-BP mRNA [10]. These data suggest that the expression of M-ASGP-BP mRNA is induced during the process of Mϕ activation or differentiation. As Mϕs have been shown to acquire enhanced bactericidal and tumoricidal ability during differentiation and activation, the induction of M-ASGP-BP mRNA expression should contribute to them.

The purpose of this study was to determine the effects of LPS on M-ASGP-BP gene expression in thioglycolate-elicited peritoneal Mϕs. We report that LPS is capable of down-regulating expression of the M-ASGP-BP gene through a transcriptional mechanism. This modulatory function of LPS appears to be mediated by the LPS receptor TLR4, involving the transcription factor NF-κB to elicit intracellular signaling events that result in decreased transcription of the M-ASGP-BP gene.

Materials and methods

Materials

Eight-week old Wistar/ST male rats, C3H/HeJ and C3H/HeN male mice were supplied by Japan SLC, Inc. (Shizuoka, Japan). LPS from *E.coli* 0111 B4 and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO). A 3% thioglycolate solution was obtained from Becton Dickinson (Cockeysville, MD). An Ultraspec-II RNA isolation system and SuperScript II RNaseH(-) Reverse Transcriptase kit were purchased from Biotex Laboratories Inc. (Edmonton, Alberta, Canada) and Invitrogen (Carlsbad, CA), respectively. Rabbit polyclonal anti-ERK1/2 and mouse monoclonal anti-phosphorylated IκB-α (5A5) antibodies were purchased from Cell Signaling (Beverly, MA). Monoclonal antibody against phosphorylated ERK1/2 (E-4) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Kinase inhibitors PD98059 and SB202190, protein synthesis inhibitor cycloheximide and anti-IκB-α rabbit polyclonal antibodies were purchased from Calbiochem (San Diego, CA). A protease inhibitor cocktail was obtained from Nacalai Tesque (Kyoto, Japan). All chemicals for gel electrophoresis and Western blot were supplied by ATTO (Tokyo, Japan), Bio-Rad (Hercules, CA), and PIERCE (Rockford, IL).

Preparation of rat and mouse thioglycolate-elicited peritoneal Mϕs

Thioglycolate-elicited peritoneal cells were obtained from Wistar/ST male rats, or C3H/HeJ or C3H/HeN male mice according the procedure described previously [7]. Briefly, rats and mice were injected with 10 and 2 ml of 3% thioglycolate broth intraperitoneally, respectively. Four days after the injection, peritoneal elicited cells were harvested by lavage with 20 ml of RPMI1640 medium. Peritoneal elicited cells were plated on 10 cm dishes ($1.0\sim2.0\times10^7$ cells/dish) in RPMI1640 medium containing 10% FCS, 3% glutamine. After 1 h incubation, the dishes were washed with PBS to remove nonadherent cells and the resultant adherent cells were used as elicited peritoneal Mϕs.

Treatment of Mϕs with LPS or various inhibitors

Thioglycolate-elicited peritoneal Mϕs were treated with 10 ng/ml LPS for 2, 6 and 24 h, respectively. Protein synthesis inhibitor cycloheximide was added at 100 nM. MEK1/2-

specific inhibitor PD98059 or anti-oxidant NF-κB inhibitor PDTC was added at the concentration of 20 or 30 μM, respectively. Each inhibitor was added 30 min prior to LPS stimulation.

RNA preparation and RT-PCR determination

Total RNA was extracted from thioglycolate-elicited peritoneal Mϕs induced by LPS for different times using the Ultraspec-II RNA isolation system. A reverse transcriptase reaction was performed with 5 μg of total RNA using the SuperScript II kit according to the manufacturer's instructions. Following cDNA synthesis, PCR was then carried out with specific primers: rat M-ASGP-BP forward, 5′-CTCA GAACAGATCTATCTGAA-3′; rat M-ASGP-BP reverse, 5′-CAGTGCGTAAAGCCTTTCT-3′; mouse M-ASGP-BP forward, 5′-TGGACCCACCTCCTCCTGTT-3′; mouse M-ASGP-BP reverse, 5′-GTTGGCCAGCTGGCACGTCA-3′; rat IL-1β forward, 5′-GACTTGGGCTGTCCAGATGA-3′; rat IL-1β reverse, 5′-TGCTCTGCTTGAGAGGTGCT-3′; rat TNF-α forward, 5′-CAAGGAGGAGAAGTTCCCA-3′; rat TNF-α reverse, 5′-CTCCTGGTATGAAGTGGAAA-3′; rat IL-6 forward, 5′-CAGAAAACAATCTGAAACT TCCA-3′; rat IL-6 reverse, 5′-AGTTCTTCGTAGAGAAC AACATA-3′; rat iNOS forward, 5′-GGTCAAGATCCA GAGGTCTT-3′; rat iNOS reverse, 5′-ACCAAGACTGT GAACCGGAT-3′; mouse iNOS forward, 5′-CCCTT CCGAAGTTTCTGGCAGCAGC-3′; mouse iNOS reverse, 5′-GGCTGTCAGAGCCTCGTGGCTTTCG-3′; rat β-actin forward, 5′-CAGAGCAAGAGAGGCATCCT-5′; rat β-actin reverse, 5′-AAGGCTGGAAGAGAGCCTCG-3′; mouse β-actin forward, 5′-AAGAGCTATGAGCTGCCTGA-3′; mouse β-actin reverse, 5′-CAGGAGGAGCAATGATCTTG-3′, respectively. 30 cycles of PCR were performed, each cycle consisting of denaturation at 94°C for 30 s, annealing at 57°C (rat primers) or 60°C (mouse primers) for 60 s, and extension at 72°C for 60 s. The PCR products were electrophoresed through an agarose gel and visualized by ethidium bromide staining.

Assaying of MEK/ERK1/2, p38 MAPK and IκB activation in LPS-induced Mϕs

To examine the tyrosine phosphorylation of ERK1/2, threonine/tyrosine phosphorylation of p38 MAPK and serine phosphorylation of IκB in thioglycolate-elicited peritoneal Mϕs, the cells were cultured in serum-free RPMI 1640 medium containing 20 μM PD98059, 2 μM SB202190, or 30 μM PDTC for 30 min and then treated with 10 ng/ml LPS or 10 and 60 min, respectively. The LPS-induced Mϕs were lysed with 1% NP40 lysis buffer, and the whole cell lysates were resolved on a 5–20% Tris-HCl gradient gel (ATTO) and then analyzed by Western blotting [12]. Western blotting specific for each phosphor protein was performed first and then the assay membrane was reprobed with ERK1/2, P38 MAPK or IκB specific antibodies. For visualization, a SuperSignal West Pico Chemiluminescent kit (PIERCE) was used with HRP-conjugated anti-mouse/rabbit IgG antibodies (Zymed).

Results

LPS down-regulates M-ASGP-BP mRNA accumulation in a time-dependent manner in thioglycolate-elicited peritoneal Mϕs In previous studies, M-ASGP-BP mRNA was expressed in elicited peritoneal Mϕs but only slightly in resident peritoneal Mϕs [9]. To study the kinetics of the LPS effects upon the mRNA levels of M-ASGP-BP and some proinflammatory cytokines (IL-6, IL-1β, and TNF-α were used as well-established LPS-up-regulated cytokines), thioglycolate-elicited rat peritoneal Mϕs were exposed to LPS (10 ng/ml) for increasing periods of time (Fig. 1). The thioglycolate-elicited rat peritoneal Mϕs without the LPS treatment expressed a high-level of M-ASGP-BP mRNA. The decrease in M-ASGP-BP gene expression was detectable as early as 6 h after exposure to LPS and became the maximum after 24 h. The LPS-induced increase in IL-6, IL-1β, TNF-α or iNOS expression exhibited more rapid kinetics and became the maximum after 2 h LPS exposure, remaining significantly elevated versus the control level until 24 h. This demonstrates that in addition to the widely known ability of LPS to induce the expression of proinflammatory cytokine genes, LPS is a powerful inhibitor of M-ASGP-BP expression in thioglycolate-elicited peritoneal Mϕs.

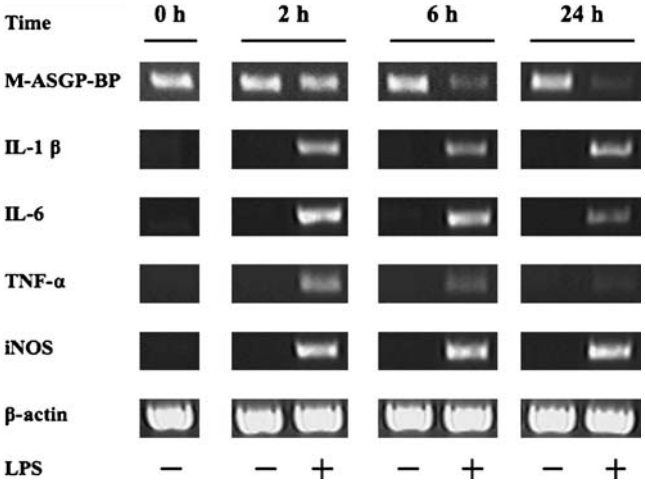

Fig. 1 Time course analysis of the down-regulatory effect of LPS on M-ASGP-BP mRNA expression. Thioglycolate-elicited rat peritoneal Mϕs were treated with LPS at 10 ng/ml for 2–24 h. At the indicated times, the Mϕs were lysed in TRIZOL and total RNA was purified. The expression of M-ASGP-BP, IL-1β, IL-6, TNF-α and iNOS mRNA was analyzed by RT-PCR as described under "Materials and methods"

Inhibition of protein synthesis does not suppress the down-regulatory effect of LPS on M-ASGP-BP mRNA accumulation in thioglycolate-elicited peritoneal Mϕs LPS exposure induces the release of various cytokines/growth factors, such as IL-1, IL-6, iNOS and TNF-α. Thus, in order to determine whether the down-regulatory effect of LPS on M-ASGP-BP mRNA was caused by LPS-induced *de novo* synthesis of proteins, thioglycolate-elicited rat peritoneal Mϕs were incubated with or without 100 nM cycloheximide (CHX), an inhibitor of protein synthesis, for 30 min, and then exposed to LPS at 10 ng/ml for 6 h. As shown in Fig. 2, RT-PCR analysis revealed that CHX did not affect the LPS-induced decrease in M-ASGP-BP mRNA. These results indicate that LPS-induced protein synthesis, such as of proinflammatory cytokines, does not down-regulate the M-ASGP-BP mRNA accumulation.

Blockade of TLR4 inhibits the suppressive effect of LPS on M-ASGP-BP mRNA accumulation in thioglycolate-elicited mouse peritoneal Mϕs TLR4 functions as a primary LPS signaling receptor. To determine whether LPS acts *via* TLR4 to down-regulate M-ASGP-BP mRNA, we compared the effects of LPS on M-ASGP-BP mRNA in thioglycolate-elicited peritoneal Mϕs derived from C3H/HeJ and C3H/HeN mice, which are well recognized to be fully histocompatible strains. The C3H/HeJ mouse has a missense mutation in the coding region for the TLR4 gene, and is characterized by hyporesponsiveness to LPS, quite similar to the TLR4-deficient mouse [13]. In contrast, the C3H/HeN mouse expresses a normal TLR4 molecule, which is apparently not a prerequisite for LPS sensitivity. As shown in Fig. 3, the thioglycolate-elicited peritoneal Mϕs derived from C3H/HeJ and C3H/HeN mice were incubated with 10 ng/ml LPS for 24 h, respectively. RT-PCR analysis revealed that the suppressive effect of LPS on M-ASGP-BP mRNA extracted from C3H/HeJ mice was completely abolished (Fig. 3a) compared with that in the case of C3H/HeN mice (Fig. 3b). These data suggest that the expression of wild-type TLR4 from C3H/HeN mice but not the mutant

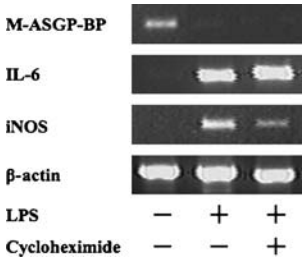

Fig. 2 Effect of cycloheximide treatment on LPS-induced suppressive expression of M-ASGP-BP mRNA. Thioglycolate-elicited rat peritoneal Mϕs were treated with cycloheximide at 100 nM for 30 min, and then incubated with LPS at 10 ng/ml for 6 h. The expression of M-ASGP-BP, IL-6, and iNOS mRNA was analyzed by RT-PCR as described under "Materials and methods"

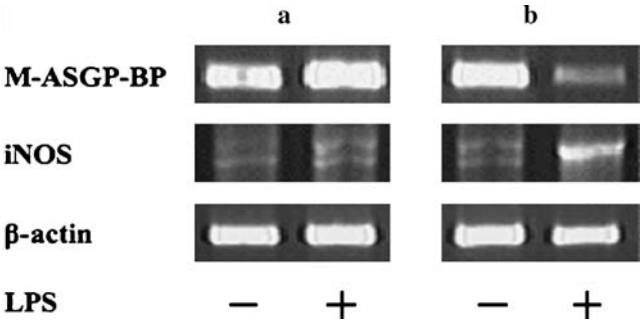

Fig. 3 Involvement of TLR4 in the LPS-induced down-regulatory effect on M-ASGP-BP mRNA expression. Thioglycolate-elicited peritoneal Mϕs derived from C3H/HeJ **a** and C3H/HeN **b** mice were induced with 10 ng/ml LPS for 24 h, respectively. The expression of M-ASGP-BP, and iNOS mRNA was analyzed by RT-PCR as described under "Materials and methods"

TLR4 from C3H/HeJ mice *via* NF-κB activation mediates the down-regulatory effect of LPS on the M-ASGP-BP gene.

Inhibition of MEK/ERK1/2 or p38 MAPK activity does not suppress the down-regulatory effect of LPS on M-ASGP-BP mRNA in thioglycolate-elicited peritoneal Mϕs LPS stimulates Mϕs, and induces the secretion of inflammatory mediators such as growth factors, chemokines, and inflammatory cytokines, which activate three distinct MAPK cascades: the MEK/ERK1/2, JNK/SAPK, and p38 MAPK pathways. To determine whether the MEK/ERK1/2 and p38 MAPK pathways mediate the down-regulatory effect of LPS on M-ASGP-BP mRNA, thioglycolate-elicited rat peritoneal Mϕs were preconditioned with MEK/ERK1/2 inhibitor PD98059 (at 20 μM) and p38 MAPK inhibitor SB202190 (at 2 μM), respectively, for 30 min, followed by further addition of LPS (at 10 ng/ml) for an additional 10 min. As shown in Fig. 4, RT-PCR analysis revealed that in the presence of either PD98059 (Fig. 4a) or SB202190 (data not shown), the down-regulatory effect of LPS on M-ASGP-BP mRNA was not inhibited. In addition, the tyrosine phosphorylation of ERK1/2 was selectively inhibited by PD98059 at 20 μM (Fig. 4b). These data indicate that neither the MEK/ERK1/2 pathway nor the p38 MAPK one mediates the down-regulatory effect of LPS on the M-ASGP-BP gene.

Inhibition of NF-κB activation blocks the suppressive effect of LPS on M-ASGP-BP mRNA expression in thioglycolate-elicited peritoneal Mϕs NF-κB is a major transcription factor that up-regulates proinflammatory cytokine expression. To determine whether the suppressive effect of LPS on M-ASGP-BP mRNA was mediated through NF-κB activation, thioglycolate-elicited rat peritoneal Mϕs were preconditioned for 30 min with NF-κB activation inhibitor PDTC (at 10 μM), followed by the addition of LPS (at 10 ng/ml) for an additional 10 or 30 min and 6 h incubation, respectively. PDTC treatment effectively prevented the LPS-induced

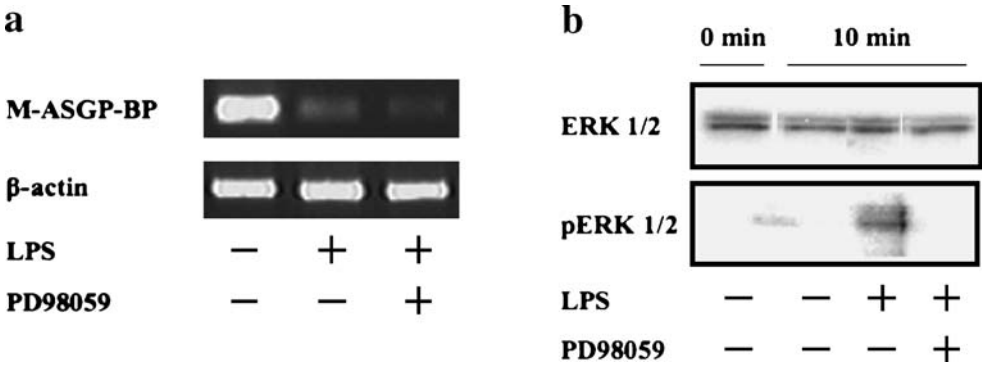

Fig. 4 Relation of the MAP kinase pathway with LPS-induced suppression of M-ASGP-BP mRNA expression. **a** Effect of an MEK/ERK1/2 inhibitor on LPS-induced suppressive expression of M-ASGP-BP mRNA. Thioglycolate-elicited rat peritoneal Mϕs were incubated with MEK/ERK1/2 inhibitor PD98059 at 20 μM for 30 min, followed by the addition of LPS at 10 ng/ml for 6 h. The expression of M-ASGP-BP mRNA was examined by RT-PCR as described under "Materials and methods." **b** Tyrosine phosphorylation of ERK1/2 induced by LPS stimulation. Thioglycolate-elicited rat peritoneal Mϕs were preconditioned with PD98059 at 20 μM for 30 min, followed by treatment with LPS at 10 ng/ml for an additional 10 min. The LPS-induced tyrosine phosphorylation of ERK1/2 was analyzed by Western blotting with specific anti-pERK1/2 antibodies, and then the assay membrane was reprobed with anti-ERK1/2 antibodies

M-ASGP-BP mRNA level decrease as well as the increase in iNOS (Fig. 5a). Furthermore, the serine phosphorylation of IκB was completely inhibited by PDTC (at 10 μM) in both 10 and 60 min (Fig. 5b). These results demonstrate that the inhibition of NF-κB is sufficient to block LPS-induced suppression of the M-ASGP-BP gene, indicating the involvement of NF-κB activation as the mechanism in this process.

Discussion

In this report, we demonstrate that exposure to the bacterial component LPS inhibits M-ASGP-BP mRNA expression in thioglycolate-elicited peritoneal Mϕs and that the effect of LPS on M-ASGP-BP mRNA is time-dependent, appearing as early as 6 h after exposure and reaching the maximum after 24 h treatment (Fig. 1). At the cellular level, LPS primarily stimulates Mϕs and other cell types to induce the expression of an impressive number of genes and to release proinflammatory agents such as cytokines [1–3]. The intracellular pathways that mediate LPS-induced effects are not yet completely understood, but certain src-related kinases, protein kinase C (PKC), mitogen-activated protein kinases (p38, p42 and p44 MAPK), TLR4, G proteins, NF-κB, and stress-activated protein kinases (SAPK/JNK) all seem to be important elements [14–17]. We report herein that the suppressive effect of LPS on M-ASGP-BP mRNA does not appear to be mediated *via* the either the MEK/ERK1/2 or p38 MAPK pathway. However, blockade of TLR4, as well as inhibition of NF-κB activation, results in suppression of the effect of LPS on M-ASGP-BP mRNA (Fig. 3).

Recently, Sato *et al*. of the Irimura group have indicated that M-ASGP-BP-positive cells migrated from the dermis to regional lymph nodes during the sensitization phase of

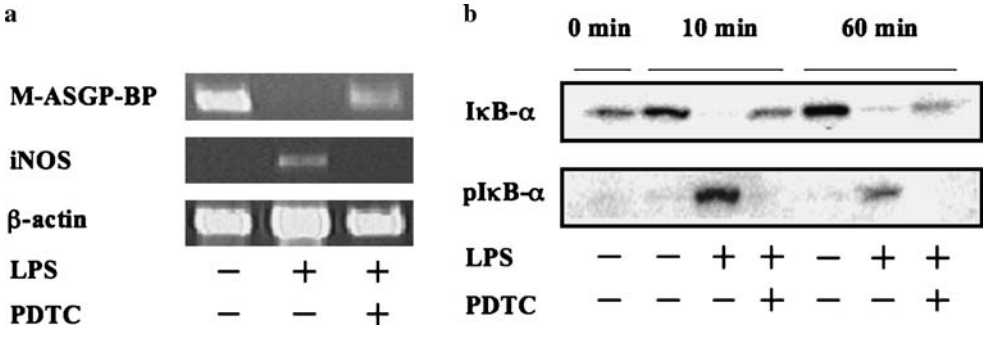

Fig. 5 Involvement of NF-κB activation in the suppressive effect of LPS on M-ASGP-BP mRNA expression **a** Effect of an NF-κB activation inhibitor on LPS-induced suppressive expression of M-ASGP-BP mRNA. Thioglycolate-elicited rat peritoneal Mϕs were incubated with NF-κB activation inhibitor PDTC at 30 μM for 30 min, followed by the addition of LPS at 10 ng/ml for 6 h. The expression of M-ASGP-BP and iNOS mRNA was analyzed by RT-PCR as described under "Materials and methods" **b** Serine phosphorylation of IκB induced by LPS stimulation. Thioglycolate-elicited rat peritoneal Mϕs were preconditioned with PDTC at 30 μM for 30 min, followed by treatment with LPS at 10 ng/ml for an additional 10 and 60 min, respectively. The LPS-induced serine phosphorylation of IκB was analyzed by Western blotting with specific anti-pIκB antibodies, and then the assay membrane was reprobed with anti-IκB antibodies

contact hypersensitivity, and that the migration was initiated by cytokine-mediated release of M-ASGP-BP-positive cells from the dermis [18, 19]. The cells homed to the boundary of the T-cell area in the regional lymph nodes, and the prevention of migration seemed to interfere with sensitization.

Proinflammatory cytokines such as IL-6, IL-1β and TNF-α contribute to the development of symptoms associated with the acute and chronic phases of various immune and regeneration disorders. LPS-stimulated cytokine-producing signals are highly dependent upon the activity of NF-κB transcription factor, which is a key signaling intermediate for LPS-induced responses in Mϕs. NF-κB dimers associate with IκB to form an inactive complex that is localized in the cytoplasm of unstimulated cells. When cells are stimulated by agonists, IκB is inactivated through enzymatic modifications such as phosphorylation and ubiquitination, followed by proteolysis. NF-κB becomes activated after dissociation from IκB and is translocated to the nucleus [20, 21]. The data obtained in this study indicate that treatment with NF-κB activation inhibitor PDTC effectively prevents the LPS-induced M-ASGP-BP mRNA level decrease, and the serine phosphorylation of IκB is completely inhibited (Fig. 5). These results strongly suggest that the inhibition of NF-κB is sufficient to block LPS-induced suppression of the M-ASGP-BP gene.

TLR4 is the signal-transducing component of the LPS recognition complex and is essential for LPS-induced septic shock. In the present study, the expression of M-ASGP-BP mRNA was down-regulated on LPS treatment. The binding of LPS with TLR 4 resulted in a decrease in M-ASGP-BP mRNA through NF-κB activation. The demonstration of LPS inhibition of M-ASGP-BP mRNA expression within hours is consistent with a transcriptional mechanism of action. Our studies implicate NF-κB in this mechanism, as suppression of NF-κB activation inhibits the effect of LPS on M-ASGP-BP gene expression and induction of NF-κB activation by itself produces a similar effect (with a similar time-course) to that of LPS, and the mechanism involves TLR4-mediated down-regulation of M-ASGP-BP mRNA expression.

Although many studies have demonstrated that LPS regulates the expression of many genes, our data show for the first time that LPS regulates the M-ASGP-BP expression level in normal LPS-responsive thioglycolate-elicited peritoneal Mϕs. Therefore, these data suggest a potentially important role for this protein in regulating host responsiveness to LPS.

However, the biologic consequences of M-ASGP-BP regulation by Mϕs in response to LPS exposure is unclear. Several groups have reported that the expression of M-ASGP-BP mRNA is induced during the process of Mϕ activation and differentiation, and the induction of M-ASGP-BP, as an endocytosis receptor, may contribute to enhancement of bactericidal and tumoricidal ability during the differentiation and activation of Mϕs [9–11]. On the other hand, most recently, Higashi *et al*., also of the Irimura group, found that M-ASGP-BP was also expressed on monocyte-derived immature dendritic cells (DCs) on serial analysis of gene expression (SAGE), and was up-regulated at the immature DC stage but down-regulated at the mature stage, suggesting it may be partly responsible for the known enhanced antigen uptake of immature DCs at distinct stages of differentiation because it contains the intracellular tyrosine motif "YENF" that is required for interaction with clathrin-coated vesicles [22, 23]. A strategy for specifically targeting immature DCs with vaccine antigens may be to employ M-ASGP-BP-mediated antigen uptake by modifying the candidate antigens with Gal/GalNAc residues. If such a strategy would target immunogenic DCs but not tolerogenic DCs, this would be a major advance in vaccinology. In fact, the activation and differentiation of Mϕs depend on the net balance between positive and negative signals. Importantly, we hypothesize, based upon these findings, that LPS suppression of M-ASGP-BP gene expression in Mϕs may play an important role during the processes of Mϕ activation and differentiation by affecting LPS cellular uptake and clearance.

Our data also suggest that engagement of M-ASGP-BP by LPS may yield a negative signal that interferes with the LPS-induced positive signals mediated by proinflammatory cytokines. Furthermore, at least three other Mϕ receptors are down-regulated on LPS treatment. Shepherd *et al*. demonstrated that LPS/PMA treatment of Mϕs results in inactivation of mannose receptor, another major endocytosis receptor, with no effect on receptor turnover or biosynthesis [24], and Van Lenten *et al*. have indicated that LPS prevents the expression of scavenger receptor activity on human monocyte-derived Mϕs [25]. Also, Weiel *et al*. showed that the Mϕ transferrin receptor is down-regulated after LPS exposure [26]. These reports support our hypothesis regarding the possible mechanism for the LPS suppression of M-ASGP-BP gene expression in thioglycolate-elicited rat peritoneal Mϕs. However, the true biologic consequences of and precise mechanisms by which these downstream signaling cascades function have yet to be elucidated.

Elucidation of the mechanisms involved in the inhibition of constitutive M-ASGP-BP gene expression requires further study. The molecular mechanisms that mediate the downstream effects of LPS may involve the activation of multiple classes of transcription factors, including NF-κB [27], Sp1 [28], c-Jun [29, 30], Ets [30], and Egr-1 [30]. Our prior work indicated that the promoter binding sites for AP-1, NF-IL6 and AP-2 exist in the promoter region of the M-ASGP-BP gene (unpublished data), suggesting that these nuclear factors might control the expression of M-ASGP-BP mRNA. Future studies will examine whether NF-κB acts through direct inhibition of M-ASGP-BP gene transcription or

through induction of an intermediary suppressor gene/protein. The identification of M-ASGP-BP is an important goal, as it should facilitate understanding of the regulatory mechanism of the immune system and aid the development of vaccination protocols. It is possible that targeting of M-ASGP-BP by attaching Gal/GalNAc residues to a candidate antigen might improve specific antigen presentation by Mϕs and immature DCs, and thus improve vaccine immunogenicity.

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