Bacterial lipopolysaccharide-induced expression of the I κ B protein MAIL in B-lymphocytes and macrophages*

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Summary. Molecule possessing ankyrin-repeats induced by lipopolysaccharide (MAIL), a recently cloned nuclear I κ B protein induced by lipopolysaccharide (LPS) stimulation in lymphoid organs, is involved in the regulation of inflammatory responses. The present *in situ* hybridization and immunohistochemical analyses revealed the distinct expression of the MAIL mRNA and protein in B-lymphocytes of the white pulp of the spleen and cortical lymphoid follicles of lymph nodes in LPS-injected mice. MAIL signals were also localized in F4/80-positive macrophages in these organs. LPS clearly induced MAIL expression in cultured B-lymphocytes and monocytes/macrophages, but only faintly so in T-lymphocytes, fibroblasts, and endothelial cells. MAIL was also induced by inflammatory cytokines such as interleukin-1 and -6, and tumor necrosis factor in cultured cells. Northern blot, Western blot, and *in situ* hybridization analyses showed that the major expression product of the *Mail* gene was a long splicing variant (MAIL-L) rather than a short one, both in lymphoid organs and cultured cells. These

results collectively indicate that LPS induces MAIL-L predominantly in B-lymphocytes and macrophages.

Introduction

Infection by Gram-negative bacteria causes numerous inflammatory responses and occasionally leads to septic shock. The virulence and pathogenic potentials of this group of bacteria are attributed mainly to lipopolysaccharide (LPS), a component of the outer bacterial membrane. Recent studies have demonstrated that the signaling cascade from the cell surface Toll-like receptor (TLR) 4 to transcriptional factors nuclear factor κ B (NF- κ B) and/or activator protein 1 (AP-1) plays crucial roles in the responses to LPS (Aderem and Ulevitch, 2000). The activities of NF- κ B and AP-1 proteins are regulated by the I κ B family proteins such as I κ B- α , β , γ /p105/NF- κ B1, δ /p100/NF- κ B2, ϵ , and BCL-3 (Bundy and McKeithan, 1997; Chen *et al.*, 1999; Na *et al.*, 1999).

We have recently identified a new nuclear I κ B protein, MAIL, as a molecule possessing ankyrin-repeat induced by lipopolysaccharide in mice (Kitamura *et al.*, 2000). MAIL mRNA expression is below detectable levels in intact mice, but — as manifested by its name — it increases dramatically and rapidly after LPS-injection in several organs, especially in the spleen and lymph node. The ectopic expression of MAIL augments interleukin (IL)-6 production in cultured fibroblasts (Kitamura *et al.*, 2000), and inhibits NF- κ B activation in macrophage-like cells and embryonic kidney cells (Yamazaki *et al.*, 2001) suggesting its potential role in the regulation of inflammatory responses. Despite these observations, it is still uncertain as to which types of cells express MAIL in LPS-induced inflammation.

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MAIL has long and short splicing variants, namely MAIL-L and MAIL-S, both of which were originally isolated by us from cDNA libraries of an intact mouse spleen (Kitamura *et al.*, 2000). Recently, MAIL-L and MAIL-S have also been reported by other research groups as INAP and I κ B- ξ , respectively (Haruta *et al.*, 2001; Yamazaki *et al.*, 2001). MAIL-S lacks the N-terminal 99 amino acids of MAIL-L, in which PEST-like sequences exist (Shiina *et al.*, 2002). Since PEST-like sequences are implicated in the rapid turnover of protein, the half-life of MAIL-S seems to be longer than that of MAIL-L. Both MAIL-L and MAIL-S enhance LPS-induced IL-6 secretion in Swiss3T3 cells, although MAIL-S is more effective for this response (Kitamura *et al.*, 2000). In Northern blot analysis, MAIL mRNA was detected as a single hybridization band in several tissues after LPS stimulation (Kitamura *et al.*, 2000). Haruta *et al.* (2001) showed that IL-1 elicits only MAIL-L/INAP expression in mouse stromal cell line OP9. On the other hand, Yamazaki *et al.* (2001) reported that MAIL-S/I κ B- ξ is a molecule induced by LPS and IL-1 in thioglycollate-elicited peritoneal macrophages, macrophage-like RAW264.7 cells, and fibroblast-like NIH3T3 cells. At present, however, the major and functionally important variant of MAIL in LPS-induced inflammation remains to be determined.

In this study, we identify cells expressing MAIL mRNA and protein using *in vivo* tissue samples, freshly isolated cells, and cultured cell lines. We also determine which variant is dominant in these tissues and cells.

Materials and Methods

Reagents

LPS (*E. coli* 055 : B5) was purchased from Sigma (St. Louis, MO, USA). Mouse recombinant IL-1 β , IL-2, IL-6, and tumor necrosis factor (TNF)- α were obtained from Pepro Tech (London, UK). A rabbit antibody raised against mouse MAIL (amino acids 108 to 403) was the kind gift of Dr. K. Todokoro (Institute of Physical and Chemical Research, Tsukuba). Rabbit anti-actin, rabbit anti-B220, and rat anti-F4/80 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cedarlane Laboratories (Ontario, Canada), and Serotec (Oxford, UK), respectively. The Alexa Fluor 488-conjugated anti-rabbit IgG antibody and Alexa Fluor 594-conjugated anti-rat IgG were from Molecular Probes (Eugene, OR, USA).

Experimental animals

Male C57BL/6 (7–8 weeks old; SLC, Shizuoka) were injected intraperitoneally with either LPS (3 mg/kg) or sterile phosphate-buffered saline (PBS). For Western blot, North-

ern blot, and *in situ* hybridization analyses, mice were sacrificed by cervical dislocation 0–24 h after the injection, and the spleen, mesenteric lymph node, and thymus were immediately removed. For immunohistochemical analysis, mice were anesthetized by a lethal dose of pentobarbital and perfused through the aorta with PBS followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) 0–12 h after the LPS injection. After perfusion-fixation, the spleen, lymph node, and thymus were immersed in the same fixative for an additional 6 h, dipped in 30% sucrose solution, and frozen in liquid nitrogen. The experimental procedure and care of animals were in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University.

Cells

Peritoneal macrophages were collected from intact C57BL/6 mice by peritoneal lavage as described previously (Elliot *et al.*, 1995). Isolation of B220-positive B-lymphocytes and Thy1.2-positive T-lymphocytes from the mouse spleen was conducted using a magnetic cell separation system (Miltenyi Biotec, Gladbach, Germany). Mouse cell lines BCL1-B20, J774.1, M1, and EL-4 were obtained from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai). P3-X63-Ag8.653, CTLL2, and Swiss 3T3 cells were from the Riken Cell Bank (Tsukuba). Cells were treated with either medium alone (RPMI 1640 medium; Invitrogen, Carlsbad, CA, USA, supplemented with 10% fetal calf serum), LPS (10 μ g/ml), IL-1 β (20 ng/ml), IL-6 (40 ng/ml), or TNF- α (20 ng/ml) for 1–3 h.

Western blotting

Tissues were homogenized in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 0.2% Nonidet P-40, and a complete protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were lysed in PBS containing 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.01% SDS, and a complete protease inhibitor cocktail. Samples were fractionated by 10% SDS-PAGE and electrotransferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20, incubated with the rabbit anti-mouse MAIL antibody for 2 h, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 2 h. The antibody complexes were visualized with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were also stained with the rabbit anti-actin antibody as a reference.

In situ hybridization

Cryostat sections, 16 μm in thickness, were prepared using fresh unfixed tissues and mounted on glass slides precoated with 3-aminopropyltriethoxysilane. They were fixed with 4% paraformaldehyde for 10 min and acetylated for 10 min in 0.25% acetic anhydride in PBS. The sections were hybridized with ^{35}S -dATP-labeled sense or antisense oligonucleotide probes corresponding to +190 to +232 bp (specific for MAIL-L) and +1,431 to +1,475 bp (common for both variants) of the coding region sequence of MAIL cDNA (accession number, AB020974). The sections were dehydrated through a graded series of ethanol, and prehybridized for 2 h in a buffer containing 50% formamide, 0.1 M Tris-HCl (pH 7.5), $4 \times \text{SSC}$, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.6 M NaCl, 0.25% SDS, 200 $\mu\text{g}/\text{ml}$ tRNA, 1 mM EDTA, and 10% dextran sulfate. Hybridization was performed at 42°C for 10 h in the prehybridization buffer supplemented with 1.0×10^7 cpm/ml of ^{35}S -labeled oligonucleotide probes. The sections were washed at room temperature for 20 min in $2 \times \text{SSC}$ containing 0.1% sarkosyl and at 55°C for 40 min in $0.1 \times \text{SSC}$ containing 0.1% sarkosyl. They were exposed to either Hyperfilm- β max (Amersham Pharmacia Biotech) for 1 week, or dipped in Kodak NTB2 nuclear track emulsion for 2 months. All sections were counterstained with hematoxylin.

Immunohistochemistry

Single immunostaining for MAIL and B-lymphocytes was carried out according to the avidin-biotin complex method using the rabbit anti-MAIL antibody and anti-B220 antibody, respectively. Frozen sections, 16 μm thick, were prepared in a cryostat and incubated in 0.3% Triton X-100 for 30 min, followed by 0.3% hydrogen peroxide in absolute methanol for 10 min. After pretreatment with 5% normal goat serum for 30 min, the sections were incubated with a primary antibody (the anti-mouse MAIL antibody diluted 1 : 500 or an anti-B220 antibody specific for B-lymphocytes diluted 1 : 400) overnight. The sections were then treated with a biotinylated goat anti-rabbit IgG (Histofine; Nichirei, Tokyo) followed by application of an avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA), each for 1 h. The antigen-antibody reaction was visualized by incubation in a 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.002% H_2O_2 . All sections were counterstained with hematoxylin.

For double staining for MAIL and macrophages, some cryostat sections were incubated with both the anti-MAIL antibody (1 : 200) and F4/80 antibody specific for macrophages (1 : 600) overnight, and subsequently with Alexa Fluor 488- and Alexa Fluor 594-conjugated antibod-

ies for 1 h. The slides were washed with PBS and observed with a confocal scanning fluorescence microscope (FLUOVIEW system; Olympus, Tokyo).

Northern blotting

For Northern blot analysis, total RNA extracted with Trizol solution (Invitrogen, Carlsbad, CA, USA) was separated on 1% agarose/formaldehyde gel, and transferred to a nylon membrane (Hybond N; Amersham Pharmacia Biotech). A common cDNA probe for MAIL-L and MAIL-S (+1,329 to +1,709 bp) and a MAIL-L specific probe (−63 to +250 bp) were labeled with α - ^{32}P -dCTP. The nylon membranes were prehybridized with a buffer containing 50% formamide, $5 \times \text{SSPE}$, 0.1% SDS, $5 \times \text{Denhardt's}$ solution, and 200 $\mu\text{g}/\text{ml}$ salmon sperm DNA at 42°C for 10 h, and then hybridized at 42°C for 12 h in the prehybridization buffer supplemented with 1.0×10^6 cpm/ml of the labeled probes. The blots were washed with $2 \times \text{SSC}$, 0.1% SDS and $0.1 \times \text{SSC}$, 0.1% SDS solutions, and were exposed to X-ray films (RX-U; Fuji Film, Tokyo) for 1 or 2 weeks. The radioactivity was quantified using a BAS-1000 bio-image analyzer (Fuji Film). The membranes were also hybridized with mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH, +566 to +1017 bp; accession number, M32599) cDNA as a reference.

Results

Western blot analysis of MAIL protein in mouse lymphoid tissues

We previously demonstrated that LPS induced MAIL mRNA expression *in vivo* in the mouse spleen, lymph node, and thymus (Kitamura *et al.*, 2000). To confirm the protein expression of MAIL, we investigated protein levels of two MAIL variants in these tissues of mice using Western blot analysis. Before LPS injection, MAIL protein was not detected in the spleen, but 1 h after LPS injection two distinct immunoreactive bands of 79 kDa and 82 kDa appeared (Fig. 1). These bands corresponded to products translated from two different in-frame translation initiation sites separated by 78 nucleotides of MAIL-L mRNA (Haruta *et al.*, 2001). We shall refer to the longer product as MAIL-L82 and the shorter one as MAIL-L79. Expression levels of MAIL-L proteins reached a peak in 2 h and returned to the basal levels at 24 h. A similar MAIL-L induction was observed in the lymph node and thymus after LPS injection, though less intense in the thymus (Fig. 1). On the other hand, no band of MAIL-S (72 kDa) was detected in these tissues even after LPS injection (Fig. 1).

Fig. 1. MAIL protein expression in mouse lymphoid organs after LPS injection. The spleen, lymph node, and thymus were taken at 0–24 h after the intraperitoneal injection of LPS. Total cell lysate (100 μ g protein) was subjected to Western blot analysis using anti-MAIL and anti-actin antibodies. The arrows show the double bands of MAIL-L proteins, namely MAIL-L79 and MAIL-L82 (see text). The arrowhead shows the band of MAIL-S. The total cell lysate of Swiss 3T3 cells transfected with MAIL-L (L) and MAIL-S (S) was used for standard samples of MAIL-L and MAIL-S, respectively. The single asterisk and double asterisks indicate nonspecific proteins cross-reacting with the antibody.

In situ hybridization analysis of MAIL mRNA in mouse lymphoid tissues

To identify the cells expressing MAIL mRNA in lymphoid organs, *in situ* hybridization was conducted using oligonucleotide probes for either a common region of the two variants or a specific region of MAIL-L. The two probes exhibited almost identical labeling patterns (Fig. 2A), whereas their sense probes did not show any specific hybridization signals. In PBS-injected control mice, MAIL mRNA signals were only faintly detected in all the tissues, whereas after LPS injection, the signals greatly increased at 0.5 h, reached a peak at 1–2 h (Fig. 2A), and were sustained

for at least 8 h (data not shown). The most remarkable signals were localized in the white pulp of the spleen, which was densely populated by B220-positive B-lymphocytes (Fig. 2B₁, B₂). The signals of MAIL mRNA in the lymph node were also intensely found in B-lymphocyte-rich regions such as cortical lymphoid follicles and medullary cords (Fig. 2C). The MAIL signals increased less intensely yet still significantly after LPS injection in the red pulp of the spleen and the medullary sinuses of the lymph node, where macrophages were profuse (Fig. 2B₁, C). In the thymus, weak MAIL signals were localized in both the cortex and medulla, predominating in the latter (Fig. 2D).

Immunohistochemical analysis of MAIL protein in mouse lymphoid tissues

To confirm the MAIL protein expression in B-lymphocytes and macrophages in LPS-treated mice, an immunohistochemical study using an antibody against MAIL was carried out. As shown in Figure 3A and C, specific MAIL immunoreactivity was undetectable in the spleen, lymph node, and thymus in the intact mice. At 1 to 12 h after LPS injection, a significant immunoreactivity for MAIL was detected in B-lymphocyte-rich regions such as lymphoid follicles in the spleen and mesenteric lymph node, where the immunoreactivity appeared to be localized to the cytoplasm (Fig. 3B, D). Another type of MAIL-immunoreactive cell, possibly a macrophage, was scattered in the red pulp of spleen, in the medullary sinuses of lymph node, and in the medulla of thymus (Fig. 3B, D, E). The MAIL immunoreactivity in macrophages was localized predominantly in their nucleus. Double staining with the macrophage F4/80-specific antibody and anti-MAIL antibody confirmed that these cells were macrophages (Fig. 3F). In accordance with previous observations of cultured cells (Kitamura *et al.*, 2000; Haruta *et al.*, 2001; Yamazaki *et al.*, 2001), MAIL signals were restricted to the nucleus in those macrophages whose cell surfaces were labeled with the F4/80 antibody (Fig. 3F). On the other hand, in T-lymphocyte-rich regions such as the periarterial lymphoid sheath of the spleen and cortex of the thymus, MAIL immunoreactivity was scarce at best (Fig. 3B, E).

Fig. 2. Localization of MAIL mRNA in mouse lymphoid organs after LPS injection. The spleen, thymus, and lymph node were taken at 2 h after the intraperitoneal injection of LPS (3 mg/kg) or PBS, and frozen tissue sections were processed for *in situ* hybridization and immunohistochemistry. **A.** Macroautoradiographic images of MAIL mRNA signals in the mouse tissues using a common probe for MAIL-L and MAIL-S, and a specific probe for MAIL-L. Microscopic views of MAIL mRNA of the spleen (**B₁**), lymph node (**C**), and thymus (**D**) after LPS injection. **B₂**. A section adjacent to **B₁** was stained with an anti-B220 antibody. WP: white pulp, RP: red pulp, Cx: cortex, MC: medullary cord, MS: medullary sinus, Pc: paracortex, Md: medulla. Bars = 50 μ m

Fig. 2. Legend on the opposite page.

Fig. 3. Legend on the opposite page.

LPS-induced MAIL mRNA expression in isolated cells

To further identify MAIL-expressing cells, we isolated peritoneal macrophages and splenic lymphocytes, and examined the effects of LPS stimulation on MAIL expression *in vitro*. Using a probe common to the two MAIL variants, a single hybridization band of about 4 kbp was detected in B-lymphocytes stimulated by LPS; this was hardly detected in PBS-treated control cells (Fig. 4). LPS-induced MAIL mRNA was also observed in macrophages but not in T-lymphocytes even after 2 h of LPS stimulation. A specific probe for MAIL-L also detected a band of the same size as that seen with the common probe.

MAIL mRNA expression in cell Lines stimulated by LPS and cytokines

The above results obtained with tissues and isolated cells indicated that B-lymphocytes and macrophages induced MAIL in response to LPS stimulation. We also investigated the effects of stimulation with LPS and inflammatory cytokines on MAIL mRNA expression in various mouse cell lines BCL1-B20 (B-lymphocytes), J774.1 (macrophages), M1 (monocytes), CTLL2 (killer T-lymphocytes), EL-4 (helper T-lymphocytes), P3-X63.Ag8.653 (myeloid cells), LEII (endothelial cells), and Swiss 3T3 (fibroblasts). In these cell lines without stimulation, the MAIL mRNA level was quite low or negligible. However, at 1-2 h after LPS stimulation, it was elevated in all the cell lines except EL-4 (Fig. 5). In particular, the B-lymphocyte (BCL1-B20), macrophage (J774.1), and monocyte (M1) cell-lines showed remarkable MAIL induction with 38-, 47-, and 31-fold increases compared with the PBS-controls, respectively. Similar to LPS, inflammatory cytokines also provoked the induction of MAIL mRNA in some cell lines: IL-1 β in J774.1, M1, EL-4 and Swiss 3T3; TNF- α in J774.1, CTLL2, and LEII; and IL-6 only in J774.1. However, the stimulatory effects of these cytokines were less than that of LPS in all the cells except EL-4, even at doses comparable to those eliciting the maximal cellular responses reported so far (Espey *et al.*, 2000; Krishnaraju *et al.*, 1998). The cDNA probes specific to MAIL-L and common to the two variants yielded almost the same results (data not shown).

Induction of MAIL-L protein in cultured cells by LPS

We investigated MAIL protein expression in isolated cells and also in some cell lines. As shown in Fig. 6A, LPS induced the two forms of MAIL-L (MAIL-L79 and MAIL-L82) to a remarkable degree in isolated B-lymphocytes and macrophages, but only slightly in T-lymphocytes. Similar to the isolated cells, LPS elicited the induction of MAIL-L proteins in BCL1-B20, J774.1, and M1, with the most intense induction in macrophage cell line J774.1 (Fig. 6B). On the other hand, the MAIL-S band of 72 kDa was not detected in any cells examined even after LPS stimulation.

Discussion

We have previously reported that the intraperitoneal injection of LPS into mice induces MAIL mRNA predominantly

Fig. 4. Effects of LPS on MAIL mRNA expression in isolated mouse cells. Splenic B-lymphocytes (B), peritoneal macrophages (M), and splenic T-lymphocytes (T) were treated with PBS (–) and LPS (+; 10 μ g/ml) for 2 h. Total RNA (15 μ g) was subjected to Northern blot analysis using probes for a common region of the two variants (upper left), a region specific to MAIL-L (upper right), and GAPDH (lower left).

Fig. 3. Immunohistochemical localization of MAIL protein in mouse lymphoid organs after LPS injection. The spleen (A, B, and F), lymph node (C and D), and thymus (E) were taken at 3 h after intraperitoneal injection of PBS (A and C) or LPS (3 mg/kg; B, D, E, and F), and frozen tissue sections were processed for immunohistochemistry. F. Double staining with MAIL (green) and F4/80 (red) antibodies. WP: white pulp, RP: red pulp, P: periarterial lymphoid sheath, Cx: cortex, MC: medullary cord, MS: medullary sinus, Pc: paracortex, Md: medulla. Bars = 50 μ m (A, B, C, and E), 100 μ m (D), 5 μ m (F).

Fig. 5. Effects of LPS and cytokines on MAIL mRNA expression in mouse cell lines. Cells were stimulated with either PBS (-), LPS (10 μ g/ml), IL-1 β (20 ng/ml), TNF- α (20 ng/ml), or IL-6 (40 ng/ml) for 1–2 h, and 30 μ g of total RNA was subjected to Northern blot analysis using a probe common to the two variants.

in the spleen and lymph node, where B-lymphocytes and macrophages are profuse (Kitamura *et al.*, 2000). The present *in situ* hybridization analysis revealed that MAIL mRNA was actually expressed in the B-lymphocyte- and macrophage-rich regions in these organs. Immunohistochemistry confirmed the localization of MAIL protein in these cell types, leading us to conclude that B-lymphocytes and macrophages are predominant cell types for MAIL production. The subcellular localization of MAIL protein differed between B-lymphocytes and macrophages, namely cytoplasmic or nucleoplasmic component. The immunolabeling of nuclei in macrophages is reasonable, since previous studies have demonstrated the predominant localization of MAIL in the nucleus of several types of cells such as fibroblasts (Kitamura *et al.*, 2000; Haruta *et al.*, 2001; Yamazaki *et al.*, 2001). The selective staining of the cytoplasm in B-lymphocytes was unexpected. However, it is known that other I κ B family proteins move between the cytoplasm and nucleus (Tam *et al.*, 2000). The present staining results in B-lymphocytes may suggest the translocation of MAIL into the cytoplasm, due to the interaction or coupling with other regulatory molecules. Consistent

Fig. 6. Effects of LPS on MAIL protein expression in mouse cells. Isolated mouse cells (A) and cell lines (B) were stimulated with either medium alone (-) or LPS (10 μ g/ml) for 1–3 h. The total cell lysate (A: 75 μ g, B: 100 μ g (BCL1-B20 and M1) and 10 μ g (J774.1)) were subjected to Western blot analysis using the anti-MAIL and anti-actin antibodies. The total cell lysate of Swiss 3T3 cells transfected with MAIL-L (L) and MAIL-S (S) was used for standard samples of MAIL-L and MAIL-S, respectively. The arrows and arrowhead show MAIL-L and MAIL-S, respectively. The asterisk indicates an LPS-induced 75 kDa product, which is distinct from MAIL-L and MAIL-S. B: splenic B-lymphocytes, M: peritoneal macrophages, T: splenic T-lymphocytes.

with these morphological findings, cultured B-lymphocytes and monocytes/macrophages expressed abundant MAIL mRNA and protein after LPS stimulation. In contrast, the expression of MAIL was either detectable or negligible in T-lymphocytes. Thus, B-lymphocytes and macrophages are the dominant cell types expressing MAIL in response to LPS.

Several studies have provided conclusive evidence that the cell surface receptor TLR4 is critical for LPS-induced inflammatory responses in B-lymphocytes and macrophages. For example, TLR4 is expressed in B-lymphocytes and macrophages (Shimazu *et al.*, 1999), and a gene knockout of TLR4 results in impaired responses to LPS (Hoshino *et al.*, 1999). We recently demonstrated that the LPS-induced MAIL expression was attenuated in human myelomonocytic cell line U937 treated with an anti-TLR4 neutralizing antibody, and also in the spleen of mice having a missense mutation in the *Tlr4* gene (Kitamura *et al.*, 2002). Thus, the cascade from TLR4 to NF- κ B activated by LPS may contribute to the induction of MAIL in B-lymphocytes and macrophages.

It has been well established that B-lymphocytes and

macrophages exhibit a wide variety of cellular responses to LPS stimulation, including proliferation and immunoglobulin secretion in B-lymphocytes, and phagocytosis and production of various cytokines in macrophages (Hoshino *et al.*, 1999; Ogata *et al.*, 2000; Zhang *et al.*, 2000). These responses to LPS are caused mainly through the activation of nuclear transcriptional regulatory factors such as NF- κ B and AP-1 (Aderem and Ulevitch, 2000). We previously indicated that MAIL potentiates LPS-induced transactivation of the *Il-6* gene (Kitamura *et al.*, 2000), whose promoter region has the binding elements for NF- κ B, AP-1, CREB, and NF-IL6 (Dendorfer *et al.*, 1994). According to our unpublished data, MAIL-L appeared to potentiate NF- κ B-dependent reporter gene activity in HeLa cells, while Yamazaki *et al.* (2001) reported a suppressive effect of MAIL-S on NF- κ B in macrophage-like RAW264.7 cells. It is thus likely that MAIL is one of the transcriptional regulators that modulate various cellular responses to LPS of B-lymphocytes and macrophages in association with some nuclear regulatory factors.

MAIL has two alternative splicing variants, MAIL-L/INAP and MAIL-S/I κ B- ξ (Kitamura *et al.*, 2000; Haruta *et al.*, 2001; Yamazaki *et al.*, 2001). MAIL-S mRNA does not contain the exon 3 region of the *Mail* gene, resulting in the deletion of 99 amino acids in the N-terminal region of MAIL-L (Shiina *et al.*, 2002). Although both variants potentiate LPS-induced IL-6 production in Swiss3T3 cells, MAIL-S is more effective in the induction of this cytokine (Kitamura *et al.*, 2000). The present *in situ* hybridization study and Northern blot analyses demonstrated similar mRNA expression patterns with a MAIL-L specific probe and a probe common to MAIL-L and MAIL-S. Moreover, Western blot analyses using an anti-MAIL antibody detected only MAIL-L. These results collectively indicate that MAIL-L is a dominant variant induced by LPS. Further studies are needed to identify the cells expressing MAIL-S, if any, and their stimulants.

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