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Cutting Edge: Cell Surface Expression and Lipopolysaccharide Signaling Via the Toll-Like Receptor 4-MD-2 Complex on Mouse Peritoneal Macrophages[1]

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The human MD-2 molecule is associated with the extracellular domain of human Toll-like receptor 4 (TLR4) and greatly enhances its LPS signaling. The human TLR4-MD-2 complex thus signals the presence of LPS. Little is known, however, about cell surface expression and LPS signaling of the TLR4-MD-2 complex in vivo. We cloned mouse MD-2 molecularly and established a unique mAb MTS510, which reacted selectively with mouse TLR4-MD-2 but not with TLR4 alone in flow cytometry. Mouse MD-2 expression in TLR4-expressing cells enhanced LPS-induced NF-κB activation, which was clearly inhibited by MTS510. Thioglycolate-elicited peritoneal macrophages expressed TLR4-MD-2, which was rapidly down-regulated in the presence of LPS. Moreover, LPS-induced TNF-α production by peritoneal macrophages was inhibited by MTS510. Collectively, the TLR4-MD-2 complex is expressed on macrophages in vivo and senses and signals the presence of LPS. ***The Journal of Immunology*****, 2000, 164: 3471–3475.**

Bacterial infection is still a major threat to humans (1). Invasion of Gram-negative bacteria elicits immune responses, a major mediator of which is endotoxin/LPS, a component of the cell wall of Gram-negative bacteria. LPS-induced activation of monocytes/macrophages leads to secretion of a number of proinflammatory cytokines such as TNF-α, IL-1, and IL-6. The defense programs are then activated, and invading bacteria are eliminated. Excessive amount of the cytokines, however, may result in fatal endotoxin shock. LPS signaling must be kept under the control during immune responses. It is therefore of particular importance to understand the molecular mechanisms underlying LPS recognition and signaling for the control of immune responses and endotoxin shock.

LPS-binding protein is the plasma protein that first interacts with and recruits LPS from bacterial membrane to another protein CD14 (2, 3). CD14, which is present in plasma as well as on the monocyte cell surface binds to LPS and facilitates LPS signaling (4, 5). CD14 is a glycosylphosphatidylinositol-anchored protein and does not have a cytoplasmic signaling domain. Another molecule therefore must transmit the LPS signal across the plasma membrane (3, 5).

Toll-like receptors (TLRs)[3] are mammalian homologues of the *Drosophila* Toll receptor and are thought to have a role in innate recognition of bacteria or fungi (6–9). TLR4 is now identified as a signaling receptor for LPS. The TLR4 gene is mutated in the LPS low responder mouse strains C3H/HeJ and C57BL/10ScCr (10–12). Human TLR4 alone, however, is not capable of sensing the presence of LPS (13, 14). Another molecule, MD-2, which is physically associated with TLR4, is required for LPS recognition (14). The TLR4-MD-2 complex thus serves as the LPS signaling receptor. These conclusions are drawn from the results using stable transfectants expressing human TLR4 and/or MD-2. It is not clear as to mouse TLR4-MD-2 complex, or about in vivo expression and LPS signaling of the TLR4-MD-2 complex. The present study deals with these issues.

Materials and Methods

Molecular cloning of mouse MD-2 and mouse TLR4

A partial sequence of mouse MD-2 cDNA was obtained through the EST database at the National Center for Biological Information (accession number AA109204). Because the sequence did not contain the 3′-end of the coding region, 3′-RACE system (Life Technologies, Rockville, MD) was conducted to obtain the full length cDNA from BALB/c mouse kidneys with a forward primer: TCCGATGGTCTTCCTGGCGAG. The full length cDNA encoding mouse TLR4 was obtained from a BALB/c spleen cDNA library with the human TLR4 probe (accession number H48602) that was obtained from Genome Systems (St. Louis, MO).

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[3] Abbreviation used in this paper: TLR, Toll-like receptor.

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Expression constructs and stable transfectants

The cDNAs were cloned into an expression vector, pEFBOS (15). The DNA fragment encoding the flag epitope followed by the His tag epitope had been introduced into the pEFBOS vector such that all expressed proteins bear the flag epitope at the C termini.

The plasmids were transfected into Ba/F3 cells (16) by electroporation. An NF-κB reporter construct, p55IgκLuc (17), was also introduced. Expression of TLR4 or MD-2 was confirmed by flow cytometry staining with cell permeabilization and immunoprecipitation and probing with the anti-flag mAb (Fig. 2, *B* and *C*, and data not shown).

Cells, reagents, and animals

The following reagents were purchased from Sigma (St. Louis, MO): a mAb against the flag epitope M2; LPS from *Escherichia coli* (0111:B4 or 055:B5); LPS or lipid A from *Salmonella minnesota* (Re595). Ba/F3 cells were fed in 10% FCS-RPMI 1640 supplemented with IL-3 and 50 μM 2-ME. Mice and rats were obtained from Japan SLC (Shizuoka, Japan). Thioglycolate-elicited peritoneal macrophages were obtained from mice that had been injected 4 days before with 2 ml 4% thioglycolate i.p. (Difco, Detroit, MI).

Establishment of a mAb to mouse TLR4

A rat was immunized with Ba/F3 cells expressing mouse TLR4 and MD-2 and used for hybridoma production. The MTS510 mAb (rat IgG2a/k) that specifically reacted with the immunized transfectant but not with the original Ba/F3 line was selected for further analyses. The mAb was purified from ascites obtained from SCID mice.

Immunoprecipitation and immunoprobing

Cells were washed and lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 2 mM $MgCl_2$, and 2 mM $CaCl_2$. After 30 min of incubation on ice, lysate was centrifuged and nuclei were removed. The *N*-hydroxysuccinimide-activated Sepharose 4FF beads (Amersham Pharmacia Biotech, Piscataway, NJ) coupled with MTS510 (4 mg/ml) were added to cell lysate and rotated for 4 h at 4°C. Beads were washed in the lysis buffer and bound proteins were subjected to SDS-PAGE (9% acrylamide under nonreduced conditions) and Western blotting. TLR4 and MD-2 were detected with the anti-flag mAb M2 (Sigma) and Supersignal chemiluminescent substrate (Pierce, Rockford, IL). To detect LPS-induced phosphorylation of IκB-α, the PhosphoPlus IκB-α Ab Kit (NEB, Beverly, MA) was used.

Luciferase assay

Stable transfectants derived from Ba/F3 were inoculated onto 96-well plates at 5×10^4/well. After 4 h stimulation, cells were harvested, washed, and lysed in 100 μl lysis buffer, and luciferase activity was measured using 10 μl lysate and 50 μl luciferase substrate (Nippon Gene, Toyama, Japan). The luminescence was quantitated as a relative light unit on a luminometer (Berthold Japan, Tokyo, Japan).

Cell surface staining

Cells were stained with an FITC-conjugated mAb to Mac-1 (M1/70) or to CD19 (1D3) and biotinylated MTS510 followed by streptavidin-PE (PharMingen, San Diego, CA). Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

ELISA

TNF-α production from peritoneal macrophages was measured by ELISA according to the manufacturer's instruction (Genzyme, Cambridge, MA).

Results

Mouse MD-2 is associated with mouse TLR4

The whole sequence of mouse MD-2 was determined and shown in Fig. 1. The longest coding region encodes 160 aa. Mouse MD-2 has 64% (103 aa) identity to human MD-2 in amino acid sequence. The identity (64%) is similar to that between human and mouse MD-1 (66%). Stable transfectants were next derived from Ba/F3 cells by transfecting expression vectors encoding mouse MD-2 or mouse TLR4. Expression of TLR4 and/or MD-2 was confirmed by cytoplasmic or cell surface staining or by immunoprecipitation and subsequent probing of the flag epitope (Fig. 2).

```
Identities = 103/160 (64%)
Positives = 129/160 (80%)
M 1   MLPFILFSTLLSPILTESEKQQWFCNSSDA
      MLPF+ FSTL S I TE++KQ W CNSSDA
H 1   MLPFLFFSTLFSSIFTEAQKQYWVCNSSDA

M 31  IISYSYCDHLKFPISISSEPCIRLRGTNGF
       ISY+YCD +++PISI+  PCI L+G+ G
H 31  SISYTYCDKMQYPISINVNPCIELKGSKGL

M 61  VHVEFIPRGNLKYLYFNLFISVNSIELPKR
      +H+ +IPR +LK LYFNL+I+VN++ LPKR
H 61  LHIFYIPRRDLKQLYFNLYITVNTMNLPKR

M 91  KEVLCHGHDDDYSFCRALKGETVNTSIPFS
      KEV+C G DDDYSFCRALKGETVNT+I FS
H 91  KEVICRGSDDDYSFCRALKGETVNTTISFS

M121  FEGILFPKGHYRCVAEAIAGDTEEKLFCLN
      F+GI F KG Y+CV EAI+G  EE LFCL
H121  FKGIKFSKGKYKCVVEAISGSPEEMLFCLE

M151  FTIIHRRDVN 160
      F I+H+ + N
H151  FVILHQPNSN 160
```

FIGURE 1. Mouse MD-2 amino acid sequence and its alignment with human MD-2. Alignment of amino acid sequences of mouse (M) and human (H) MD-2 was conducted with the BLAST2 program at the National Center for Biological Information. The nucleotide sequence of mouse MD-2 will appear in the DNA Data Base in Japan/EMBL/GenBank nucleotide sequence databases with the accession number AB018550.

A novel mAb MTS510 was generated that, in flow cytometry, specifically reacted with the immunized Ba/F3 line expressing TLR4 and MD-2 but not with the original Ba/F3 line (Fig. 2*Ab*). To further determine the specificity of the mAb, immunoprecipitation with MTS510 and subsequent immunoprobing of TLR4 or MD-2 with the anti-flag mAb were conducted (Fig. 2*C*). A significant amount of TLR4 was precipitated from Ba/F3 cells expressing TLR4 alone (*lane 3*), whereas MD-2 was not (*lane 2*). The mAb is thus directed against TLR4 but not MD-2.

We then used the MTS510 mAb to see whether the physical association of TLR4 and MD-2, which had been previously shown in human (14), is conserved in mice. TLR4 was immunoprecipitated from Ba/F3 cells expressing TLR4 and MD-2 with the MTS510 mAb. TLR4 and MD-2 were probed with the anti-flag mAb. The MTS510 mAb was able to precipitate, in addition to TLR4, additional signals around 30 kDa (*lane 4*). These 30-kDa signals were similar in size to MD-2 that was precipitated with an anti-flag mAb from Ba/F3 cells expressing MD-2 alone or TLR4-MD-2 (*lanes 6* and *8*). Similar signals around 30 kDa were not detected with the MTS510 immunoprecipitation from Ba/F3 cells expressing TLR4 alone (*lane 3*) or from Ba/F3 cells expressing TLR4 with the flag epitope and MD-2 with another epitope tag (data not shown), as was shown in the previous human study (14). Mouse MD-2 was thus coprecipitated with mouse TLR4.

We noticed that an even larger amount of TLR4 was precipitated with MTS510 from Ba/F3 cells expressing TLR4 and MD-2 than from those expressing TLR4 alone (*lanes 3* and *4*). Expression of TLR4 protein in these Ba/F3 lines was shown by immunoprecipitation and probing with the anti-flag mAb (*lanes 7* and *8*). The Ba/F3 line expressing TLR4 alone was comparable with that expressing TLR4 and MD-2 with respect to TLR4 protein expression. MTS510 mAb seemed to react better with TLR4-MD-2 than with TLR4 alone. Flow cytometry analyses further revealed poor reactivity of the MTS510 mAb with TLR4 alone. In cell surface staining, MTS510 easily detected TLR4 on Ba/F3 cells expressing TLR4 and MD-2, but not on those expressing TLR4 alone (Fig. 2*A*, *a* and *b*). We also conducted cell-permeabilized staining of Ba/F3 cells expressing TLR4 alone, to detect intracellular TLR4 that might account for the majority of TLR4 protein expressed. MTS510 did not show significant binding to TLR4

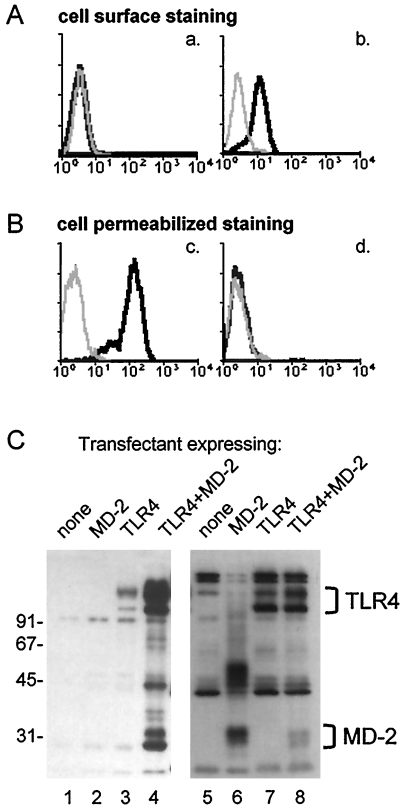

FIGURE 2. Physical association of TLR4 with MD-2 and preferential recognition of TLR4-MD-2 than of TLR4 alone by the MTS510 mAb (*A*) Ba/F3 cells expressing either mouse TLR4 alone (*a*) or mouse TLR4 and MD-2 (*b*) were used for cell surface staining with the MTS510 mAb followed by goat anti-rat IgG-FITC. Gray lines depict histograms stained with the second Ab alone. *B*, Ba/F3 cells expressing TLR4 alone were used for cell-permeabilized staining with the anti-flag mAb (*c*) or the MTS510 mAb (*d*) followed by goat anti-mouse IgG-FITC. Gray lines depict histograms stained with the second Ab alone. *C*, Immunoprecipitation with MTS510 (*lanes 1-4*) or with the anti-flag mAb (*lanes 5–8*) was conducted. After SDS-PAGE (9% polyacrylamide under nonreduced condition) and electroblotting, precipitated TLR4 and MD-2 were probed with the anti-flag mAb M2. Cells examined were: the original Ba/F3 line (*lanes 1* and *5*); Ba/F3 cells expressing MD-2 alone (*lanes 2* and *6*), TLR4 alone (*lanes 3* and *7*), and TLR4 + MD-2 (*lanes 4* and *8*). Precipitates from $\sim 3 \times 10^7$ cells were loaded for each lane.

alone (Fig. 2*Ad*), abundant expression of which was seen with the anti-flag mAb (Fig. 2*Ac*). Taken together with the immunoprecipitation results, the MTS510 mAb preferentially reacts, especially in flow cytometry, with TLR4 that is associated with MD-2.

LPS signaling via mouse TLR4 is augmented by MD-2

A role for MD-2 in the LPS signaling via TLR4 was addressed. The Ba/F3 transfectants were stimulated with LPS. NF-κB activation was examined by measuring luciferase activity from a reporter construct that had been transfected (Fig. 3*A*) or by immunoprobing with the Ab specific for phosphorylated IκB-α (Fig. 3*B*). The transfectant expressing mouse MD-2 alone did not respond to LPS even at 10 μg/ml, whereas the TLR4-expresing transfectant showed a significant response to LPS as judged by phosphorylation of IκB-α as well as by luciferase activity (Fig. 3, *A* and *B*). In the luciferase assay, the TLR4-MD-2-expressing transfectant showed one or two orders of magnitude higher sensitivity and 2- to 3-fold stronger LPS response than the cells expressing TLR4 alone to all LPS and lipid A (Fig. 3*A*). Moreover, LPS-induced

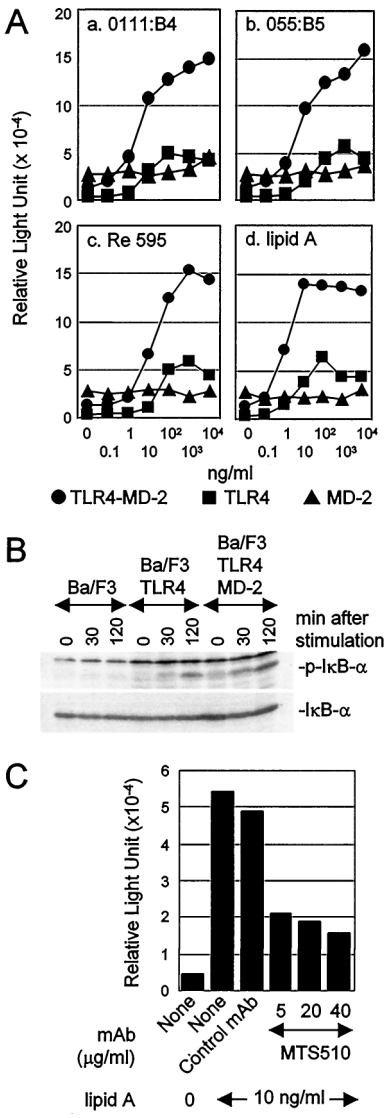

FIGURE 3. Mouse MD-2 enhances the LPS signaling via TLR4. (A) The Ba/F3 cells expressing TLR4 alone, MD-2 alone, or TLR4-MD-2 were stimulated with graded doses of LPS or lipid A as indicated in each panel. After 4 h, cells were harvested, and luciferase activity was measured. *B*, Ba/F3 cells or those expressing TLR4 alone or TLR4-MD-2 (1×10^5 cells/lane) were stimulated with lipid A (100 ng/ml) and harvested after the indicated time points. IκB-α was probed with the Abs against either phosphorylated (p) IκB-α or IκB-α. *C*, The Ba/F3 cells expressing TLR4-MD-2 were stimulated with lipid A (10 ng/ml). MTS510 mAb (doses indicated on the abscissa) as well as a control mAb (20 μg/ml) was included during stimulation. After 4 h, cells were harvested, and luciferase activity was measured. The results represent three independent experiments.

NF-κB activation was specifically inhibited by the MTS510 mAb (Fig. 3*C*). The enhancing effect by MD-2 coexpression was also demonstrated by increased phosphorylation of IκB-α (Fig. 3*B*). MD-2 thus enhances LPS signaling via TLR4 in an in vitro cell line.

Cell surface expression and LPS signaling of TLR4-MD-2 on thioglycolate-elicited peritoneal macrophages

The mAb MTS510 allowed us to examine expression of the TLR4-MD-2 complex on cells in vivo as well as in vitro. Among in vitro cell lines, TLR4-MD-2 was detected on B cell lymphomas such as BCL_1 and a macrophage line J774, but not on T cell lines or fibroblast lines (data not shown). Cell surface TLR4-MD-2 was

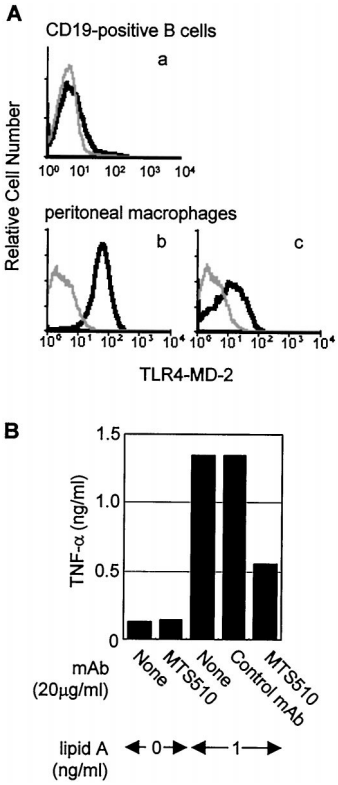

FIGURE 4. Cell surface expression and LPS signaling of TLR4-MD-2 on thioglycolate-elicited peritoneal macrophages. *A*, Spleen cells were doubly stained with FITC-conjugated anti-CD19 mAb and biotinylated MTS510 followed by avidin-PE. Expression of the MTS510 epitope on CD19-positive B cells is shown in *a*. Thioglycolate-elicited macrophages from C3H/HeN mice were collected and stimulated for 2 h with (*c*) or without (*b*) LPS from *S. minnesota* (1 μg/ml). Cells were harvested and used for staining with an anti-Mac-1 mAb conjugated with FITC and biotinylated MTS510 followed by streptavidin-PE. Expression of the MTS510 epitope on Mac-1-positive cells was shown. Histograms of cells stained without the MTS510 mAb were shown with gray lines as negative controls. The results represent four independent experiments. *B*, Thioglycolate-elicited macrophages were stimulated with lipid A (1 ng/ml) for 18 h with MTS510 or a control mAb. Supernatants were measured with an ELISA kit for TNF-α. TNF-α production reached a maximum at 1 ng/ml lipid A, and that concentration was therefore used. Data are expressed as an average from duplicate wells. Similar results were obtained from three independent experiments.

hardly detectable on CD19-positive B cells in spleen, bone marrow cells, or thymocytes (Fig. 4*A* and data not shown). Intracellular staining by the saponin detergent did not make any difference (data not shown). Apparent expression was observed on thioglycolate-elicited peritoneal macrophages (Fig. 4*Ab*). The expression was not observed on peritoneal macrophages from mice lacking TLR4, confirming the specificity of MTS510 (18). Interestingly, TLR4-MD-2 was rapidly down-regulated by 2 h stimulation with LPS (Fig. 4*Ac*). Interestingly, the down-regulation of TLR4-MD-2 was similarly observed in C3H/HeJ mice, which harbor the mutated TLR4 gene (18).

LPS signaling via native TLR4-MD-2 was then examined with MTS510. We measured LPS-dependent TNF-α production from peritoneal macrophages, and the mAb was included during LPS stimulation. MTS510 alone was not agonistic. As was seen with the stable transfectant, MTS510 acted antagonistically to LPS on peritoneal macrophages (Fig. 4*B*).

Discussion

A newly established mAb MTS510 showed reactivity to the Ba/F3 line expressing TLR4 and MD-2 in both immunoprecipitation and cell surface staining. In immunoprecipitation using Ba/F3 cells expressing TLR4 alone or MD-2 alone, the mAb was able to precipitate TLR4 but not MD-2 (Fig. 2C, *lane 2* and *3*). The MTS510 mAb is therefore directed against TLR4. From Ba/F3 cells expressing TLR4 and MD-2, MD-2 was coprecipitated with TLR4, demonstrating conserved association of TLR4 and MD-2 in mice. Interestingly, MD-2 greatly influences the reactivity of MTS510 with TLR4. An even larger amount of TLR4 was precipitated from Ba/F3 cells expressing TLR4 and MD-2 than from those expressing TLR4 alone, despite an equal amount of TLR4 protein in these two Ba/F3 transfectants (Fig. 2*C*, *lane 7* and *8*). Moreover, in flow cytometric analyses, the binding of MTS510 to TLR4 was observed in Ba/F3 cells expressing TLR4 and MD-2 but scarcely detectable in those expressing TLR4 alone (Fig. 2*B*). Collectively, MTS510 discriminates between TLR4 alone and TLR4 that is associated with MD-2. A conformational change likely occurs to TLR4 by MD-2 association.

As was shown with human MD-2, mouse MD-2 enhanced LPS signaling via TLR4. Two, not mutually exclusive mechanisms would account for the enhancing effect of MD-2. First, the TLR4-MD-2 complex is more sensitive than TLR4 alone with regard to LPS responses. Ba/F3 cells expressing human TLR4 alone, despite apparent expression on the cell surface, does not signal LPS, whereas TLR4-MD-2 does (14). Similarly, association of mouse MD-2 may augment the ability of mouse TLR4 to signal LPS. The MTS510 mAb suggested that a conformational change occurs to TLR4 with MD-2 association. Such a conformation may correlate with higher sensitivity and stronger response to LPS. The second possibility is that augmentation of LPS signaling by MD-2 may be attributed to increased expression of cell surface TLR4. This possibility is based on the fact that mouse TLR4 alone, in contrast to the human counterpart, is able to signal LPS (Fig. 3). Unfortunately, the MTS510 mAb failed to detect TLR4 alone in flow cytometry (Fig. 2, *A* and *B*). Although human MD-2 does not change cell surface expression of human TLR4 (14), we do not know whether mouse MD-2 influences cell surface expression of TLR4. In either mechanism, the present study clearly demonstrated that mouse MD-2, as the human counterpart does, enhances the LPS signaling via TLR4.

The MTS510 mAb allowed us to see TLR4-MD-2 expression in vivo with flow cytometry staining. TLR4-MD-2 was demonstrated on peritoneal macrophages and, interestingly, rapidly down-regulated in the presence of LPS. Moreover, LPS-induced TNF-α production was pronouncedly inhibited by MTS510. TLR4-MD-2 is thus expressed in vivo on peritoneal macrophages and signals LPS.

Down-regulation of TLR4-MD-2 can be attributed to disrupted association of TLR4 and MD-2, shedding from the cell surface, or internalization. In this regard, recent studies showed that LPS is rapidly internalized and transported to the Golgi apparatus (19, 20). Rapid down-regulation of TLR4-MD-2 therefore may be due to colocalization and cointernalization with LPS. Interestingly, C3H/HeJ mice, which are defective in LPS internalization (21), did not reveal a defect in down-regulation of TLR4-MD-2 (18). We must directly compare the distribution of TLR4-MD-2 and LPS in peritoneal macrophages.

B cells in spleen hardly expressed TLR4-MD-2 (Fig. 4*A*). This does not necessarily deny expression of TLR4 on B cells. Considering that C3H/HeJ B cells show hyporesponsiveness to LPS, TLR4 must be expressed and signal LPS in B cells. In keeping

with this, one of our mAbs to human TLR4 demonstrated expression of TLR4 on a subpopulation of B cells in peripheral blood or a tonsil (our unpublished observation). Preliminary studies with Northern hybridization revealed that most B cell lymphomas as well as spleen cells express mRNA transcripts encoding mouse MD-2 as well as TLR4 (Ref. 14 and data not shown). It must be determined whether B cells express the MD-2 protein. The B cell sensitivity to LPS is about two orders of magnitude lower than that of macrophages. It would be interesting to learn whether low LPS responsiveness in B cells is due to low expression of the MD-2 protein or to a lack of association with TLR4. We are currently examining this issue with newly established mAbs to mouse MD-2.

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