

# MD-2, a Molecule that Confers Lipopolysaccharide Responsiveness on Toll-like Receptor 4

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## Summary

Toll-like receptor 4 (TLR4) is a mammalian homologue of *Drosophila* Toll, a leucine-rich repeat molecule that can trigger innate responses against pathogens. The TLR4 gene has recently been shown to be mutated in C3H/HeJ and C57BL/10ScCr mice, both of which are low responders to lipopolysaccharide (LPS). TLR4 may be a long-sought receptor for LPS. However, transfection of TLR4 does not confer LPS responsiveness on a recipient cell line, suggesting a requirement for an additional molecule. Here, we report that a novel molecule, MD-2, is requisite for LPS signaling of TLR4. MD-2 is physically associated with TLR4 on the cell surface and confers responsiveness to LPS. MD-2 is thus a link between TLR4 and LPS signaling. Identification of this new receptor complex has potential implications for understanding host defense, as well as pathophysiologic, mechanisms.

Key words: leucine-rich repeat • RP105 • MD-1 • nuclear factor  $\kappa$ B • signaling

Exclusion of invading nonself pathogens is one of the basic homeostatic mechanisms for multicellular organisms. In organisms as divergent as plants, flies, and humans, leucine-rich repeat (LRR)<sup>1</sup> molecules have been implicated in the defense system against pathogens (1–5). LRR, a protein motif that has been implicated in protein–protein interaction (6), is thought to directly recognize a pathogen or its products. Toll, a *Drosophila* receptor molecule with extracellular LRR, has a role in triggering innate defenses against bacteria or fungi (2). Human homologues were recently cloned and constitute the Toll-like receptor (TLR) family, in which five TLRs (TLR1 to TLR5) have so far been isolated (7–9). TLRs are similar to *Drosophila* Toll in that they have extracellular domains containing LRR and cytoplasmic portions homologous to the intracellular signaling domain of the type 1 IL-1 receptor. The extracellular LRR domains are expected to recognize products from pathogens, and the cytoplasmic domains trigger activation of the transcription factor nuclear factor (NF)- $\kappa$ B, leading to the induction of a number of proinflammatory genes. Recent reports showed that TLR2 and TLR4 may recognize and signal LPS, a constituent of the outer membrane of the cell walls of gram-negative bacteria. Transfection of

cell lines with TLR2 confers on them the ability to respond to LPS with activation of NF- $\kappa$ B (10, 11). The TLR4 gene is defective in C3H/HeJ and C57BL/10ScCr mice, both of which have been well characterized as hyporesponders to LPS (12, 13). TLR4, as well as TLR2, may be a long-sought receptor for LPS (14). However, contrary to this prediction, transfection of cell lines with TLR4 did not confer the ability to respond to LPS but constitutively activated NF- $\kappa$ B (11). These studies imply a lack of a factor or a subunit in transfected cells that is indispensable for LPS signaling via TLR4, as suggested by Wright (14). In this study, we described a novel molecule, designated as MD-2, that is associated with TLR4 and requisite for its LPS signaling.

## Materials and Methods

**Cells and Reagents.** An expression plasmid, pEFBOS (15), and p55Ig $\kappa$ Luc, a reporter construct with three tandemly repeated  $\kappa$ B motifs upstream of a minimal IFN- $\beta$  promoter (16), were gifts from Drs. Shigekazu Nagata (Osaka University Medical School, Osaka, Japan) and Takashi Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), respectively. The human kidney cell line 293T was provided by Dr. T. Hirano (Osaka University Medical School, Osaka, Japan). LPS and lipid A were purchased from Sigma Chemical Co. The rat mAb against mouse CD14 (17) was provided by Dr. Shunsuke Yamamoto (Oita Medical University, Oita, Japan). An mAb against the flag epitope M2 was purchased from Sigma Chemical Co., and a rat mAb against the protein C epitope was established in our own laboratory.

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R. Shimazu and S. Akashi contributed equally to this study.

<sup>1</sup>Abbreviations used in this paper: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LRR, leucine-rich repeat; NF, nuclear factor; RT, reverse transcriptase; TLR, Toll-like receptor.

**cDNA Cloning and Sequencing.** The Est clone encoding human MD-2 (sequence data available from EMBL/GenBank/DBJ under accession no. AA099571) was purchased from Genome Systems, Inc. Sequencing was conducted with an ALExpress DNA sequencer and a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Ltd.).

**Northern Hybridization.** Total RNA was extracted from various cell lines or tissues with Isogen (Nippon Gene) and subjected to agarose electrophoresis (20  $\mu$ g/lane). After transfer to a sheet of nylon membrane (Hybond N<sup>+</sup>; Amersham Pharmacia Biotech, Ltd.), RNA was hybridized to a probe that had been labeled by random priming of a cDNA clone. The hybridization buffer consisted of 10% dextran sulfate (Pharmacia Biotech), 1 M NaCl, 1% SDS, and 50 mM Tris/HCl, pH 7.5. Hybridization was conducted at 65°C for 20 h. Washing was carried out in 2 $\times$  SSC and 0.1% SDS at 65°C. Radioactive signals were visualized with an image analyzer (BAS2000; Fuji Film Co., Ltd.). The same membrane was reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

**Stable Transfectants.** The IL-3-dependent line Ba/F3 (18) was transfected with the pEFBOS expression vector encoding human TLR4. Expression of TLR4 was confirmed by intracellular staining for the flag epitope, which had been attached to the COOH terminus of TLR4. The established line was then transfected with the p55 $\kappa$ B reporter construct for measuring NF- $\kappa$ B activity with luciferase activity (16). Cell lines expressing TLR4 and the reporter construct were screened by measuring spontaneous luciferase activity, which was expected to be high due to TLR4 expression (11, 19). One of the selected lines was further transfected with the expression vector encoding human MD-2 with either the flag epitope or another epitope tag, protein C (Boehringer Mannheim) at the COOH terminus. Cells expressing human MD-2 on the cell surface were screened by staining corresponding epitope tags. We also transfected human MD-2 with the flag epitope into the original Ba/F3 line, and transfectants expressing MD-2 were selected by permeabilized cell staining of the flag epitope.

**Surface and Permeabilized Cell Staining.** Cells were incubated with indicated mAbs. After washes with staining buffer (PBS containing 2% FCS and 0.1% azide), goat anti-mouse IgG-FITC (Chemicon International, Inc.) or goat anti-rat IgG-PE (Southern Biotechnology Associates, Inc.) was added. Propidium iodide was included in the second incubation to exclude dead cells. Cells were analyzed on a FACScan<sup>TM</sup> (Becton Dickinson). Permeabilized cell staining was conducted as described by Veis et al. (20). In brief, staining was conducted with the staining buffer containing saponin detergent (0.03%; Sigma Chemical Co.).

**Confocal Microscopy.** The transfectant line expressing TLR4 and MD-2 was stained as above with the mouse anti-human TLR4 mAb and the rat anti-protein C mAb, which recognized MD-2. The second Abs were goat anti-mouse IgG-FITC for the HTA125 mAb and goat anti-rat IgG-PE for the anti-protein C mAb. These second reagents did not react with the other first reagent, as judged on a FACScan<sup>TM</sup> (data not shown). Stained cells were viewed on a scanning confocal microscope system, Fluoview (Olympus Corp.).

**Immunoprecipitation.** 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, 1mM PMSF, 10  $\mu$ g/ml soybean trypsin inhibitor, 2 mM MgCl<sub>2</sub>, and 2 mM CaCl<sub>2</sub>. After 30-min incubation on ice, lysate was centrifuged and nuclei were removed. The N-hydroxysuccinimide-activated Sepharose 4FF beads (Amersham Pharmacia Biotech, Ltd.) coupled with HTA125 (4 mg/ml) were added to cell lysate and rotated for 2 h

at 4°C. Beads were washed in the lysis buffer, and bound proteins were subjected to SDS-PAGE (9% acrylamide under the nonreduced condition) and Western blot analysis. TLR4 and MD-2 were detected with the anti-flag mAb, M2 (Sigma Chemical Co.) and Supersignal<sup>®</sup> chemiluminescent substrate (Pierce Chemical Co.).

**Transient Transfection.** Transient transfection was conducted according to the report by Kaisho et al. (21). The human kidney cell line 293T was plated onto a 24-well plate at 1.5  $\times$  10<sup>5</sup> cells/well on the day before transfection. DNA was diluted in 100  $\mu$ l deionized water, and 2 M calcium chloride (14  $\mu$ l) was added to DNA. An equal amount (114  $\mu$ l) of 2 $\times$  HEPES-buffered saline (280 mM NaCl, 50 mM HEPES, and 1.5 mM sodium phosphate, pH 7.05) was added slowly in a dropwise manner. After 30-min incubation at room temperature, coprecipitates were added to 293T cells. Medium was changed on the following day, and the cells were cultured for another day. Cells were harvested with PBS containing 1 mM EDTA and used for luciferase assays.

**Luciferase Assay.** In transient transfection, the pEFBOS expression vector encoding  $\beta$ -galactosidase (a gift from Dr. Masato Ogata, Osaka University Medical School, Osaka, Japan) was used as an internal control for transfection. After transient transfection, cells were lysed in 50  $\mu$ l lysis buffer, and luciferase activity was measured using 10  $\mu$ l lysate and 50  $\mu$ l luciferase substrate (Nippon Gene). The luminescence was quantitated by a luminometer (Berthold Japan). Luciferase activity was normalized with the  $\beta$ -galactosidase activity, which was measured with 2-nitro-phenyl- $\beta$ -d-galactopyranoside (Boehringer Mannheim).

Stable transfectants were inoculated onto 48-well plates (2  $\times$  10<sup>5</sup>/well). LPS and lipid A were added together. After 4 h of stimulation, cells were harvested and lysed in 50  $\mu$ l lysis buffer, and luciferase activity was measured as above.

**The Anti-TLR4 mAb HTA125.** BALB/c mice were immunized with the Ba/F3 line expressing TLR4, and spleen cells were fused with SP2/0 myeloma cells. An mAb was chosen that stained the line used for immunization. A more detailed description of the mAbs and expression/function of TLR4 will be published elsewhere.

## Results and Discussion

RP105 was cloned in our laboratory as the first LRR molecule expressed on lymphocytes, the extracellular LRR of which had similarity to *Drosophila* Toll (22, 23). We have recently isolated MD-1 as a molecule that is physically associated with RP105 on the cell surface (24). MD-1 itself is a secretory molecule but tethered to the cell surface by coexpressed RP105. MD-1 is likely to interact with LRR of RP105, which constitutes an entire region of the extracellular portion of RP105. As the extracellular LRR of RP105 is similar to *Drosophila* Toll or homologous TLRs, we hypothesized that TLRs might also be associated with a molecule like MD-1. The hypothesis prompted us to seek a molecule with similarity to MD-1. A computer search using the MD-1 amino acid sequence retrieved a human cDNA clone from an expressed sequence tag database (Sequence data available from EMBL/GenBank/DBJ under accession no. AA099571). The whole nucleotide sequence was determined and is shown in Fig. 1 A. After an in-frame stop codon, the longest open reading frame codes for 160 amino acids, including a 16-residue, NH<sub>2</sub>-terminal hydrophobic stretch that may function as a signal peptide. We re-

**A**

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1  GCGGGCCCGCTCCCACTTCGGCACGAGGGGACAGGTAATCTTTCTGCTTACTGAAA
61  AGGAAGAGTCTGATGATTAGTTACTGATCCTCTTTGCATTTGTAAAGCTTTGGAGATATT
121  GAATCATGTTACCATTTCGTGTTTTTCCACCCCTGTTTCTCCATATTTACTGAAGCTC
1   M L P F L F F S T L F S S I E T E A

181  AGAAGCAGTATTGGGTCTGCAACTCATCCGATGCAAGTATTTATACACCTACTGTGATA
19   Q K Q Y W V C N S S D A S I S Y T Y C D

241  AAATGCAATACCAATTTCAATTAATGTTAACCCCTGTATAGAAATGAAAGGATCCAAAG
39   K M Q Y P I S I N V N P C I E L K G S K

301  GATTATTGCACATTTTCTACATTTCCAAGGAGAGATTTAAAGCAATATATTTCAATCTCT
59   G L L H I F Y I P R R D L K Q L Y F N L

361  ATATAACTGTCAACACCATGAATCTTCCAAGCGCAAGAAGTATTTGCCGAGGATCTG
79   Y I T V N T M N L P K R K E V I C R G S

421  ATGACGATTACTCTTTTTCAGAGCTCTGAAGGGAGAGACTGTCAATAACAATATCAT
99   D D D Y S F C R A L K G E T V N T T I S

481  TCTCCTCAAGGGAATAAAATTTCTAAGGGAATAACAATGTGTTGTTGAAGCTATTT
119  F S F K G I K F S K G K Y K C V V E A I

541  CTGGGAGCCCAAGAAATGCTCTTTTCTGGAGTTTGTTCATCTACACCAACCTAATT
139  S G S P E E M L F C L E F V I L H Q P N

601  CAAATTAGAATAAATTCAGTATTTAAAAA*****
159  S N *

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**B**

Identities = 27/113 (23%)  
Positives = 49/113 (42%)

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huMD-2 24  VCNSSDASIS  YTYCDKMQYP  ISINVNPCIE  LKGSKGLLHI  FYIPRRDLKQ
          VC+ S + Y CD +Q +V C + S + I R D+K+
huMD-1 32  VCSDSGLEVL  YQSCDPLQ-D  FGFVSVEKCSK  QLKSNINIRF  GIILREDIKE

huMD-2 74  LYFNLYITVN  TMNLPKRKEV  ICRGSDDDYS  FCRALKGETV  NTTISFSPFK
          L+ +L + ++ IC + +S FC KGE + +
huMD-1 81  LFLDLALMSQ  GSSVLNFSYP  ICEAALPKFS  FCGRRKGEQI  YYAGPVNNPE

huMD-2 124  IKFSKGYKIC  VVE 136
          +G+Y+ ++E
huMD-1 131  FTIIPQGEYQV  LLE 143

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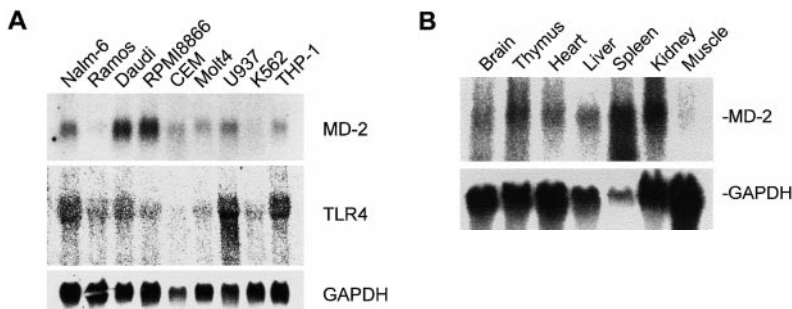
**Figure 1.** The nucleotide sequence of human MD-2 and its similarity to human MD-1. (A) The nucleotide sequence is shown with a deduced amino acid sequence. The signal peptide and canonical *N*-glycosylation sites are underlined. The stop codon is denoted by an asterisk. The nucleotide sequence of MD-2 will appear in the EMBL/GenBank/DBJ nucleotide sequence databases under accession no. AB018549. (B) Amino acid sequences of human MD-1 and MD-2 were aligned using the BLAST2 program at the National Center for Biological Information (Bethesda, MD).

fer to a mature molecule as MD-2. Significant similarity (23% identity) to MD-1 was observed over the mature polypeptide (Fig. 1 B). Notably, five out of seven cysteines of MD-2 are shared by MD-1. Expression of the MD-2 transcript was examined with Northern hybridization (Fig. 2). The size of the transcript was ~0.7 kb, which is consistent with the size of the cDNA clone. The transcript was

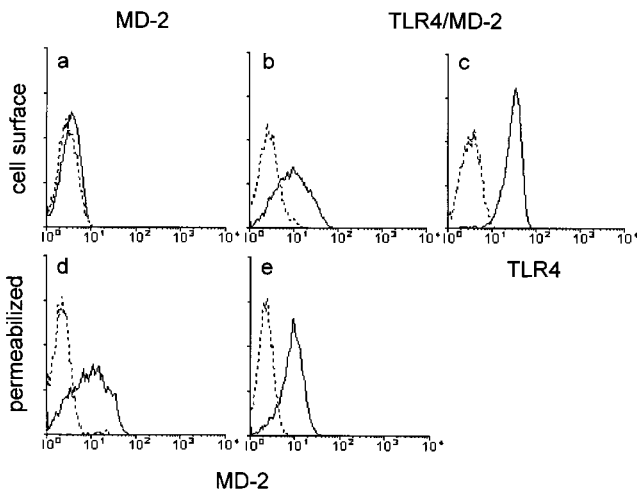
demonstrable in all human lines studied (Fig. 2 A). Three B lymphoma cell lines (Nalm-6, Daudi, and RPMI8866) showed relatively high expression. We also studied distribution of the MD-2 transcript in mouse tissues, using the mouse MD-2 probe that was cloned in our laboratory (Shimazu, R., and K. Miyake, unpublished data). Mouse MD-2 was similar to a human homologue in the size of mRNA. The transcript was also ubiquitously observed in all mouse tissues examined, among which spleen and kidney showed pronounced expression (Fig. 2 B).

We then studied interaction of MD-2 and RP105 or TLRs. RP105 seemed unlikely to interact with MD-2, as transfection of MD-2 with RP105, contrary to the case with MD-1, did not result in cell surface expression of MD-2 (data not shown). We next studied interaction with TLRs, among which TLR4 is of particular interest, because it most resembled RP105 in the extracellular LRR domain, and cells expressing the TLR4 transcript were also positive for MD-2 mRNA (Fig. 2 A). The expression vector encoding MD-2 was transfected into the Ba/F3 line. Although the precursor was demonstrable inside the cell (Fig. 3 d), MD-2 was not detectable on the cell surface (Fig. 3 a). In sharp contrast, MD-2 appears on the cell surface in a stable line expressing TLR4 (Fig. 3 c) as well as MD-2 (Fig. 3 b) and seemed to be colocalized with TLR4 on a scanning confocal microscope (data not shown). These results are consistent with membrane anchoring of MD-2 via physical association with TLR4.

To confirm the association, immunoprecipitation experiments were conducted using the transfectants expressing TLR4 and MD-2 with the newly made mAb to TLR4 (HTA125; see Materials and Methods). It should be noted that the HTA125 mAb was established by immunizing cells expressing TLR4 alone and recognizes TLR4 but not MD-2 (Fig. 4 A). Coprecipitation of MD-2 with the HTA125 mAb therefore demonstrates physical interaction of MD-2 with TLR4. We used two different transfectants, one of which expressed the flag epitope on both TLR4 and MD-2 (Fig. 4 B, lanes 1 and 2). The other line, which had the flag epitope on TLR4 but not on MD-2, was used as a control (Fig. 4 B, lane 3). Precipitates were detected with the anti-flag mAb. TLR4 was specifically precipitated from either line (Fig. 4 B, lanes 2 and 3) with HTA125 as a 120-kD band, which is consistent with a previous report (19). The signal just below presumably represents an intracellular precursor. Another species of ~25–30 kD was detected from



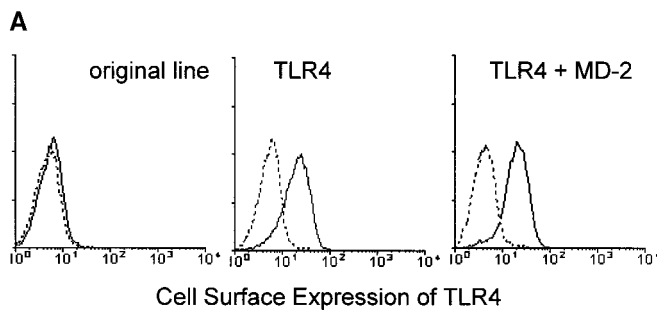
**Figure 2.** Expression of the MD-2 transcript. (A) Total RNA (20  $\mu$ g/lane) from the indicated human cell lines was electrophoresed and hybridized with probes for human MD-2, TLR4, and GAPDH as indicated. Nalm-6, Ramos, Daudi, and RPMI8866 are of B lymphocyte lineage. CEM and Molt4 are of T cell origin. U937 and THP-1 are of monocytic origin. K562 is the erythroleukemia line. (B) Total RNA (20  $\mu$ g/lane) from the indicated mouse tissues was used for Northern hybridization. The cDNA clone encoding mouse MD-2 was used as a probe. For GAPDH, the rat cDNA clone was used.



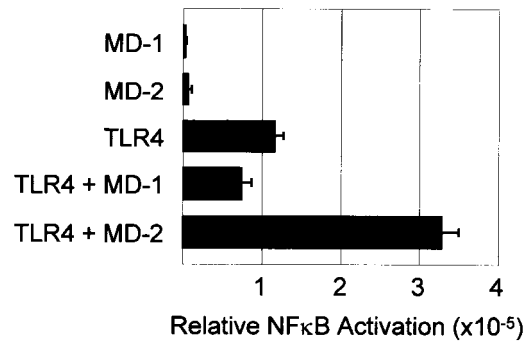
**Figure 3.** Membrane anchoring of MD-2 via TLR4. A stable line expressing MD-2 with the flag epitope was stained with the anti-flag mAb, followed by goat anti-mouse IgG-FITC (a and d). Staining with cell permeabilization is shown in d. Another stable transfectant was used that expressed MD-2 with the protein C epitope and TLR4 in b, c, and e. Cell surface MD-2 and TLR4 are shown with the rat anti-protein C mAb or the mouse anti-human TLR4 mAb HTA125 (b and c, respectively). MD-2 expression in permeabilized cells is shown in e. The second reagents used were goat anti-rat IgG-PE or goat anti-mouse IgG-FITC, respectively. Control histograms stained with isotype-matched mouse or rat mAb are shown (dotted lines).

the transfectant expressing MD-2 with the flag epitope (Fig. 4 B, lane 2). The size is similar to that of MD-1 (24) and is within a range expectable from the MD-2 amino acid sequence, consisting of 160 amino acids with two *N*-glycosylation sites (Fig. 1 A). On the other hand, this signal was not observed in the control precipitate, in which MD-2 did not bear the flag epitope (Fig. 4 B, lane 3). MD-2 is thus physically associated with TLR4.

We next explored a possible role for MD-2 in TLR4-dependent signaling. As shown in Fig. 5, expression of TLR4 alone conferred the triggering of NF- $\kappa$ B activation in 293T cells, which confirmed previous reports (11, 19). Interestingly, expression of MD-2 enhanced TLR4-dependent activation of NF- $\kappa$ Bs by 2–3 fold. Transfection of MD-1 with TLR4 did not have such an effect. Physical as-



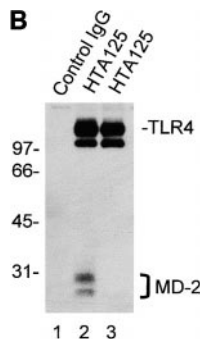
MD-2 were subjected to immunoprecipitation with control mouse IgG (lane 1) or the anti-TLR4 mAb, HTA125 (lanes 2 and 3). After blotting, precipitated molecules were detected with an anti-flag mAb, M2. The precipitates shown in lanes 1 and 2 are from the transfectant expressing TLR4 and MD-2, both of which were tagged with the flag epitope. In lane 3, we used the control line in which the flag epitope was on TLR4 but not on MD-2.



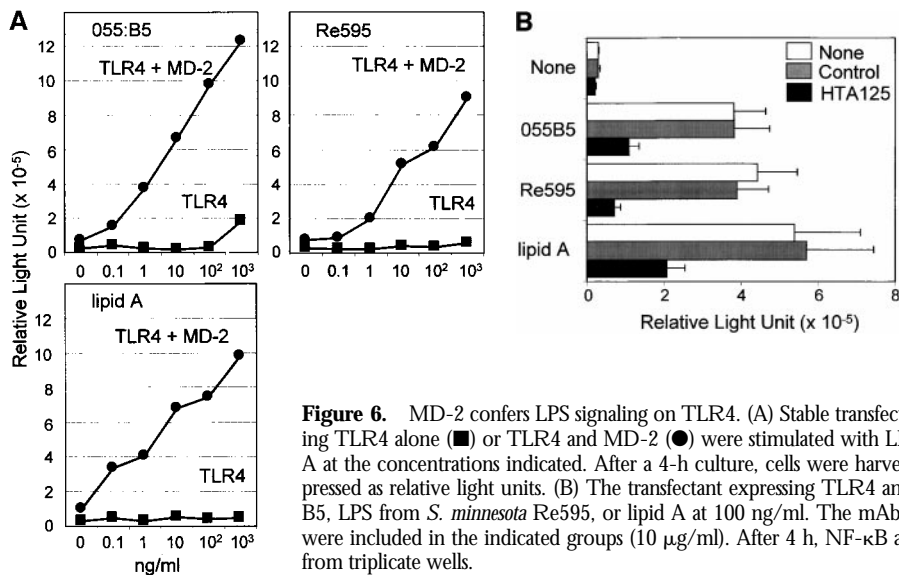
**Figure 5.** MD-2 enhances ligand-independent signaling via TLR4. A human kidney cell line, 293T, was transfected with expression vectors encoding the molecules indicated. A reporter plasmid for NF- $\kappa$ B activity and an expression vector encoding  $\beta$ -galactosidase was also transfected (see Materials and Methods). Relative NF- $\kappa$ B activity was calculated by normalizing luciferase activity with  $\beta$ -galactosidase activity. Data are shown as mean values from triplicate wells. Error bars, SD.

sociation of MD-2 therefore influences the signaling via the transmembrane TLR4 molecule. Preliminary studies suggested that MD-2 forms a homodimer or a larger complex on the cell surface. Such a complex may have multiple binding sites for TLR4 and facilitate cross-linking of TLR4, leading to higher NF- $\kappa$ B activity. Further studies are underway.

TLR4 may be an LPS receptor, as its gene is mutated in low-responder mice C3H/HeJ and C57BL/10ScCr (12, 13). Transfection of TLR4, however, did not confer LPS responsiveness on recipient cell lines (11), suggesting a requirement for another molecule that linked TLR4 to LPS signaling. We hypothesized that MD-2 might be such a link, as it interacts with TLR4 and influences the signaling of TLR4. To address this possibility, we studied LPS responsiveness of stable transfectants expressing TLR4 alone or with MD-2 by measuring NF- $\kappa$ B activity (see Materials and Methods). The stable transfectant line that expressed TLR4 alone did not respond to LPS from *Escherichia coli* 0111:B4, *E. coli* 055:B5 or *Salmonella minnesota* Re595 or to lipid A (Fig. 6 A), which is consistent with the report by Kirschning et al. (11). The Ba/F3 line, like the 293 cell line (11), might lack a molecule indispensable for LPS signaling



**Figure 4.** MD-2 is coprecipitated with TLR4. (A) Stable transfectants expressing MD-2 alone or with TLR4 were stained with or without cell permeabilization. The intracellular MD-2 precursor was stained with the anti-protein C mAb. The specificity of the anti-TLR4 mAb was shown by cell surface staining of stable transfectants. Goat anti-mouse IgG-FITC was used as the second reagent. Dotted lines correspond to histograms stained with the second reagent alone. Panels correspond to the original Ba/F3 line, a transfectant expressing TLR4 alone, and a transfectant expressing TLR4 + MD-2, respectively. (B) Stable transfectants expressing TLR4 + MD-2 were subjected to immunoprecipitation with control mouse IgG (lane 1) or the anti-TLR4 mAb, HTA125 (lanes 2 and 3). After blotting, precipitated molecules were detected with an anti-flag mAb, M2. The precipitates shown in lanes 1 and 2 are from the transfectant expressing TLR4 and MD-2, both of which were tagged with the flag epitope. In lane 3, we used the control line in which the flag epitope was on TLR4 but not on MD-2.



**Figure 6.** MD-2 confers LPS signaling on TLR4. (A) Stable transfectants (see Materials and Methods for details) expressing TLR4 alone (■) or TLR4 and MD-2 (●) were stimulated with LPS from *E. coli* O55:B5, *S. minnesota* Re595, or lipid A at the concentrations indicated. After a 4-h culture, cells were harvested, and luciferase activity was determined and expressed as relative light units. (B) The transfectant expressing TLR4 and MD-2 was stimulated with LPS from *E. coli* O55:B5, LPS from *S. minnesota* Re595, or lipid A at 100 ng/ml. The mAb HTA125 (black bars) or a control mAb (gray bars) were included in the indicated groups (10 μg/ml). After 4 h, NF-κB activity was determined. Data represent mean values from triplicate wells.

via TLR4. The mRNA of a candidate molecule MD-2 was not expressed in either cell line (data not shown). The stable line expressing TLR4 and MD-2 was then examined. Transfection of MD-2 conferred on the line strong NF-κB responses to either LPS or lipid A at concentrations as low as 0.1 ng/ml (Fig. 6 A). No response was observed to detoxified LPS from which the fatty acid side chains of the lipid A moiety were removed (data not shown). Receptor activity acquired by introducing MD-2 was triggered through TLR4, as the anti-TLR4 mAb HTA125 specifically inhibited the responses (Fig. 6 B). MD-2 thus confers LPS signaling on TLR4.

We found, by reverse transcriptase (RT)-PCR expression of the transcript of TLR2, another LPS receptor in the parental line Ba/F3. TLR3, TLR4, and TLR5 were not detected by RT-PCR or Northern hybridization (data not shown). In spite of the expression of the TLR2 transcript, stable transfectants expressing the NF-κB reporter gene alone (data not shown) or with TLR4 (Fig. 6 A) did not show any significant LPS response. The amount of the cell surface TLR2 protein, if any, may be too small to sense the presence of LPS, or mouse TLR2 may not respond to LPS as effectively as its human counterpart. Taken together with specific inhibition with the anti-TLR4 mAb, LPS responses in stable transfectants expressing TLR4 and MD-2 are mediated by the cell surface complex of TLR4-MD-2 but not by TLR2. The TLR4-MD-2 receptor complex efficiently senses the presence of bacterial endotoxin.

CD14, another LRR molecule capable of binding to

LPS, is able to enhance LPS signaling via TLR2 (10, 11). Mouse CD14 was not demonstrable by cell surface staining of the Ba/F3 line (data not shown), but it is still possible that soluble CD14 in FCS of culture medium contributes to LPS signaling via TLR4-MD-2. Further study is of importance and underway concerning a role of soluble and membrane CD14 in LPS signaling of TLR4-MD-2.

Another finding with the new receptor complex TLR4-MD-2 is that it has broader specificity than that recently described for TLR2 (10). TLR2 recognizes the LPS from *S. minnesota* Re595 much better than that from *E. coli* O55:B5. On the other hand, the TLR4-MD-2 complex responded equally to the two different types of LPS (Fig. 6 A). LPS is a complex glycolipid composed of hydrophilic polysaccharides of the core and O-antigen structures, as well as a hydrophobic domain called lipid A. Lipid A is a common component, whereas considerable diversity of structure is noted among the O-antigens. Because both TLR2 and TLR4/MD-2 receptors responded well to lipid A (reference 10 and Fig. 6 A), the core and O-antigen from *E. coli* O55:B5 must selectively affect recognition by TLR2. Studies using stable transfectants expressing each TLR would reveal further difference in recognition specificity of each TLR. MD-2 might associate with other TLR family members and confer the ability to respond to a broader spectrum of pathogens, including gram-positive bacteria and fungi. Such fundamental information concerning innate recognition of pathogens may also suggest new treatments for infectious diseases and endotoxin shock.

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