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*J Immunol* published online 30 April 2010
http://www.jimmunol.org/content/early/2010/04/30/jimmunol.0903543
Identification of a Novel Human MD-2 Splice Variant That Negatively Regulates Lipopolysaccharide-Induced TLR4 Signaling

Pearl Gray,* Kathrin S. Michelsen,† Cherilyn M. Sirois,‡ Emily Lowe,* Kenichi Shimada,* Timothy R. Crother,* Shuang Chen,* Constantinos Brikos,‡ Yunca Bulut,* Eicke Latz,‡,§ David Underhill,† and Moshe Arditi*

Myeloid differentiation factor 2 (MD-2) is a secreted gp that assembles with TLR4 to form a functional signaling receptor for bacterial LPS. In this study, we have identified a novel alternatively spliced isoform of human MD-2, termed MD-2 short (MD-2s), which lacks the region encoded by exon 2 of the MD-2 gene. Similar to MD-2, MD-2s is glycosylated and secreted. MD-2s also interacted with LPS and TLR4, but failed to mediate LPS-induced NF-κB activation and IL-8 production. We show that MD-2s is upregulated upon IFN-γ, IL-6, and TLR4 stimulation and negatively regulates LPS-mediated TLR4 signaling. Furthermore, MD-2s competitively inhibited binding of MD-2 to TLR4. Our study pinpoints a mechanism that may be used to regulate TLR4 activation at the onset of signaling and identifies MD-2s as a potential therapeutic candidate to treat human diseases characterized by an overly exuberant or chronic immune response to LPS. The Journal of Immunology, 2010, 184: 000–000.

D etection of microbial pathogens and instigation of an appropriate innate and subsequent adaptive immune response is highly reliant on TLRs (1). TLR4 is one of the most widely studied of the family and recognizes a varied repertoire of ligands, such as heat shock protein 60 (2), respiratory syncytial virus fusion protein (3), and LPS (4–8)—a major component of the outer membrane of Gram-negative bacteria. Host sensitivity to LPS is enhanced by the accessory proteins LPS-binding protein (9) and CD14 (10), but for LPS recognition to occur, TLR4 requires the coreceptor myeloid differentiation factor (MD)-2 (11–13). Upon LPS binding, a receptor multimer composed of two copies of the TLR4–MD-2–LPS complex is formed (14), which triggers a downstream signaling cascade, culminating in the activation of transcription factors such as NF-κB and the IFN regulatory factors, which in turn induce various immune and inflammatory genes.

 Tight regulation of TLR4 signaling is imperative to prevent an overactivated immune response that could contribute to the pathogenesis of autoimmune, chronic inflammatory, and infectious diseases, such as diabetes (15), asthma (16), and sepsis (4). One method of downregulating TLR4 signaling involves the production of inhibitory isoforms by alternatively splicing specific genes encoding essential signaling components. To date, several such splice variants have been identified, examples include smTLR4 (17, 18), Myd88s (19), TRAM adaptor with GOLD domain (TAG) (20) and murine MD-2B (21).

In this study, we report the identification and characterization of a novel alternatively spliced isoform of human MD-2, which we have named MD-2 short (MD-2s). Similar to full-length MD-2, this protein was glycosylated and secreted. However, despite its ability to interact with TLR4 and LPS, MD-2s failed to mediate NF-κB activation and IL-8 production after LPS exposure. We also determined that MD-2s competitively inhibited binding of full-length MD-2 to TLR4 and identified this short isoform as a negative regulator of LPS-mediated TLR4 activation. We show that MD-2s is an inducible protein that can function as a negative feedback inhibitor. Our results therefore define a novel mechanism used by the short human isoform of MD-2 that can curtail excessive activation of the innate immune response at the initiation of the TLR4 signal transduction pathway.

Materials and Methods

Cell culture and biological reagents

Immortalized human dermal microvascular endothelial cells (HMECs) were cultured in MCDB-131 medium, supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin. The HEK293 cell line, mouse RAW 264.7 macrophage cell line, and mouse aortic endothelial cells (MAECs) were cultured in DMEM and supplemented with 10% heat-inactivated FBS and 2 mM glutamine. LPS (TLR grade) was purchased from Alexis, and Biotin-LPS (Ultrapure) was purchased from Invitrogen (Carlsbad, CA).

RT-PCR and real-time quantitative PCR

Total cellular RNA was isolated from cells using the RNeasy mini kit (Qiagen, Valencia, CA) and treated with DNase. RNA from human lung, pancreas, thymus, kidney, spleen, liver, heart, and placenta was purchased from Ambion (Austin, TX). After reverse transcription with Omniscript cDNA synthesis kit (Qiagen), PCR analysis was performed using primers specific
for human MD-2 (5’-ATGTTACATTCTGTTG-3’, 5’-CTAATTGGAAATGTTG-3’) or mouse MD-2 (5’-TCGCAACCTCCGGATG-3’, 5’-GGCGGTAGATGTGGTGA-3’). The PCR was performed using Taq DNA polymerase (Invitrogen). GAPDH served as a loading control. For real-time quantitative PCR (QPCR) the following primer and probe set was used to detect MD-2s: 5’-ATT GGG TCT GCA ACT CAT CC-3’, 5’-TTC TTT GCG CTT TGG AAG AT-3’, and 5’-CACCCTA CTG TGG GAG AGA TTT AAA GCA-3’. The comparative cycling threshold method (ΔΔCT) was used for relative quantification after normalization with GAPDH expression.

**Immunoprecipitation and immunoblotting**

HEK293 cells were seeded into 100-mm dishes (1.5 × 10⁶) 24 h prior to transfection. Transfections were performed using lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. For coimmunoprecipitations, 4 μg of each construct was transfected. For competition experiments in which the effect of increasing MD-2s expression on complex formation between MD-2 and TLR4 was examined, 3 μg of each signaling molecule expression plasmid was transfected in the presence of increasing amounts (10 or 15 μg) of the MD-2s expression plasmid. The total amount of DNA in each sample was kept constant by using empty vector cDNA. Cells were harvested 24 h after transfection in 600 μl lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40 containing protease inhibitor mixture, and 1 mM sodium orthovanadate). For immunoprecipitations, the indicated Abs were incubated with the cell lysates for 2 h or overnight at 4°C. Subsequently, TrueBlot IgG beads (Ebioscience, San Diego, CA) were added and the samples were incubated at 4°C for 1 h. The immune complexes were then washed and the associated proteins were eluted from the beads by boiling in 35 μl sample buffer and then fractionated by SDS-PAGE. For immunoblotting, primary Abs were detected using HRP-conjugated secondary Abs, followed by ECL (Amersham Biosciences, Piscataway, NJ).

**Reporter gene assays**

HEK293 cells were transiently transfected with the expression vectors noted in combination with constructs encoding the NF-κB-luciferase reporter gene and the pRL-TK reporter gene to normalize for transfection efficiency. In all cases, total DNA concentration was kept constant by supplementation with empty vector control. After overnight incubation, cells were stimulated for 6 h with 50 ng/ml LPS and lysed, and luciferase activity was measured. For the inhibition studies, HEK293 cells were transfected with a constant amount (3 μg) of MD-2 expression plasmid alone or in combination with increasing concentrations of a plasmid expressing MD-2s (5, 10, 20). In all cases, total DNA concentration was kept constant by supplementation with empty vector control. Supernatants derived from these cells (5 ml final volume) were then transferred (100 μl per well) to HEK293 cells stably transfected with TLR4 and a NF-κB reporter gene. These supernatant-treated cells were then stimulated with LPS (5 ng/ml) for 6 h at 37°C and lysed, and luciferase activity was measured. Data are shown as mean ± SD of three or more independent experiments and are reported as a percentage of LPS-stimulated NF-κB promoter activity.

**ELISA of human IL-8**

After LPS stimulation, supernatants were collected and the production of human IL-8 was measured with an ELISA kit following the manufacturer’s directions (R&D Systems, Minneapolis, MN).

**Flow cytometric analysis**

HEK293T cells were retrovirally transduced to generate a cell line stably expressing TLR4-mCitrine. TLR4-mCitrine cells were seeded into 12-well dishes, transfected with 500 ng plasmid encoding Myc-tagged MD-2 or MD-2s using GeneJuice lipofection reagent (Novagen). Cells were harvested 24 h after transfection. Cells were transfected with up to 300 ng plasmid encoding myc-tagged MD-2 or MD-2s using GeneJuice lipofection reagent (Novagen) and cultured for ~24 h. Cells were stained with anti-myc Alexa-647 Ab (Abd Serotec) in culture medium at 4°C for 20 min. Cells were subsequently imaged at room temperature using an SP2 AOBs confocal laser scanning microscope (Leica Microsystems, Deerfield, IL) running LCS software (Leica Microsystems).

**Results**

Identification of a novel human MD-2 splice variant

We examined the expression profile of MD-2 in human monocyte THP-1 cells by RT-PCR analysis and detected two cDNA products, suggesting that the human MD-2 gene is alternatively spliced (Fig. 1A). RT-PCR amplification using cDNA derived from HMECs also yielded two fragments. Sequencing of the larger cDNA fragment determined that it corresponded to the published sequence of full-length human MD-2. Sequence analysis of the smaller cDNA fragment, subsequently referred to as MD-2s, revealed that this was a novel splice variant of human MD-2, lacking the region encoded by exon 2 (Fig. 1B, 1C). The open reading frame of MD-2s consists of 390 bp, which translates into a predicted protein of 130 aas.

Excision of exon 2 resulted in an in-frame deletion of 90 bp and an amino acid substitution (D38G) at the junction between exons 2 and 3. The existence of MD-2s was further confirmed; a full-length mRNA sequence, deposited in the NCBI database, corresponded to MD-2s (accession code: BM918324; www.ncbi.nlm.nih.gov). Table I depicts the 3’ and 5’ splice-site sequences at the intron-exon boundaries for MD-2 and MD-2s.

We also examined the expression profile of murine MD-2 by performing RT-PCR on cDNA derived from the murine monocytic cell line RAW246.7 and MAECs. Using primers specific for murine MD-2, only the larger RT-PCR product was detected (Fig. 1D, lanes 1 and 2, respectively). To confirm that the absence of the smaller fragment was not specific to the murine cell lines selected, we also amplified cDNA from murine bone marrow-derived dendritic cells and liver tissues obtained from C57BL/6 mice. Again, only the larger cDNA fragment was observed, suggesting that MD-2s is not expressed in mice (Fig. 1D, lanes 3 and 4). Furthermore, of the four murine MD-2 splice variants that have been deposited in the NCBI database, exon 2 is transcribed in all isoforms identified.

To determine why the human but not the mouse MD-2 gene alternatively skips exon 2, we compared the gene structures and sequences of the two species. Both the human and murine MD-2 genes are organized into five exons and four introns, and each species encodes a predicted full-length MD-2 protein of 160 aas. Alignment of the human and mouse genomic regions revealed that the exons and coding sequences were well conserved. However, analysis of the noncoding regions revealed a number of differences. In particular, it was noted that intron 1 of human MD-2 is composed of 13,241 bp, whereas the mouse has 3,064. The longer intron 1 might increase the probability for alternative lariat formation during the splicing process of human MD-2. In addition, after comparing the sequences at the 3’ end of intron 1, we found that murine MD-2 has more pyrimidines than its human ortholog, which could lead to a more stable lariat formation in murine MD-2, thereby preventing alternative splicing of exon 2 in this species.

Expression and regulation of human MD-2s

To further characterize the expression profile of human MD-2s, we performed RT-PCR analysis on a variety of human tissues. As shown in Fig. 2A, MD-2s mRNA was expressed in all tissues examined. We also observed that, although the ratio between MD-2 and MD-2s varies in different tissues, full-length MD-2 is the predominant form detected. Previous studies have indicated that MD-2 mRNA is

**Expression and regulation of human MD-2s**

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upregulated after exposure to IFN-γ (22, 23) or IL-6 (13). We therefore investigated what effect these stimuli would have on MD-2s mRNA levels. RNA was extracted from THP-1 cells stimulated with IFN-γ or IL-6, and QPCR was performed with primers specific for MD-2s and the reference gene GAPDH. Notably, we observed induction of MD-2s mRNA after treatment with both IL-6 and IFN-γ (Fig. 2B). We next examined what effect LPS stimulation would have on MD-2s expression. Interestingly, MD-2s expression was also upregulated in response to LPS (Fig. 2C). This finding suggests that MD-2s play an important regulatory role during immune responses and TLR signaling.

**MD-2s is glycosylated and secreted**

To further characterize MD-2s, we amplified the smaller RT-PCR fragment and cloned it into an expression vector encoding for the Myc tag. When this plasmid was transfected into human embryonic kidney (HEK293) cells, overexpressed MD-2s migrated with different electrophoretic mobilities after SDS-PAGE analysis (Fig. 2D, lane 1). MD-2 displays a similar expression profile because of its tertiary structure was likely to be different from that of MD-2, which could result in occlusion of these known glycosylation sites. Nevertheless, we found that after treatment with the peptide-N-glycosidase F (PNGase F), the slowest migrating forms of MD-2s were no longer evident in the sample treated with PNGase F (Fig. 2D, lane 2). Thus, MD-2s is also a gp.

MD-2 belongs to the MD-2–related lipid recognition family, the signature sequence of which is a secretion signal (25). This signal is located at the N-terminus of MD-2, and studies have shown that MD-2 is secreted (26, 27). MD-2s also contains this leader sequence. We therefore examined whether MD-2s exists as a stably secreted protein. Myc-tagged proteins were immunoprecipitated from culture supernatants collected from HEK293 cells transiently expressing MD-2s–Myc. As shown in Fig. 2E, lane 2, a secreted product corresponding to MD-2s was detected.

**MD-2s fails to induce NF-κB activation following LPS stimulation**

We investigated the requirement for MD-2s in LPS mediated NF-κB activation. It is known that the soluble form of full length MD-2 confers LPS responsiveness to cells expressing TLR4 (26, 28, 29); therefore, we investigated whether soluble MD-2s was also bioactive. Culture supernatants were collected from HEK293 cells transiently expressing control vector, MD-2, or MD-2s, and incubated with HEK293 cells stably transfected with TLR4 and an NF-κB–dependent luciferase reporter gene. Consistent with published results, soluble MD-2 conferred the ability of these reporter

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**Table I. Intron-exon boundaries of the human MD-2 gene**

<table>
<thead>
<tr>
<th>Isoform</th>
<th>3' Splice-Acceptor Site</th>
<th>Exon (bp)</th>
<th>5' Splice-Acceptor Site</th>
<th>Intron (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD-2</td>
<td>TACACCTCTGTTGtaagtaaaa</td>
<td>1 (228)</td>
<td>TACACCTCTGTTGtaagtaaaa</td>
<td>1 (13,241)</td>
</tr>
<tr>
<td>MD-2s</td>
<td>TACACCTCTGTTGtaagtaaaa</td>
<td>1 (129)</td>
<td>TACACCTCTGTTGtaagtaaaa</td>
<td>1 (18,446)</td>
</tr>
<tr>
<td>MD-2</td>
<td>TTTCTACATTCCAA</td>
<td>2 (90)</td>
<td>TTCTACATTCCAA</td>
<td>2 (5115)</td>
</tr>
<tr>
<td>MD-2s</td>
<td>AAGTTCAA</td>
<td>3 (2114)</td>
<td>AAGTTCAA</td>
<td>3 (16,659)</td>
</tr>
<tr>
<td>MD-2</td>
<td>CTCTGAAGGGAGagtaagttca</td>
<td>2 (16,659)</td>
<td>CTCTGAAGGGAGagtaagttca</td>
<td>3 (13,241)</td>
</tr>
<tr>
<td>MD-2s</td>
<td>CTCTGAAGGGAGagtaagttca</td>
<td>2 (129)</td>
<td>CTCTGAAGGGAGagtaagttca</td>
<td>2 (16,659)</td>
</tr>
<tr>
<td>MD-2</td>
<td>R D L</td>
<td>4 (53)</td>
<td>R D L</td>
<td>4 (2114)</td>
</tr>
<tr>
<td>MD-2s</td>
<td>R D L</td>
<td>3 (129)</td>
<td>R D L</td>
<td>3 (2114)</td>
</tr>
<tr>
<td>MD-2</td>
<td>T V N</td>
<td>5 (139)</td>
<td>T V N</td>
<td>5 (2114)</td>
</tr>
<tr>
<td>MD-2s</td>
<td>T V N</td>
<td>4 (117)</td>
<td>T V N</td>
<td>4 (117)</td>
</tr>
</tbody>
</table>

The intronic and exonic sequences are in lowercase and uppercase letters respectively. The splice-acceptor sites are in bold underlined letters. The single letter amino acid translations are located below the middle nucleotide of the codon.
cells to respond to LPS by inducing both NF-κB activation (Fig. 3A) and IL-8 secretion (Fig. 3B). In contrast, the secreted form of MD-2s could not activate either of these responses (Fig. 3A, 3B). We next assessed the capacity of MD-2s to mediate signal transduction triggered by LPS after transient transfection of the MD-2s expression vector. HEK293TLR4 cells stably expressing the NF-κB–dependent luciferase reporter gene were transiently transfected with plasmids encoding CD14 in combination with either MD-2 or MD-2s, and subsequently stimulated with LPS. Although full-length MD-2 activated NF-κB and led to secretion of IL-8 in the presence of LPS, overexpressed MD-2s remained inactive, even at higher concentrations (Fig. 3C, 3D). These results suggest that it is essential for MD-2 to contain the region encoded by exon 2 to mediate LPS-induced TLR4 signaling.

FIGURE 2. Expression and regulation of MD-2s. A, MD-2 and MD-2s were amplified by RT-PCR from the indicated human tissues. B, QPCR analysis of RNA from untreated, IL-6–, and IFN-γ–treated THP-1 cells using primers specific for MD-2s. Expression is normalized to the reference gene GAPDH and is presented relative to that of untreated controls. C, RT-PCR analysis of RNA from THP-1 cells treated with LPS for 4, 6, 8, and 22 h. Primers specific for MD-2s were used. Expression is normalized to the reference gene GAPDH and is presented relative to that of untreated controls. D, Myc-tagged proteins were IP with an anti-Myc Ab from cell lysates prepared from HEK293 cells transiently transfected with a plasmid encoding Myc–MD-2s. Immunoprecipitates were either left untreated (lane 1) or treated with PNGase F (lane 2). Samples were subsequently analyzed by SDS-PAGE and IB with an anti-Myc Ab. E, Myc–MD-2s was immunoprecipitated from culture supernatants that were obtained from HEK293 cells transiently transfected with a plasmid encoding Myc–MD-2s. Samples were subsequently analyzed by SDS-PAGE and immunoblotted with an anti-Myc Ab. Mock transfected culture supernatants were used as a negative control. IB, immunoblotted; IP, immunoprecipitated.

FIGURE 3. MD-2s fails to mediate LPS-dependent NF-κB activation and IL-8 secretion. A, HEK293 cells stably transfected with TLR4 and an NF-κB reporter gene were treated with supernatants containing either MD-2 or MD-2s. Cells were left untreated or stimulated with LPS (250 ng/ml) for 6 h. Mean relative stimulation of luciferase activity ± SD for a representative experiment, each performed in triplicate, is shown. B, Supernatants were collected and measured for IL-8 secretion. C, HEK293 cells stably transfected with TLR4 and an NF-κB reporter gene were transiently transfected with plasmids encoding for CD14 and either MD-2 (25 ng) or MD-2s (wedge represents 25–200 ng). Twenty-four hours later, cells were left untreated or stimulated with LPS (50 ng/ml) for 6 h. Mean relative stimulation of luciferase activity ± SD for a representative experiment, each performed in triplicate, is shown. D, Supernatants were also collected and measured for IL-8 secretion.
**MD-2s interacts with TLR4**

Although MD-2 lacks transmembrane and intracellular regions, it can be membrane-bound through its association with the extracellular portion of TLR4 (30). Given that MD-2s failed to mediate LPS signaling, we next asked whether this might be due to an inherent inability to interact with TLR4. To address this possibility, we first investigated whether MD-2s was located on the surface of TLR4-expressing cells. We transiently transfected TLR4-mCitrine-expressing cells with plasmids encoding either MD-2 or MD-2s and performed flow cytometric analysis. We observed that MD-2s was bound to the cell surface of TLR4-expressing cells (Fig. 4A). We then examined the cellular distribution of MD-2s in regard to TLR4 by confocal microscopy. We found that MD-2s exhibited the same cellular distribution as MD-2 and colocalized with TLR4 on the cell surface (Fig. 4B). We next assessed whether MD-2s was anchored to the membrane via association with TLR4. We observed that MD-2s immunoprecipitated together with TLR4 (Fig. 4C, lane 4). Furthermore, as has been reported with MD-2, both the nonglycosylated and glycosylated forms of MD-2s immunoprecipitated with TLR4. This finding indicates that although MD-2s failed to confer LPS responsiveness to cells in the absence of MD-2, this cannot be attributed to an inability to associate with TLR4.

**MD-2s interacts with LPS and inhibits TLR4 signaling by preventing the formation of an active MD-2–TLR4 complex**

Having determined that MD-2s cannot reconstitute LPS signaling in cells lacking MD-2, we next investigated the effect of soluble MD-2s on TLR4 activation. HEK293 cells stably expressing TLR4 and an NF-κB–dependent luciferase reporter gene were incubated with supernatants collected from HEK293 cells that contained a constant amount of MD-2, but increasing concentrations of MD-2s. We noted that soluble MD-2s blocked NF-κB activation in response to LPS in a dose-dependent manner (Fig. 5A). We also observed that overexpressed MD-2s inhibited LPS-induced IL-8 secretion (Fig. 5B). These findings indicate that MD-2s functions as a negative regulatory protein in the TLR4 signaling pathway, suggesting that MD-2s can be used as a therapeutic or preventative agent for modulating an overactivated MD-2–TLR4 immune response.

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**FIGURE 4.** MD-2s colocalizes and immunoprecipitates with TLR4. A, Cells stably expressing TLR4-mCitrine were transiently transfected with plasmids encoding for full-length MD-2 (upper panel) or MD-2s (lower panel) and surface-stained with anti-myc Alexa 647-conjugated Ab. Cells were analyzed by flow cytometry and gated to select single cells and the TLR4-positive population. Specific staining of TLR4 cells expressing MD-2 (upper panel, red line) or MD-2s (lower panel, red line) could be distinguished from nonspecific staining of untransfected cells (black line). B, Cells stably expressing TLR4-mCitrine (green) were transiently transfected with plasmids encoding wild-type MD-2 (upper panels) or MD-2s (lower panels). Twenty-four hours later, cells were incubated with an anti-Myc Ab and cross-liked with Alexa 647-conjugated anti-mouse polyclonal secondary Ab. Colocalization of TLR4 and MD-2 isoforms on the cell surface is visualized in yellow. C, HEK293 cells were transiently transfected with a plasmid encoding for Flag-tagged TLR4 (lanes 1–4) and cotransfected with either a Myc-tagged MD-2 (lane 3) or a Myc-tagged MD-2s (lanes 1 and 4) expressing plasmid. Coimmunoprecipitation experiments were then performed using an anti-Flag Ab (lanes 2–4) or an IgG isotype control Ab (lane 1). Samples were fractionated by SDS-PAGE, and immunoblotting with an anti-Myc Ab was performed. Experiment shown is representative of four separate experiments.
We next analyzed the published structural models of MD-2 to ascertain the structural effect of deleting exon 2. MD-2 consists of a β-cup fold with two anti-parallel β-sheets, one composed of six β-strands (numbered 1/2/9/8/5/6) and the other of three (numbered 3/4/7) (31, 32). The missing exon 2 of MD-2s encodes the first two β-strands of the three-stranded β-sheet (β3 and β4 strands; Fig. 5C). The hinges connecting β-strands 2 to 3 and 4 to 5 are also partially lost. Furthermore, the disulphide bond between Cys25 and Cys51, which assists in closing the MD-2 cavity and stabilizing the cuplike structure, is disrupted. The β6 and β7 strands

Table II. Comparison of the MD-2 splice variants, MD-2s and MD-2B

<table>
<thead>
<tr>
<th></th>
<th>MD-2s (Human)</th>
<th>MD-2B (Murine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deleted region</td>
<td>Exon 2</td>
<td>54 bp of Exon 3 at 5’ end</td>
</tr>
<tr>
<td>Glycosylated</td>
<td>Yes</td>
<td>ND but contains multiple bands</td>
</tr>
<tr>
<td>Secreted</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>Mediate LPS-induced NF-κB activation</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Upregulated</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>Immunoprecipitates with TLR4</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Immunoprecipitates with LPS</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>Inhibits LPS signaling</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Inhibits TLR4 surface expression</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Inhibits formation of the MD-2–TLR4 complex</td>
<td>Yes</td>
<td>Yes, on the cell surface</td>
</tr>
</tbody>
</table>
that line the entrance to the deep hydrophobic cavity are still encoded by the mRNA of MD-2s.

Elucidation of the TLR4–MD-2–LPS complex revealed that LPS binding instigates the formation of a receptor multimer, which is composed of two copies of the complex arranged symmetrically (14). Eleven residues are involved in the main dimerization interface of the TLR4–MD-2–LPS complex, 10 of which are still present in MD-2s. However, deletion of exon 2 in MD-2 implied that the ligand-binding pocket of MD-2s could be severely disrupted, suggesting that it might be unable to bind LPS efficiently. To address this possibility, HEK293 cells were transiently transfected with plasmids encoding either MD-2–Myc or MD-2s–Myc. Culture supernatants were then incubated with biotinylated-LPS, and Myc-tagged proteins were immunoprecipitated. In agreement with previous studies (33–35), secreted MD-2 bound readily to LPS (Fig. 5D, lane 4). Under similar conditions, an interaction between secreted MD-2s and LPS was also detected (Fig. 5D, lane 6), suggesting that soluble MD-2s can sequester LPS from binding to the MD-2–TLR4 complex and thus diminish TLR4 signal transduction.

Given that MD-2s inhibits LPS-induced TLR4 signaling, but still interacts with LPS and TLR4, we hypothesized that it competed with MD-2 to form an interaction with TLR4. To investigate this possibility, we increased the expression level of MD-2s–Myc, but kept the concentration of the constructs for HA–MD-2 and Flag–TLR4 constant. As expected, both the glycosylated and non-glycosylated forms of MD-2 immunoprecipitated with TLR4 in HEK293 cells transiently transfected with plasmids encoding both proteins (Fig. 5E, lane 2). However, overexpression of MD-2s competitively inhibited binding of MD-2 to TLR4 (Fig. 5E, compare lanes 3 and 4 to lane 2), indicating that MD-2s down-regulates TLR4 signaling by inhibiting the formation of an active MD-2–TLR4 signaling complex (see Table II for a summary of the functions of MD-2s).

Discussion

An inappropriately mounted or dysregulated immune response can cause considerable morbidity and mortality in a number of diseases. One such example is sepsis, which is among the most common causes of death in the United States, with >750,000 cases presenting annually, of which more than one quarter are fatal (36). Excessive inflammation is the hallmark of a number of related infectious pathologies as well, including sepsis, acute respiratory distress syndrome, and multiple organ failure (37). LPS derived from bacterial sources can contribute to these diseases, and it does so by interacting with MD-2 and TLR4. To circumvent an overactivated host immune response to LPS, it is imperative that TLR4 signal transduction be tightly regulated, but the precise molecular mechanisms by which this is accomplished are only partly understood.

In this study, we further elucidate the complexities involved in averting a prolonged and dysregulated immune response to LPS by the identification of a naturally occurring alternatively spliced isoform of human MD-2, which we have termed MD-2s. We report that human MD-2s is generated by skipping exon 2 of the MD-2 gene, which leads to an in-frame deletion of 30 aas spanning positions 39–69, and one amino acid substitution (D38G). Under similar conditions and using primers specific to the murine MD-2 gene, we could not detect a corresponding murine splice variant. A previous study reported an alternatively spliced version of murine MD2 (MD-2B) (21), which lacks the first 54 bases of exon 3 and downregulates LPS signaling. However, there are no data available to determine whether it is secreted or inducible, and the human relevance is unknown (see Table II for comparison between MD-2s and MD-2B).

Our results identify MD-2s as an important negative regulatory component of the TLR4 signaling pathway. The mRNA expression profile of MD-2s in human tissues revealed that it is ubiquitously expressed, suggesting that this isoform performs a widespread role in modulating TLR4-mediated responses. Previous studies have shown that IFN-γ exerts anti-inflammatory responses by inducing specific secreted inhibitors, such as the IL-1R antagonist (IL-1Ra) (38) and IL-18 binding protein (IL-18BP) (39). These molecules suppress the activity of IL-1 and IL-18, respectively. Many LPS-inducible negative regulators have also been identified, such as smlTR4 and MyD88s. We determined that MD-2s is upregulated in response to IFN-γ, IL-6, and LPS, indicating that MD-2s might be a key component involved in the negative regulation of TLR4 signaling.

Similar to full-length MD-2, MD-2s is a secreted gp. However, MD-2s overexpression failed to trigger NF-κB activation and IL-8 secretion following LPS treatment, indicating the importance of exon 2 for MD-2 function. Importantly, we also observed that MD-2s negatively regulated both NF-κB activation and IL-8 secretion following LPS stimulation, suggesting that MD-2s can be used as a therapeutic or preventative agent for modulating endotoxemia. Whereas the murine isoform MD-2B inhibited TLR4 from being expressed on the cell surface (21), MD-2s is anchored to the cell surface of TLR4 expressing cells, and both proteins localize together on the cell membrane. We also determined that MD-2s and TLR4 immunoprecipitated together. This may have been predicted, given that MD-2s retains most of the residues reported to be essential in mediating a MD-2–TLR4 interaction, with the exception of I66 and R68 (31, 40). In addition, MD-2s immunoprecipitated with LPS; this is consistent with a study demonstrating that a 15-residue peptide fragment of MD-2, encompassing the F126 loop (positions 119–132), still binds LPS (41). Additional studies have verified that residues within this fragment of MD-2 are essential for LPS binding (34, 40, 42). This region is preserved in MD-2s and most likely confers the ability of MD-2s to associate with LPS. Furthermore, although the hydrophobic pocket of MD-2s is predicted to be disrupted, all the MD-2 residues that have been shown to be involved in the main dimerization interface of the TLR4–MD-2–LPS complex, are preserved in MD-2s, with the exception of K58. Although it appears that these residues are sufficient to form an effective interaction between MD-2s and LPS, the binding affinity is most likely affected.

Several studies have shown that negative regulators can control TLR signal transduction by inhibiting the formation of active signaling complexes, including the recently identified splice variant TAG (20), IFN regulatory factor 4 (43), RP105 (44), and the IL-1Ra (45–47). Based on our results, we propose that MD-2s functionally modulates TLR4 signaling by inhibiting the formation of an active MD-2–TLR4 signaling complex. In addition, it is possible that MD-2s may behave like a decoy coreceptor by binding LPS and TLR4 to form a nonfunctional complex that does not activate NF-κB, thereby negatively regulating signaling.

Collectively our results define an important mechanistic role for MD-2s in modulating the LPS–TLR4 signal transduction pathway at the initial phase of activation. MD-2s therefore represents a prospective target for pharmacologic intervention and the development of new therapeutic and preventive strategies for sepsis and other diseases that result from an overexuberant MD-2–TLR4-induced immune response.

Acknowledgments

We thank P. Sun for technical assistance, K. Miyake (Tokyo University) for the plasmid encoding Flag MD-2, F.J. Candal (Centers for Disease Control and Prevention, Atlanta, GA) for the HMECs, and Terence M. Doherty (Cedars-Sinai Medical Center) for editorial assistance.
Disclosures

The authors have no financial conflicts of interest.

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