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CUTTING EDGE

# Cutting Edge: Gln[22] of Mouse MD-2 Is Essential for Species-Specific Lipopolysaccharide Mimetic Action of Taxol[1]

Kiyoshi Kawasaki, Kazunori Gomi, and Masahiro Nishijima[2]

**MD-2 associates with the extracellular domain of Toll-like receptor 4 (TLR4) and greatly enhances LPS signaling via TLR4. Taxol, which mimics the action of LPS on murine macrophages, induces signals via mouse TLR4-MD-2, but not via human TLR4-MD-2. Here we investigated the molecular basis for this species-specific action of Taxol. Expression of mouse MD-2 conferred both LPS and Taxol responsiveness on human embryonic kidney 293 cells expressing mouse TLR4, whereas expression of human MD-2 conferred LPS responsiveness alone, suggesting that MD-2 is responsible for the species-specificity as to Taxol responsiveness. Furthermore, mouse MD-2 mutants, in which Gln[22] was changed to other amino acids, showed dramatically reduced ability to confer Taxol responsiveness, although their ability to confer LPS responsiveness was not affected. These results indicated that Gln[22] of mouse MD-2 is essential for Taxol signaling but not for LPS signaling.** *The Journal of Immunology,* **2001, 166: 11–14.**

The immediate defensive responses against infection are due to the innate immune system. Understanding of the molecular bases of innate immune responses has advanced well over the past few years since the discovery of mammalian Toll-like receptors (TLRs)[3] (1). TLRs are structurally similar to *Drosophila* Toll, which encodes a membrane protein involved in *Drosophila* immunity against fungi (2); TLRs constitute a transmembrane protein family that has leucine-rich repeats in the extracellular portion and a cytoplasmic portion homologous to the intracellular signaling domain of type I IL-1 receptor (3, 4). Involvement of TLR4 in LPS responses was first suggested by the discovery that TLR4 from spontaneous LPS-hyporesponsive mutant mice, C3H/HeJ, has a point mutation that causes an inability to activate NF-$\kappa$B (5, 6, 7). Subsequently, using generated TLR4-deficient mice, TLR4 was unequivocally demonstrated to be involved in LPS responses (7). Furthermore, TLR4 was suggested to be involved in ligand-specific recognition of LPS (8, 9), although direct binding of TLR4 with LPS on the cell surface has not been demonstrated. However, expression of TLR4 alone is not sufficient for LPS responsiveness to be conferred on a mouse pro B cell line, Ba/F3 cells (10). In addition to TLR4, the expression of MD-2, which associates with the extracellular domain of TLR4, is required for LPS responsiveness to be conferred on Ba/F3 cells (10). In fact, there is a TLR4-MD-2 complex on the surface of mouse peritoneal macrophages, and the complex on the macrophages has been shown to be involved in LPS responses (11).

Taxol, a diterpene purified from the bark of the Western yew (*Taxus brevifolia*) (12), is an antitumor agent that blocks mitosis by binding and stabilizing microtubules (13, 14). Although the structure of Taxol is quite different from that of the LPS lipid A moiety, which is responsible for many LPS responses (15), Taxol has been shown to exhibit many LPS-mimetic activities as to murine macrophages, such as induction of TNF secretion (16), NO production (17), and NF-$\kappa$B activation (18). Taxol was suggested to share a receptor and/or signaling molecule with LPS because these LPS-mimetic activities of Taxol were not detected in macrophages from LPS-hyporesponsive C3H/HeJ mice (16–18). Interestingly, the LPS-mimetic activities of Taxol were species specific; Taxol did not mimic the action of LPS on human LPS-responsive cells including macrophages (19, 20). By using stable transfectants expressing MD-2 and/or TLR4, we found that mouse TLR4-MD-2 mediates LPS-mimetic signal transduction by Taxol and that the species-specific LPS-mimetic action of Taxol was based on the species difference between mouse and human TLR4-MD-2 (21). In this study, we investigated the molecular basis for the species-specific LPS-mimetic action of Taxol and found that Gln[22] of mouse MD-2 is essential for LPS-mimetic Taxol signaling but not for LPS signaling.

## Materials and Methods

### *Reagents*

Taxol from *Taxus brevifolia* was purchased from Sigma (St. Louis, MO). LPS prepared from *Escherichia coli* 0111:B4 was purchased from List

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; HEK, human embryonic kidney; cMD-2, chimeric MD-2.



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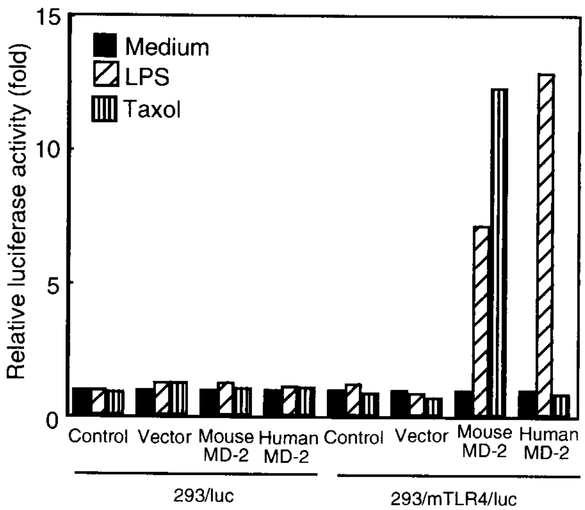


**FIGURE 1.** Human MD-2 confers LPS responsiveness, but not Taxol responsiveness, on mouse TLR4. 293/luc and 293/mTLR4/luc cells were transfected without (control) or with an expression construct for mouse MD-2 or human MD-2, or with a control vector pEFBOS(−) (vector). Three days after transfection, the cells were cultivated in culture medium containing 0.5% DMSO (medium) or in culture medium containing 0.5% DMSO and 100 ng/ml LPS or 10 $\mu$M Taxol for 6 h, and then luciferase activity was measured. Luciferase activity was normalized as to the activity in the cells cultivated in the culture medium containing 0.5% DMSO, and are presented as fold induction. The columns indicate the averages for duplicate wells.

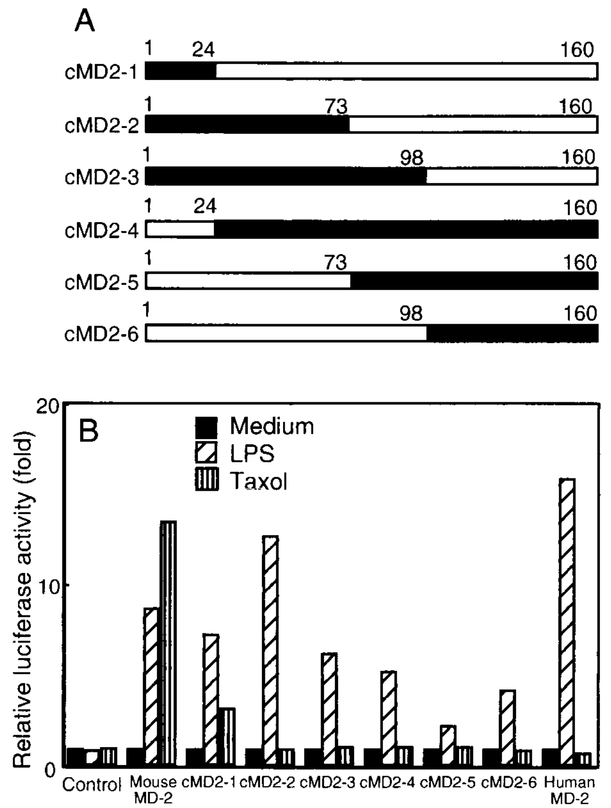


**FIGURE 2.** Reduced ability of human/mouse cMD-2 proteins to confer Taxol responsiveness on mouse TLR4. *A*, Schematic drawing of cMD-2 proteins. Black boxes indicate the MD-2 region of human origin, and the white boxes indicate the MD-2 region of mouse origin in different chimeric proteins. *B*, Expression constructs, each designed to express a cMD-2 protein in *A*, were generated, and then 293/mTLR4/luc cells were transfected without (control) or with an expression construct for mouse MD-2, cMD-2 proteins (cMD2-1, cMD2-2, cMD2-3, cMD2-4, cMD2-5, cMD2-6), or human MD-2. Luciferase activity was measured and presented as in Fig. 1. The columns indicate the averages for duplicate wells.

Biological Laboratories (Campbell, CA). All other chemicals used were of reagent grade or better.

### *Stable transfectants and cell culture*

Human embryonic kidney (HEK) 293 cells stably expressing a recombinant mouse TLR4 bearing a FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and a 6× His epitope at its C terminus were generated by introducing the mouse TLR4 expression construct prepared as described previously (11). Expression of the recombinant mouse TLR4 was confirmed by immunoblotting with Tetra-His Ab (Qiagen, Chatsworth, CA). The stable HEK293 transfectant expressing the recombinant mouse TLR4 and HEK293 cells were transfected with p55Ig$\kappa$luc, an NF-$\kappa$B-dependent luciferase reporter construct (22), the resultant stable transfectants being named 293/mTLR4/luc and 293/luc, respectively. Introduction of p55Ig$\kappa$luc was confirmed by the increase of luciferase activity on PMA stimulation. The transfectants were purified by limited dilution. The stable HEK293 transfectants were maintained in DMEM (Sigma) supplemented with 10% heat-inactivated FBS, penicillin G (100 U/ml), and streptomycin sulfate (100 $\mu$g/ml) at 37°C.

### *Transient transfection*

Cells (3 × $10^4$) were seeded into the wells of a 24-well dish (Costar, Cambridge, MA), containing 1 ml of cell culture medium. After cultivation overnight, the cells were transfected with plasmids (0.3 $\mu$g/well) using FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN). The plasmids used for transfection were purified with a Wizard PureFection Plasmid DNA Purification System (Promega, Madison, WI).

### *Luciferase assay*

Luciferase activity in cell lysates was measured with a luciferase assay system (Promega) as described previously (21).

### *Expression constructs*

Human (10) or mouse (11) MD-2 cDNA was cloned into the *Xho*I and *Not*I sites of an expression vector, pEFBOS (23), by PCR-based introduction of restriction sites. Human/mouse chimeric MD-2 (cMD-2) cDNAs and mutant MD-2 cDNAs were generated by PCR-based overlap extension (24). The sequences of the PCR primers are available upon request. cMD-2 cDNAs and mutant MD-2 cDNAs were cloned into the *Xho*I and *Not*I sites of pEFBOS. The inserts of the expression constructs were verified by sequencing with an ABI PRISM Genetic Analyzer (Applied Biosystems, Foster City, CA). The stuffer region of pEFBOS was eliminated by digestion with *Xba*I, and the resulting construct was named pEFBOS(−).

## Results and Discussion

### *Stable HEK293 transfectant expressing mouse TLR4 acquires Taxol responsiveness through the expression of mouse MD-2, but not through that of human MD-2*

Coexpression of mouse TLR4 and mouse MD-2 conferred LPS and Taxol responsiveness on a mouse pro-B cell line, Ba/F3 cells, but coexpression of human TLR4 and human MD-2 conferred LPS responsiveness alone (21). To further explore the molecular basis for this species-specific action of Taxol, we previously examined whether or not a Ba/F3 stable transfectant expressing mouse TLR4 (named Ba/mTLR4) acquired Taxol or LPS responsiveness on the additional expression of human MD-2. However, the effects of human MD-2 expression on Taxol and LPS responsiveness in Ba/mTLR4 cells were unclear (21), partly because NF-$\kappa$B was slightly activated when Ba/mTLR4 cells were stimulated with Taxol or LPS (11, 21). Because we found that LPS and Taxol were incapable of inducing any NF-$\kappa$B activation in HEK293 cells expressing mouse TLR4 (293/mTLR4/luc) (Fig. 1), we examined whether

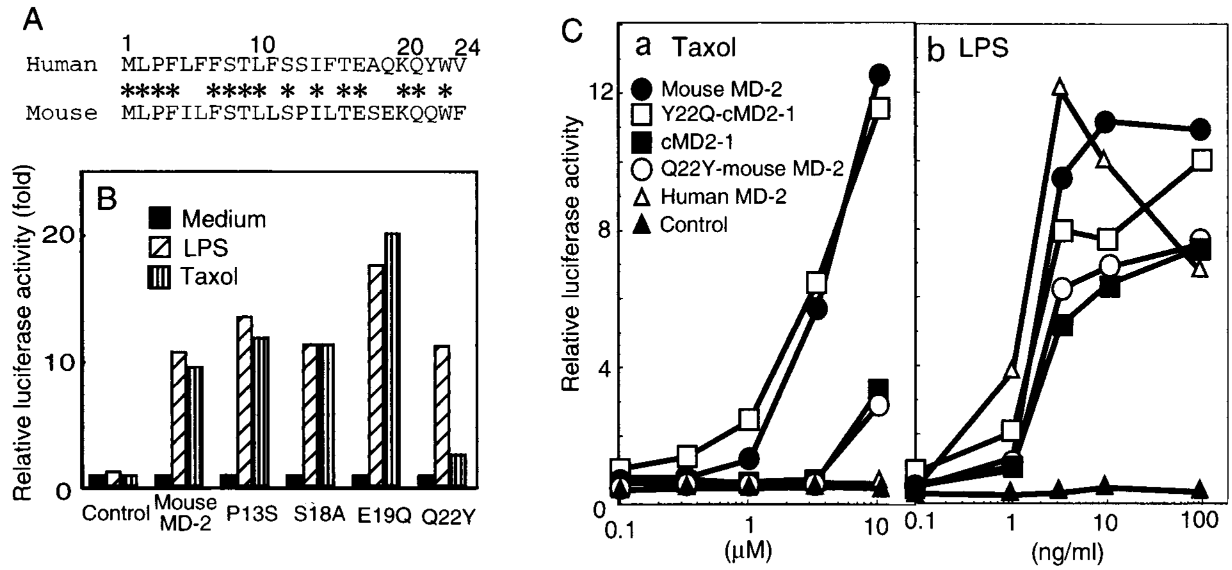


**FIGURE 3.** Gln[22] of mouse MD-2 is involved in the species-specific action of Taxol. *A*, Alignment of the first 24 aa of human MD-2 and mouse MD-2. *B*, Expression constructs, each designed to express a mutant mouse MD-2 protein in which Pro[13], Ser[18], Glu[19], or Gln[22] was changed to the corresponding amino acid residue of human MD-2 were generated, the mutant mouse MD-2 proteins being named P13S, S18A, E19Q, and Q22Y, respectively. 293/mTLR4/luc cells were transfected without (control) or with an expression construct for mouse MD-2 or mutant mouse MD-2 proteins (P13S, S18A, E19Q, and Q22Y). Luciferase activity was measured and presented as in Fig. 1. The columns indicate the averages for duplicate wells. *C*, 293/mTLR4/luc cells were transfected without (control) or with an expression construct for mouse MD-2, human MD-2, cMD2-1, Q22Y-mouse MD-2, or Y22Q-cMD2–1 that encodes a mutant cMD2–1 protein in which Tyr[22] was changed to Gln. Three days after transfection the cells were cultivated in culture medium containing 0.5% DMSO and the indicated amount of Taxol (a) or LPS (b) for 6 h, and then luciferase activity was measured. The results shown are the averages for duplicate wells.

expression of human or mouse MD-2 confers LPS or Taxol responsiveness on 293/mTLR4/luc cells by measuring NF-κB activation. As shown in Fig. 1, 293/mTLR4/luc cells expressing mouse MD-2 responded well to both Taxol and LPS stimulation, but 293/mTLR4/luc cells expressing human MD-2 responded to LPS stimulation but not to Taxol stimulation. These results show that human MD-2 does not have the ability to confer Taxol responsiveness on mouse TLR4, although it has the ability to confer LPS responsiveness on mouse TLR4.

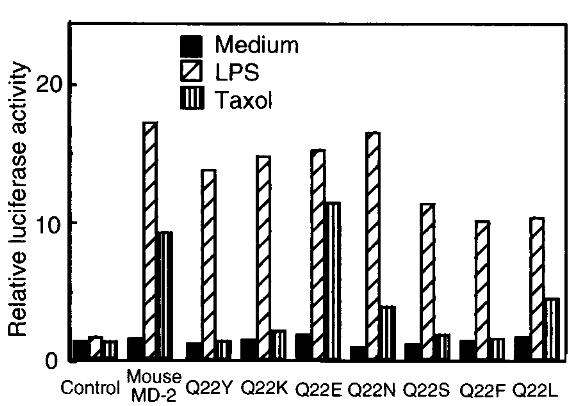


**FIGURE 4.** Gln[22] is involved in Taxol signaling, but not in LPS signaling. Expression constructs, each designed to express a mutant mouse MD-2 protein in which Gln[22] was changed to Lys, Glu, Asn, Ser, Phe, or Leu, were generated, the mutant mouse MD-2 proteins being named Q22K, Q22E, Q22N, Q22S, Q22F, and Q22L, respectively. 293/mTLR4/luc cells were transfected without (control) or with an expression construct for mouse MD-2 or mutant mouse MD-2 proteins (Q22Y, Q22K, Q22E, Q22N, Q22S, Q22F, and Q22L). Three days after transfection, the cells were cultivated in culture medium containing 0.5% DMSO (medium) or in culture medium containing 0.5% DMSO and 33 ng/ml LPS or 3.3 μM Taxol for 6 h, and then luciferase activity was measured. The columns indicate the averages for duplicate wells.

*Mouse/human cMD-2 shows reduced ability to confer Taxol responsiveness*

The amino acid sequence (160 aa) encoded by mouse MD-2 cDNA exhibited 64% identity with that (160 aa) encoded by human MD-2 cDNA (11). To determine whether there is a domain(s) of mouse MD-2 responsible for conferring Taxol responsiveness on mouse TLR4, constructs for expressing mouse/human cMD-2 proteins (Fig. 2*A*) were generated, and their ability to confer Taxol responsiveness was examined. The 293/mTLR4/luc cells expressing cMD2-2, -3, -4, -5, or -6 did not respond to Taxol stimulation, and those expressing cMD2-1 slightly responded to Taxol stimulation (Fig. 2*B*). In contrast, all of the transfectants expressing cMD-2 proteins responded to LPS stimulation (Fig. 2*B*). These results suggested that the ability of mouse MD-2 to confer Taxol responsiveness on mouse TLR4 is not due to a particular domain of mouse MD-2.

*Gln[22] of mouse MD-2 is an essential residue for Taxol signaling*

Although only nine amino acid residues were different between the amino acid sequences of mouse MD-2 and cMD2–1 (Figs. 2*A* and 3*A*), the ability of mouse MD-2 to confer Taxol responsiveness was apparently different from that of cMD2–1, the fold induction of reporter activity on Taxol stimulation being 14- and 3.2-fold in 293/mTLR4/luc cells expressing mouse MD-2 and cMD2–1, respectively (Fig. 2*B*). To determine which of the nine different amino acid residues were important for Taxol signaling, we generated mutant mouse MD-2 expression constructs in which Pro[13], Ser[18], Glu[19], or Gln[22] was replaced with the corresponding amino acid residue of human MD-2, and then their ability to confer Taxol and LPS responsiveness was examined (Fig. 3*B*). Although P13S-, S18A-, and E19Q-mouse MD-2 exhibited similar abilities to confer Taxol responsiveness on 293/mTLR4/luc cells to that of mouse MD-2, Q22Y-mouse MD-2 had an apparently lower ability to confer Taxol responsiveness than mouse MD-2, the fold induction of reporter activity on 10 $\mu$M Taxol stimulation being 9.4- and 2.7-fold in 293/mTLR4/luc cells expressing mouse MD-2 and Q22Y-mouse MD-2, respectively (Fig. 3*B*). With regard to the effect of the Taxol concentration on NF-$\kappa$B activation, 1 $\mu$M Taxol induced NF-$\kappa$B activation in the cells expressing mouse MD-2, whereas 10 $\mu$M Taxol was required to induce NF-$\kappa$B activation in the cells expressing Q22Y-mouse MD-2, just like in the cells expressing cMD2–1 (Fig. 3*C*). Furthermore, the cells expressing Y22Q-cMD2–1, in which $Tyr^{22}$ of cMD2–1 was replaced with mouse type $Gln^{22}$, showed similar sensitivity to Taxol to that of the cells expressing mouse MD-2 (Fig. 3*C*). In contrast, the sensitivity to LPS was similar between the cells expressing mouse MD-2 and those expressing Q22Y-mouse MD-2 (Fig. 3, *B* and *C*). These results, taken together, indicate that $Gln^{22}$ of mouse MD-2 is involved in the species-specific LPS-mimetic action of Taxol.

To determine whether $Gln^{22}$ is essential for Taxol signaling or $Tyr^{22}$ specifically inhibits Taxol signaling, several mutants of mouse MD-2 were generated by replacing $Gln^{22}$ with other amino acids and then their ability to confer Taxol and LPS responsiveness was examined. The 293/mTLR4 cells expressing Q22K-, Q22S- and Q22F-mouse MD-2 did not respond to Taxol (Fig. 4). In contrast, the level of Taxol-induced NF-$\kappa$B activation in the 293/mTLR4 cells expressing Q22E-mouse MD-2 was similar to that in those expressing mouse MD-2 (Fig. 4). The structural similarity between glutamic acid and glutamine may explain why Q22E-mouse MD-2 has the ability to confer Taxol responsiveness. It is noteworthy that the level of LPS-induced NF-$\kappa$B activation was similar among these cells expressing mutant mouse MD-2 proteins. These results, taken together, suggest that $Gln^{22}$ is an essential residue for Taxol signaling.

In this study, we showed that human MD-2 did not have the ability to confer Taxol responsiveness on mouse TLR4 and that the ability of mouse MD-2 was not due to a particular domain of mouse MD-2. Furthermore, we found that $Gln^{22}$ of MD-2 is essential for Taxol signaling but not for LPS signaling.

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