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(54) **ISOLATED COMPLEXES OF COVALENTLY CROSS-LINKED ENDOTOXIN AND MODIFIED MD-2**

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This patent is subject to a terminal disclaimer.

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(51) **Int. Cl.**

A61K 45/00 (2006.01)
A61K 38/00 (2006.01)
A61P 31/00 (2006.01)

(52) **U.S. Cl.** **424/278.1**; 514/2.1

(58) **Field of Classification Search** None
See application file for complete search history.

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(57) **ABSTRACT**

Applicants have produced and isolated chemically cross-linked complexes of endotoxin and modified MD-2, and have developed methods of using these complexes.

25 Claims, 23 Drawing Sheets

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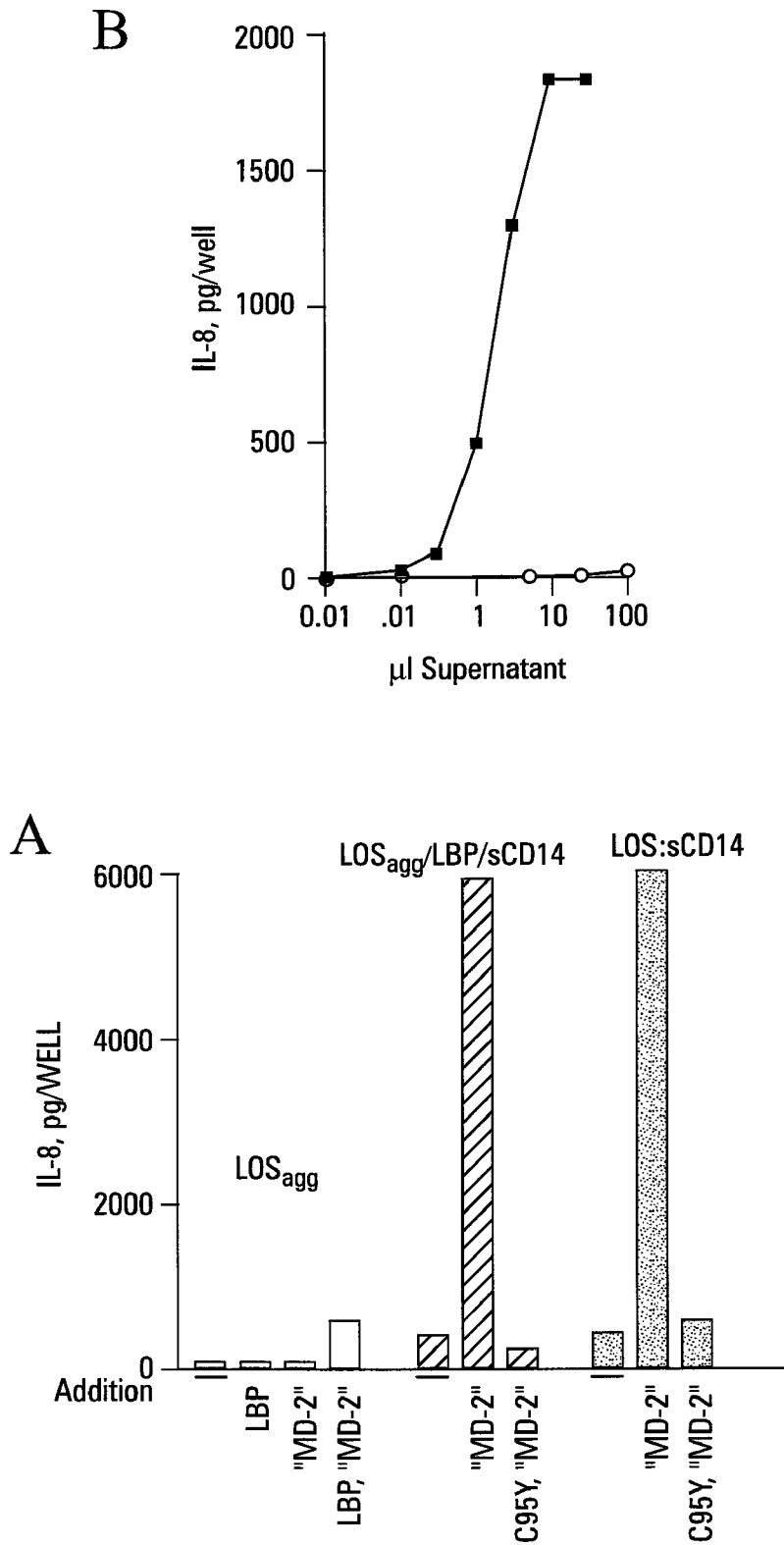


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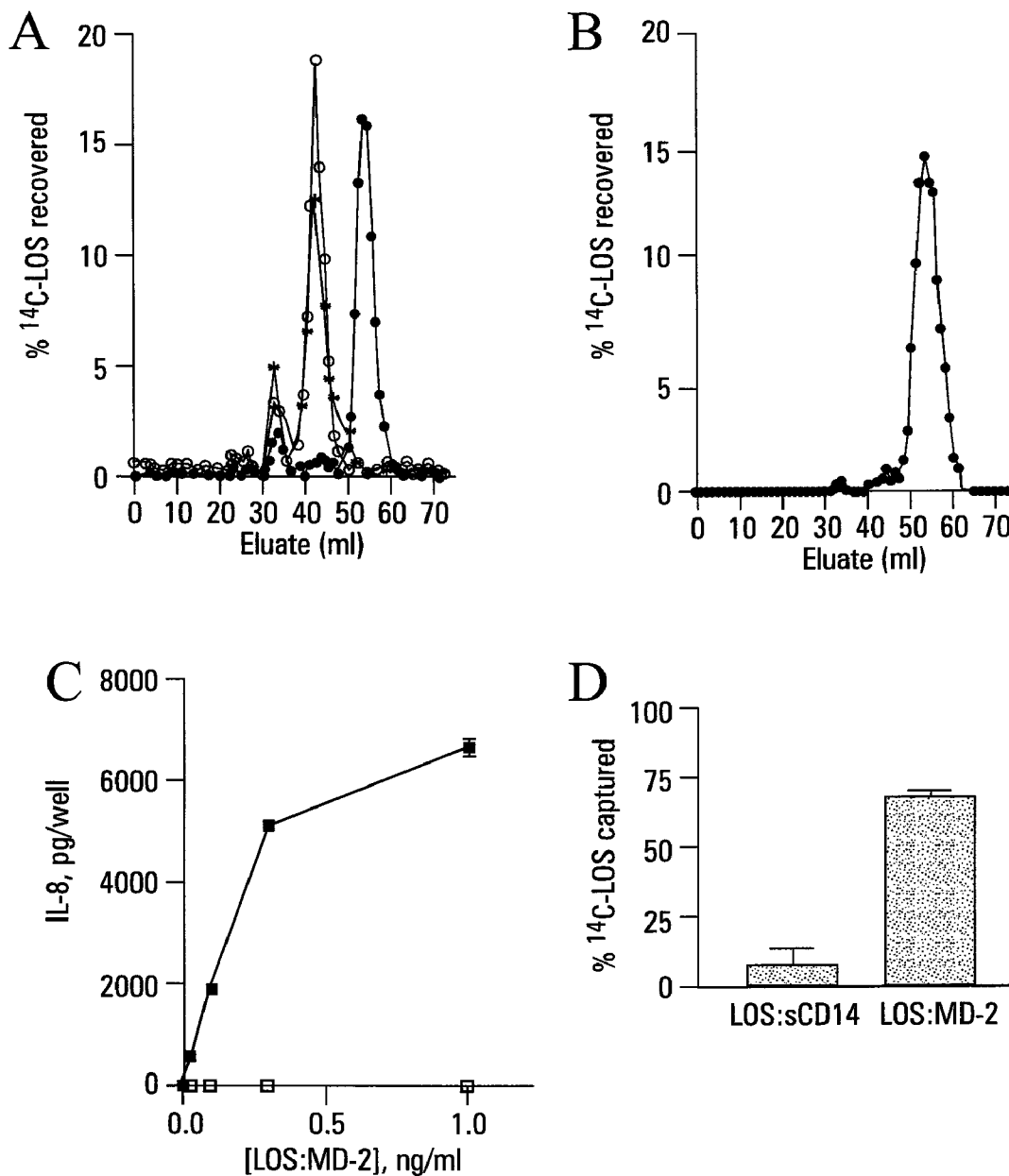


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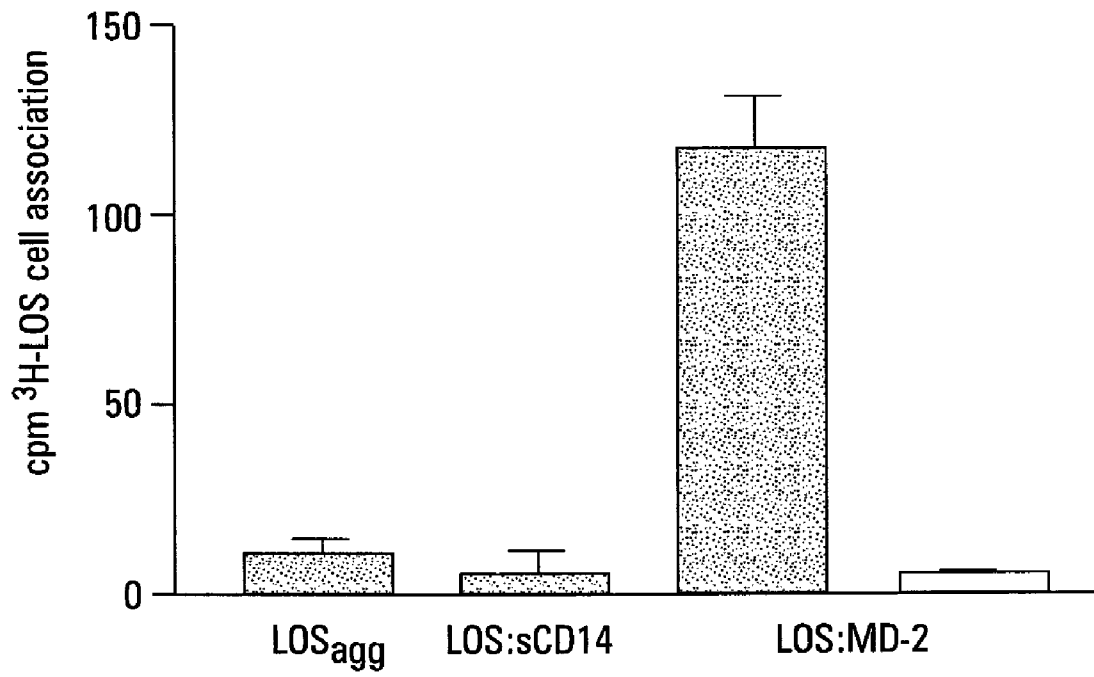


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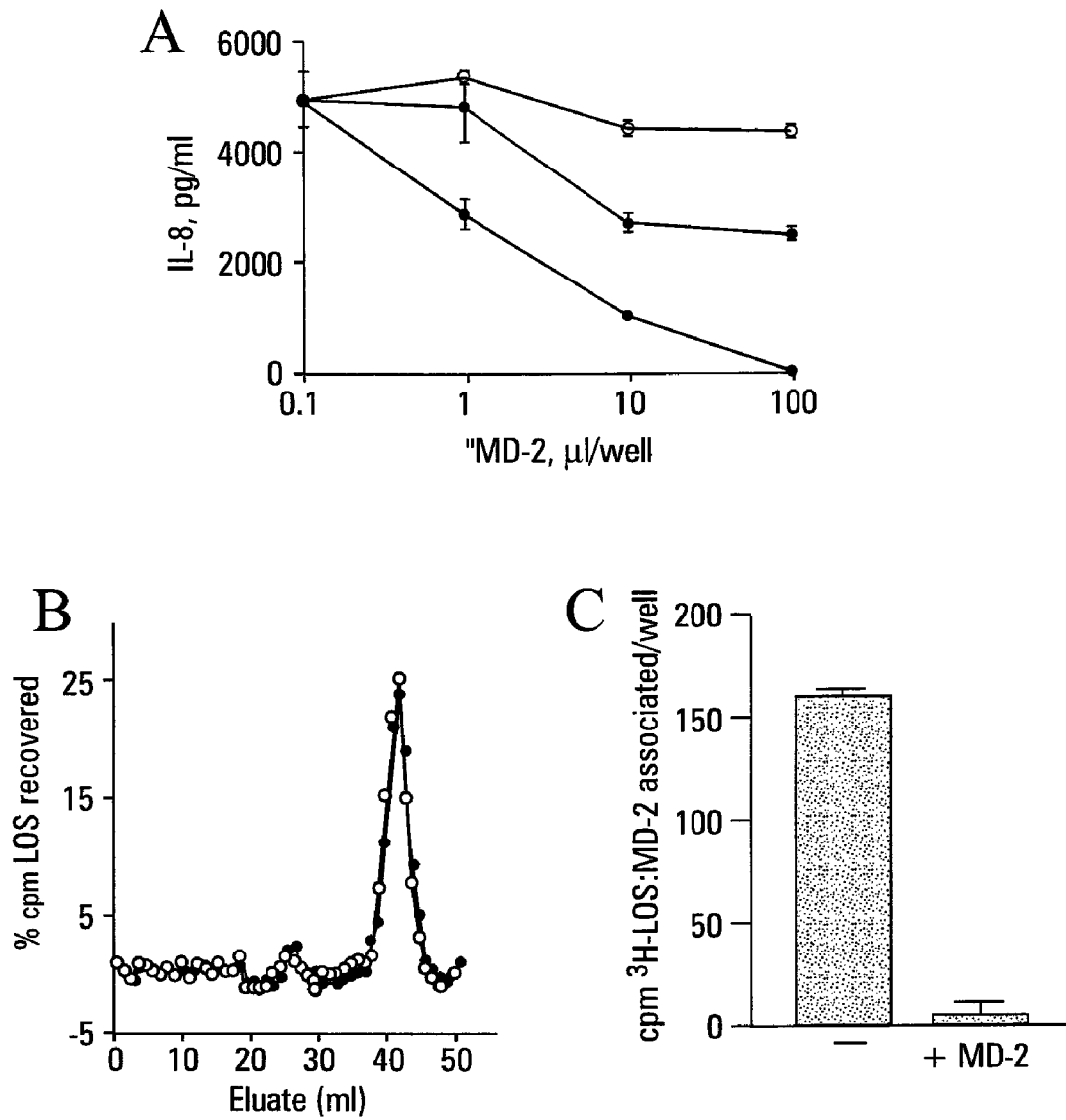


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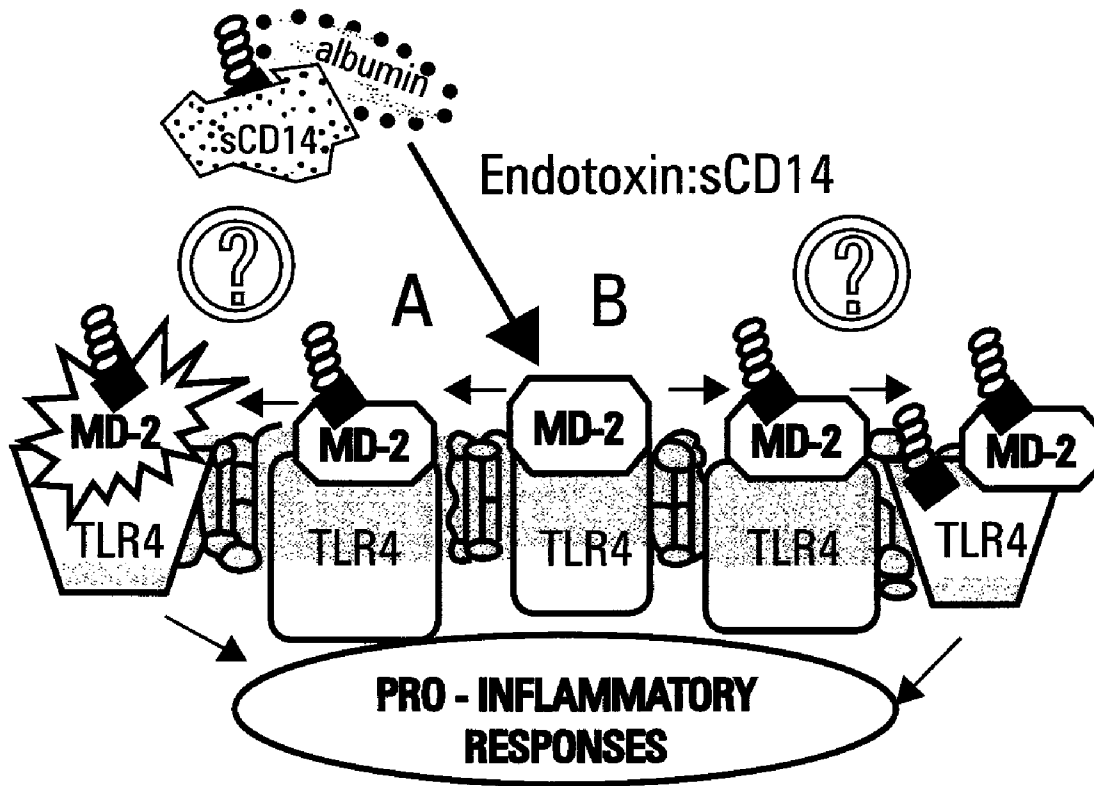


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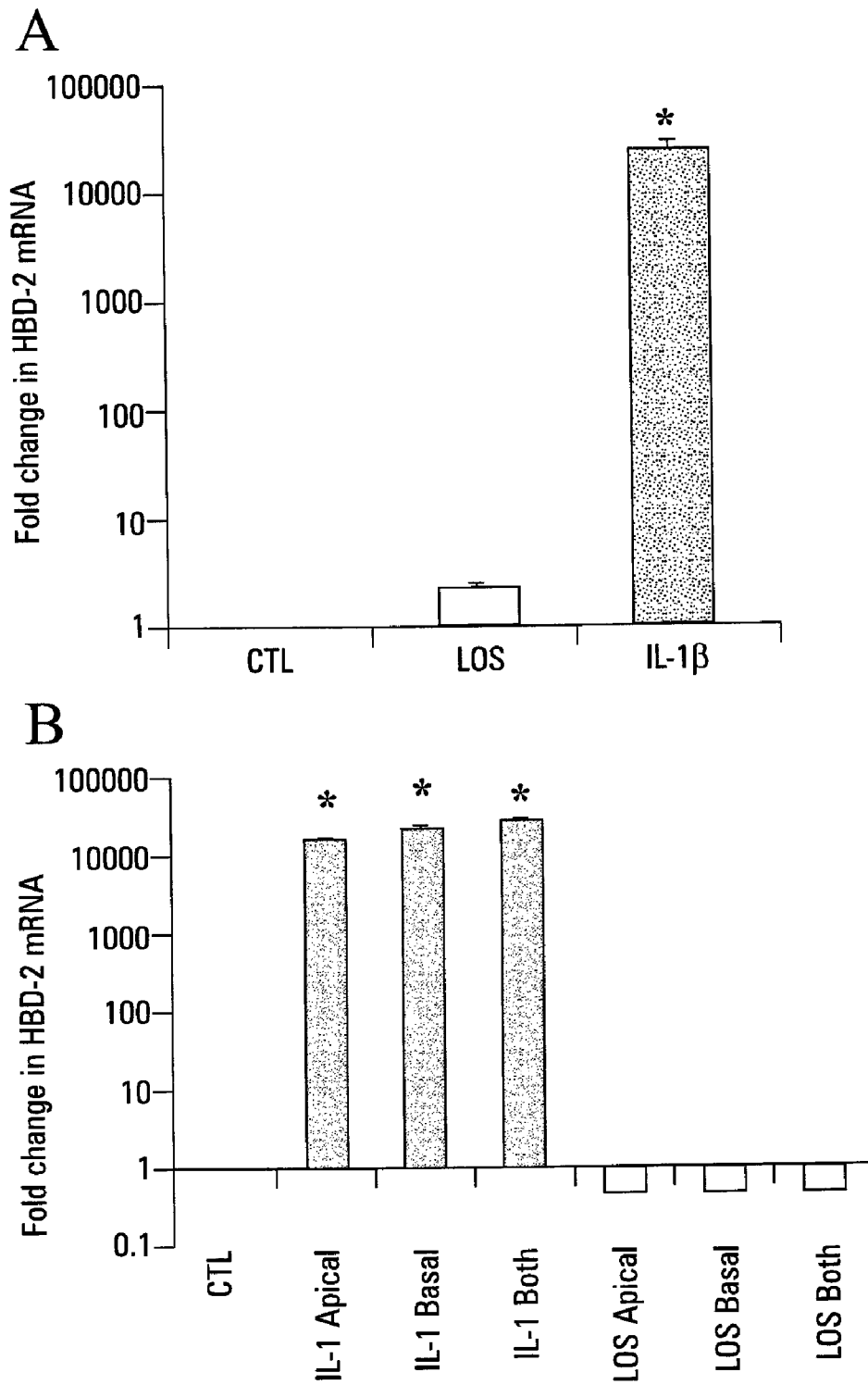


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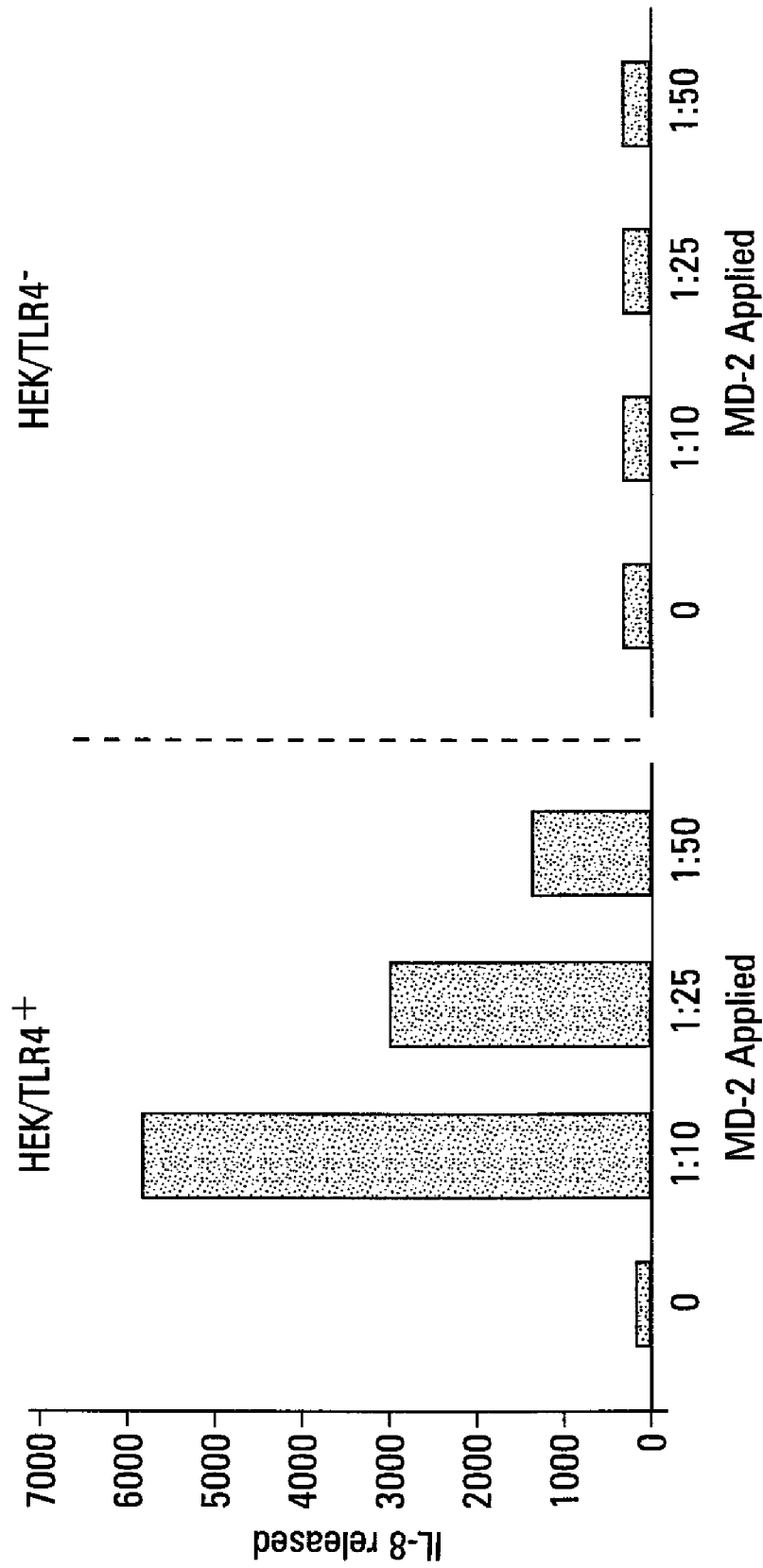


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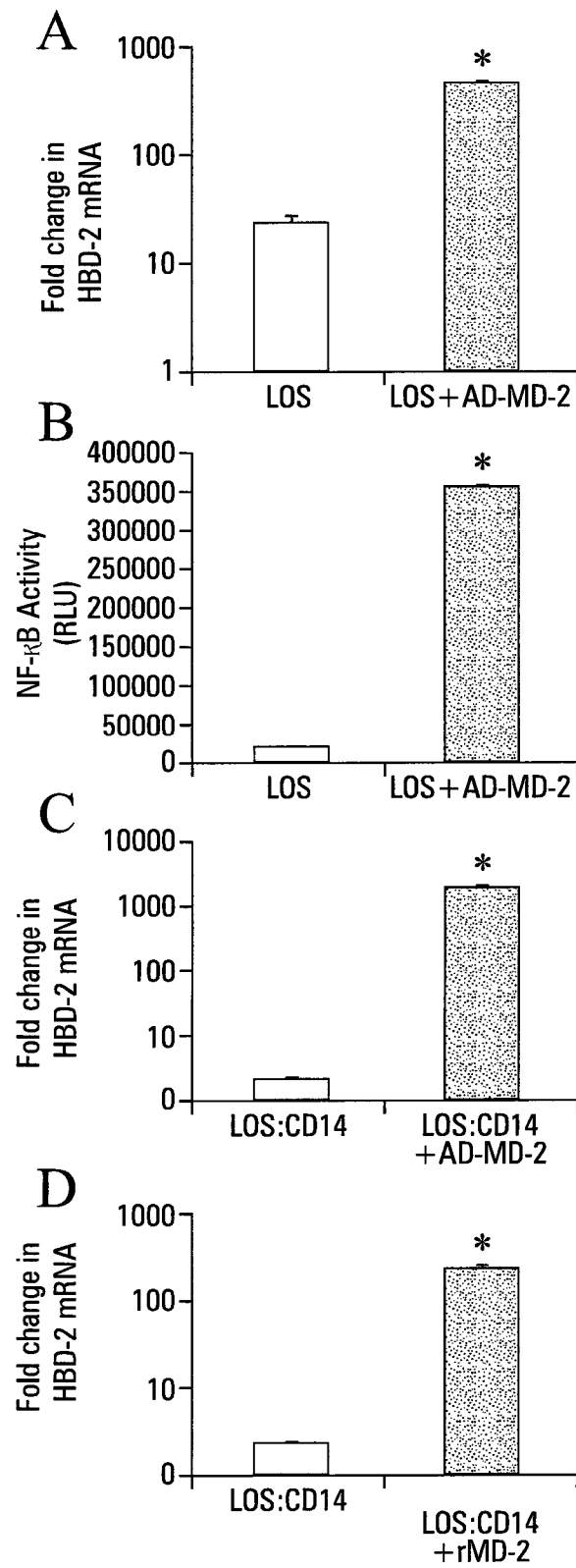


Fig. 8

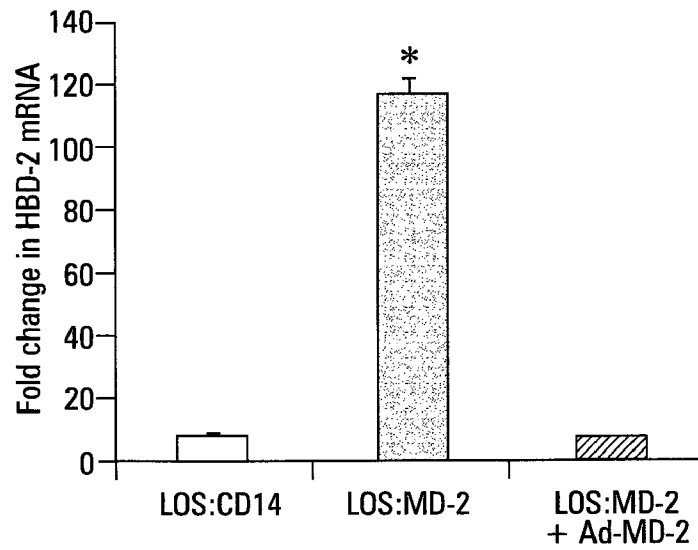


Fig. 9

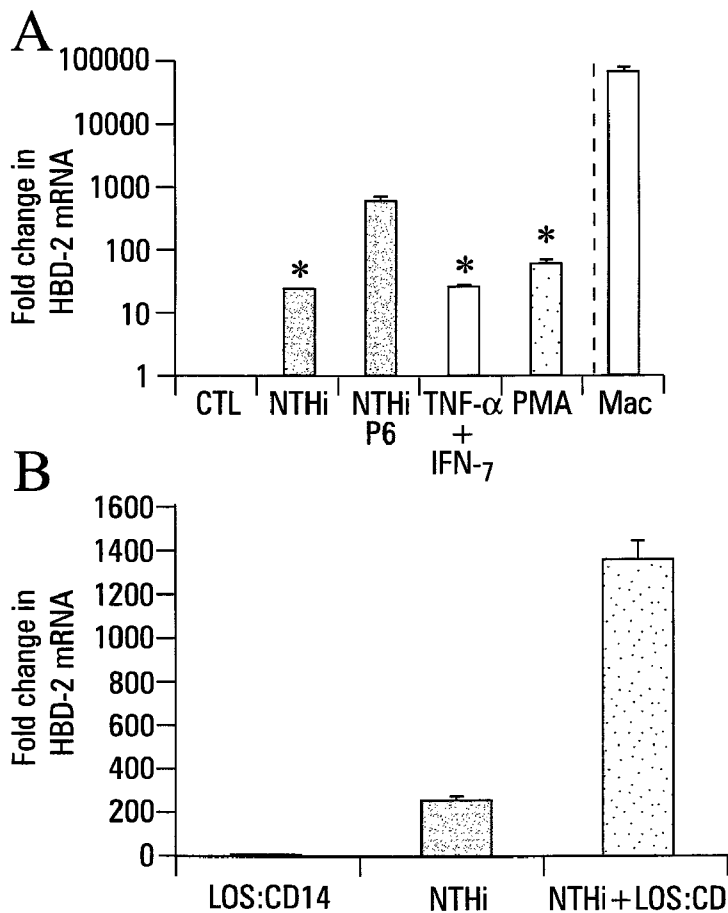


Fig. 10

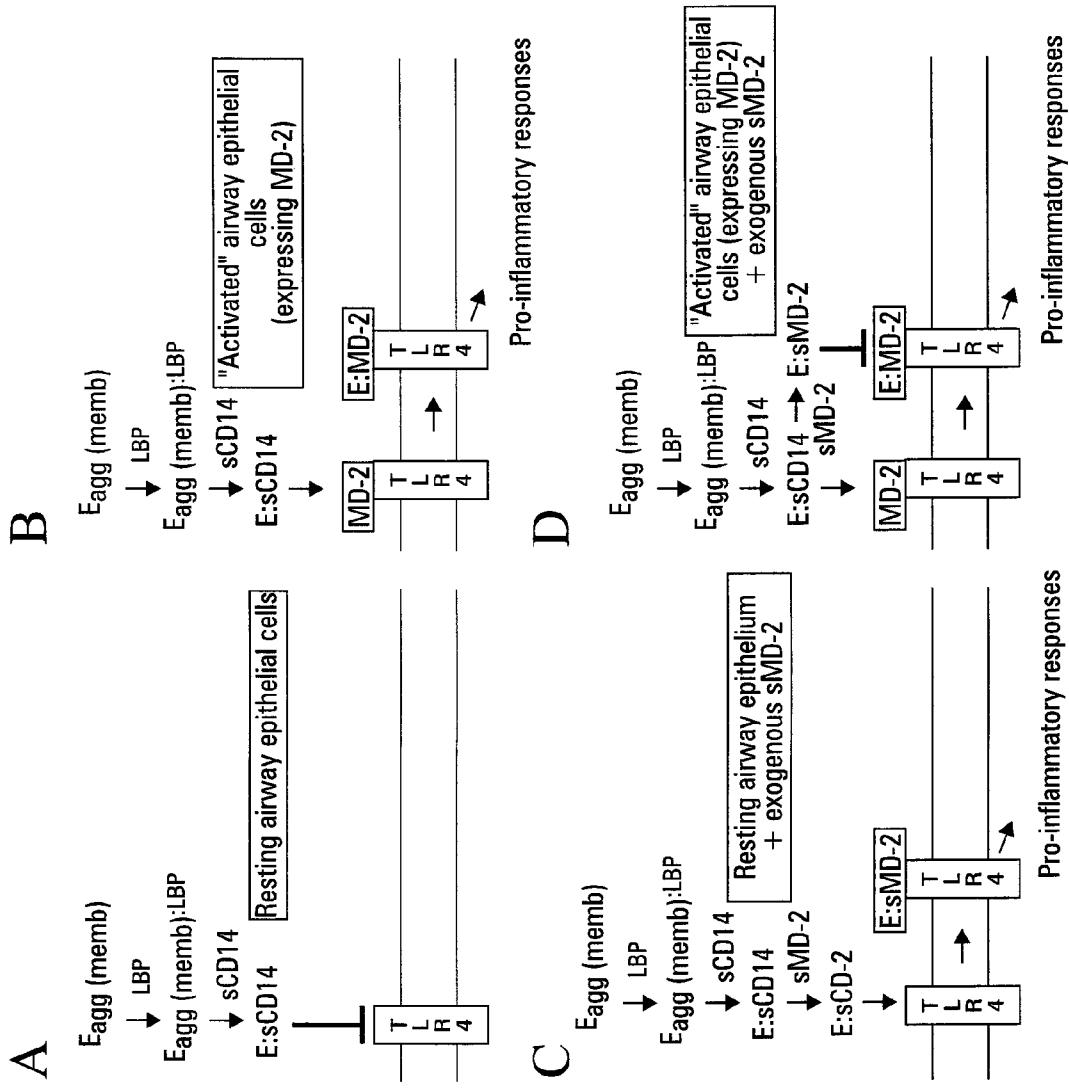


Fig. 11

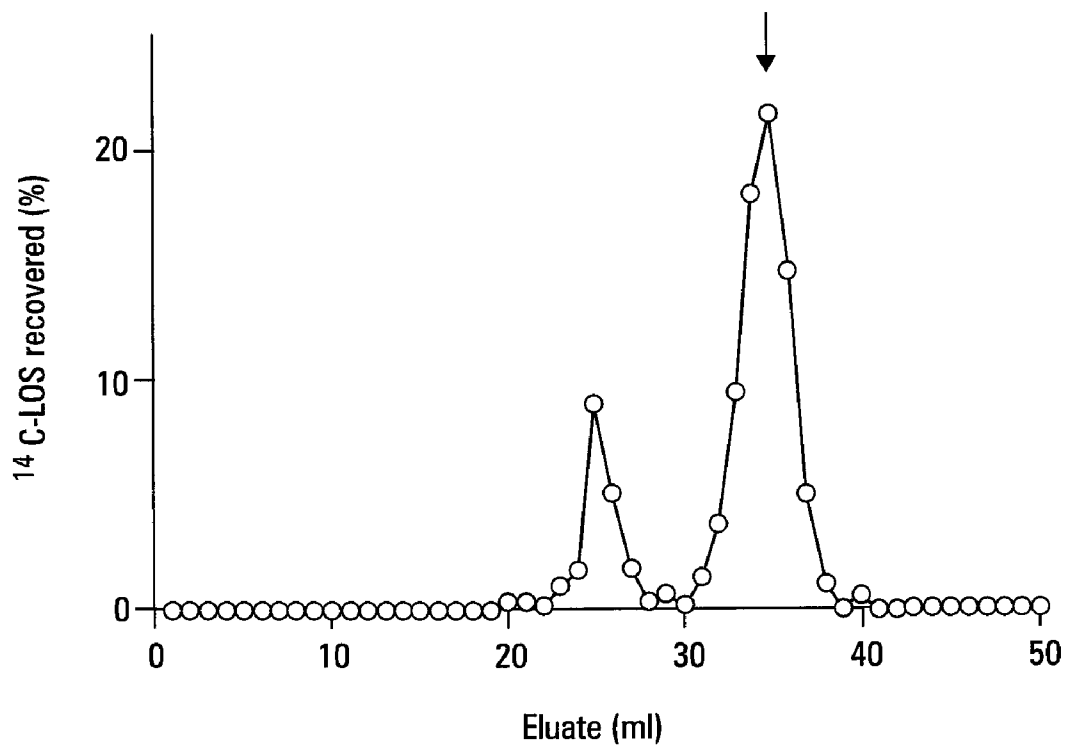


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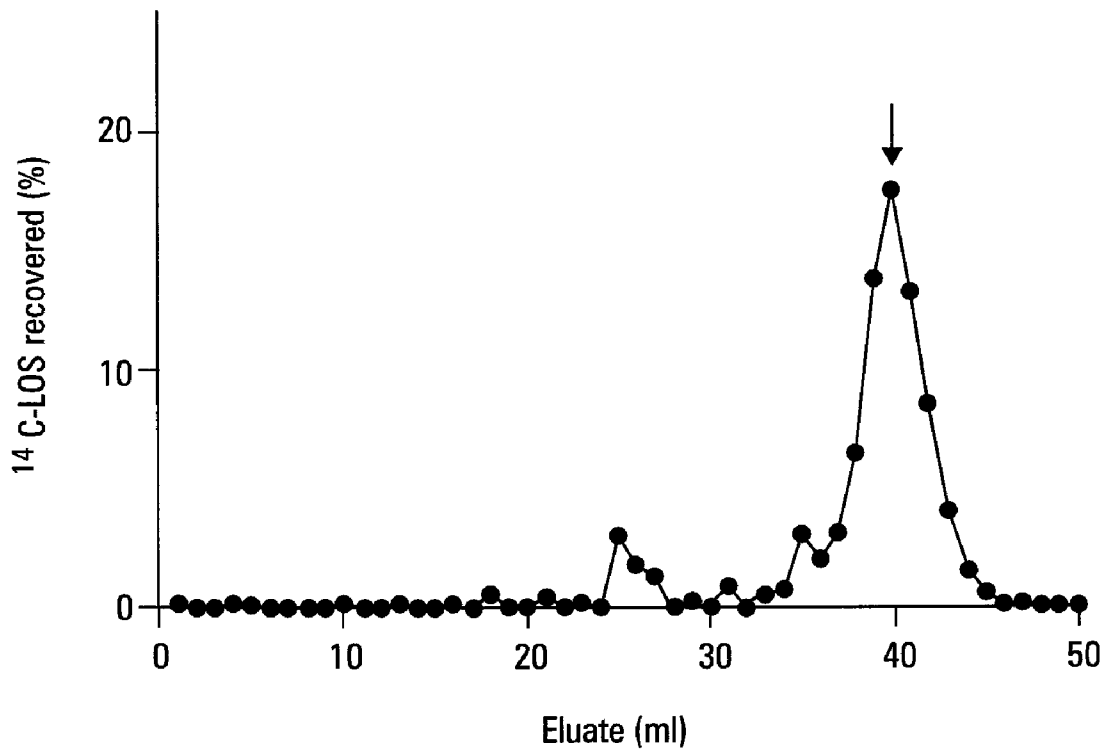


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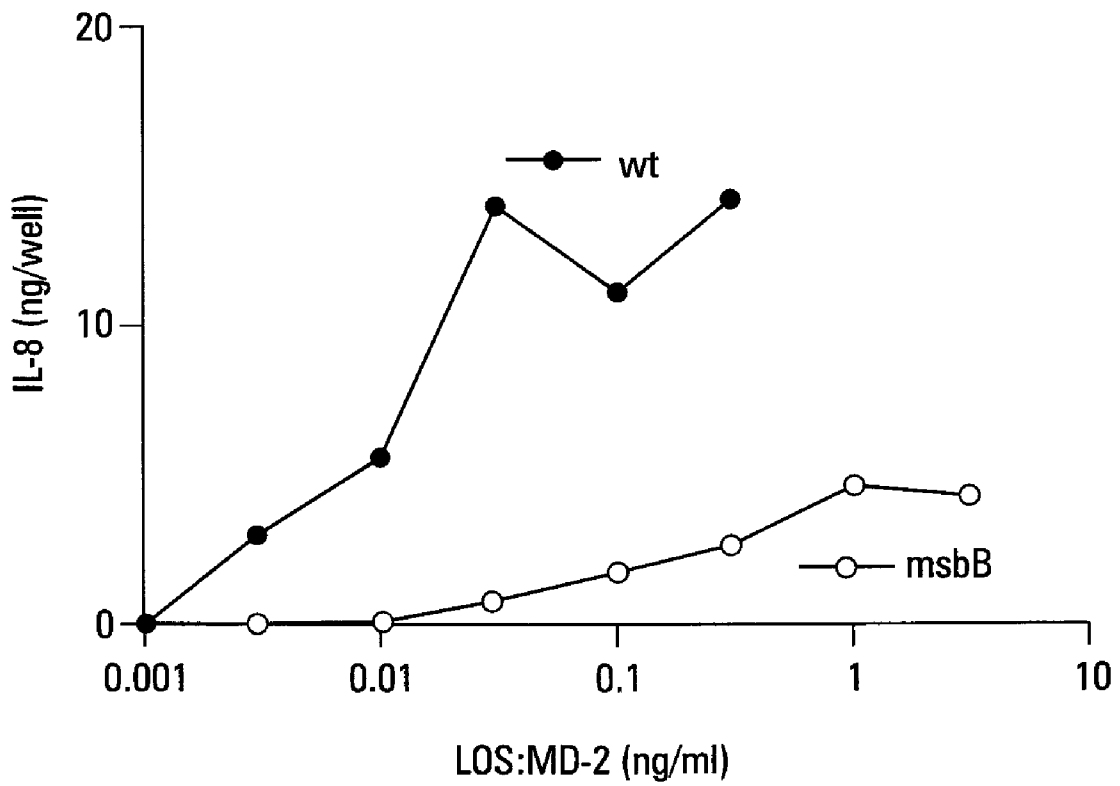


Fig. 14

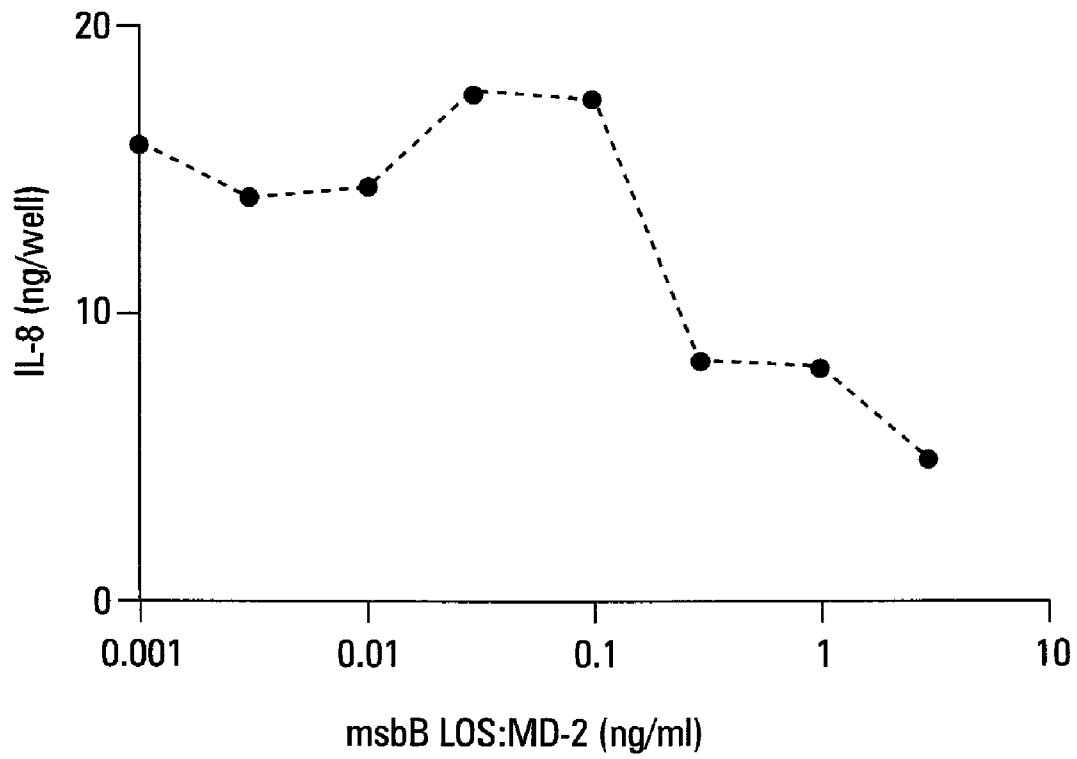
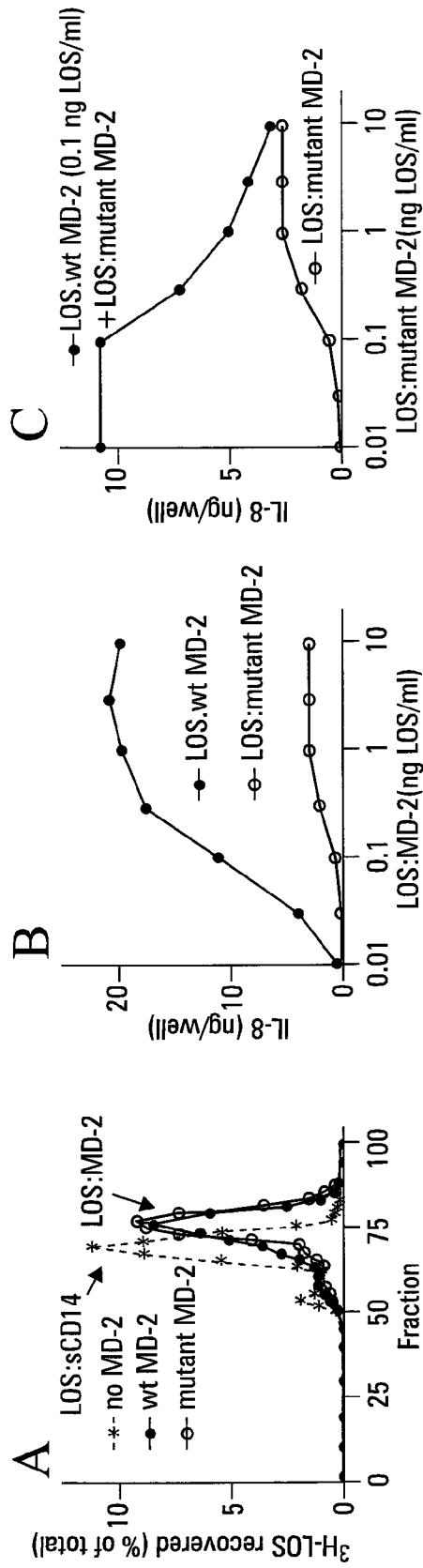
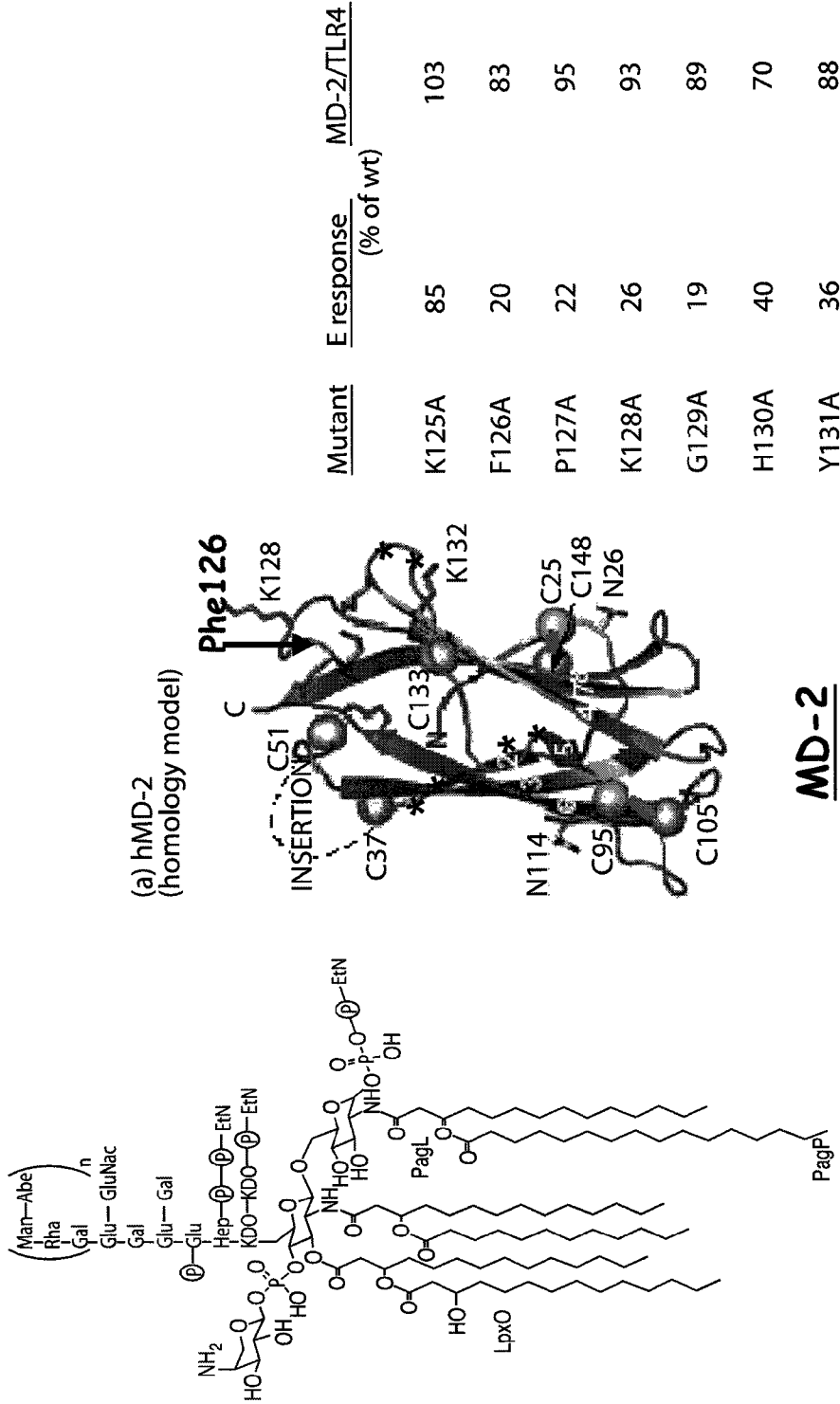


Fig. 15

Fig. 16





Mutant	E response (% of wt)	MD-2/TLR4
K125A	85	103
F126A	20	83
P127A	22	95
K128A	26	93
G129A	19	89
H130A	40	70
Y131A	36	88

Fig. 18

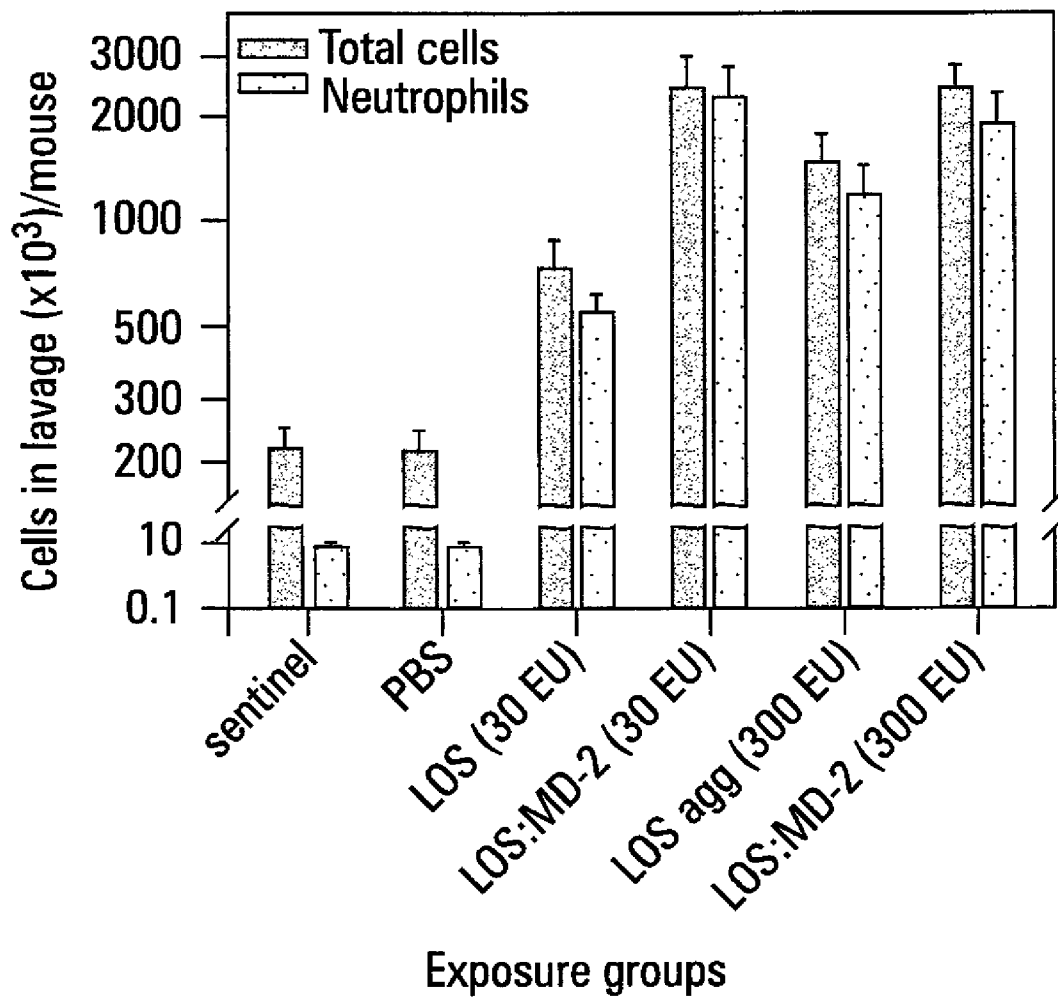


Fig. 19

Fig. 21A

Purification of X-linked [¹⁴C]LOS:MD-2

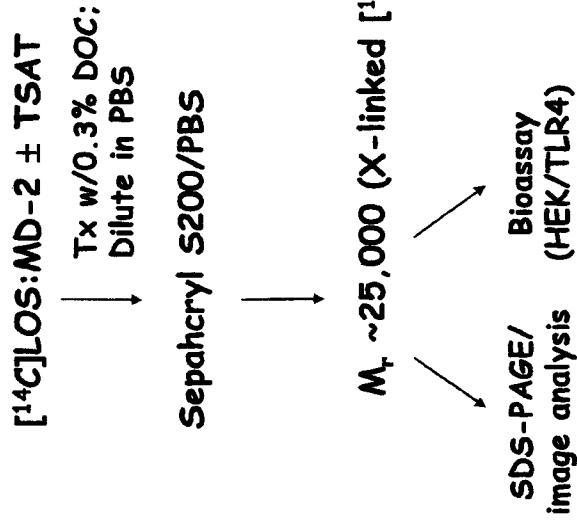


Fig. 21B

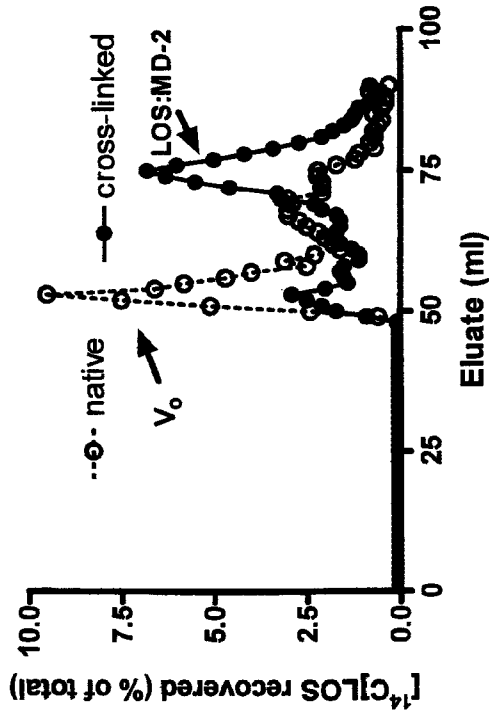
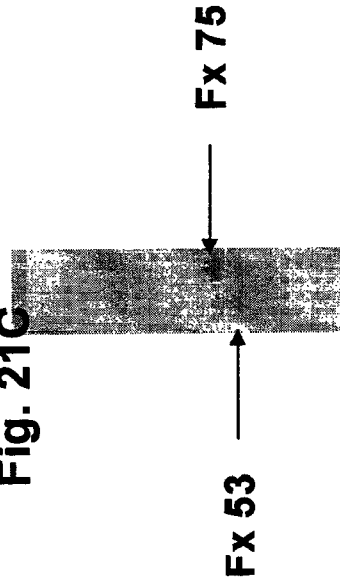


Fig. 21C



X-linked E:MD-2 acts like TLR4 antagonist

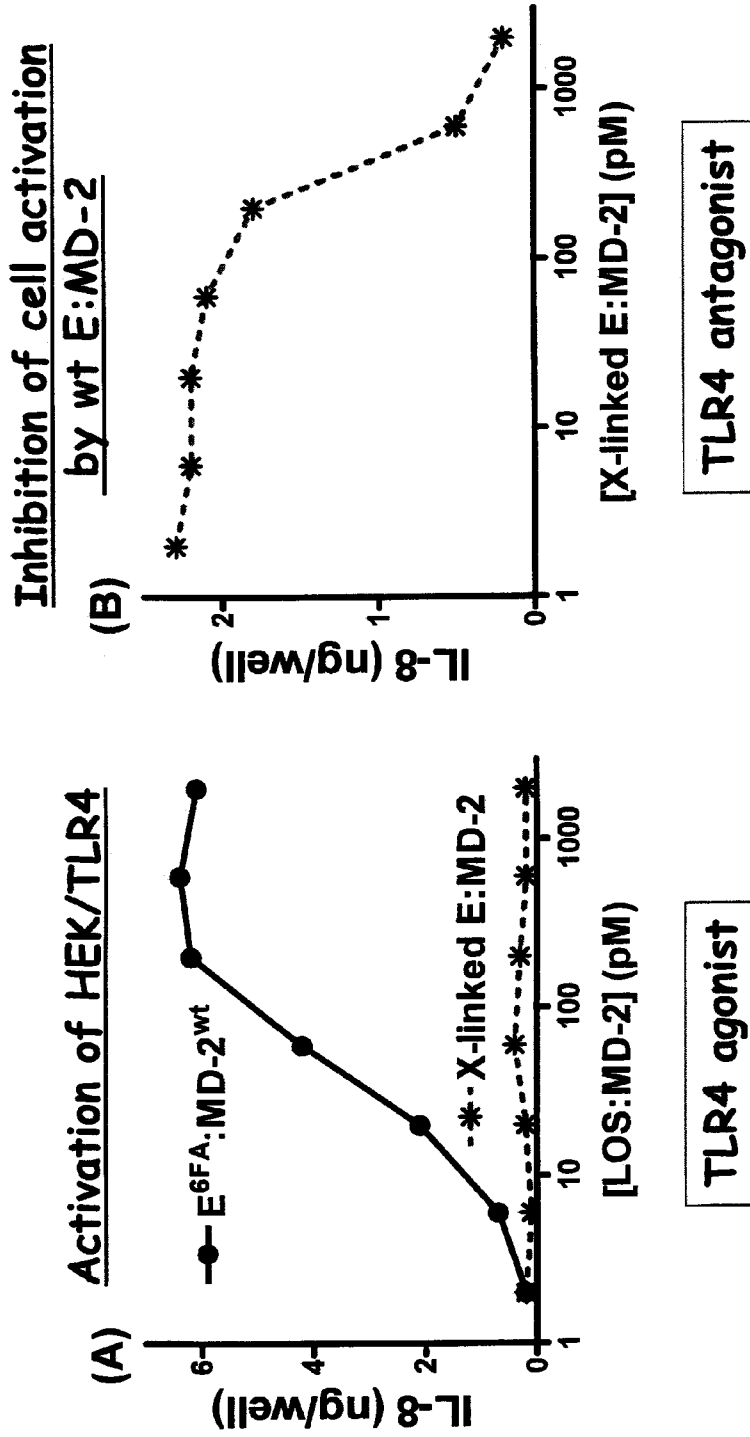


Fig. 22A

Fig. 22B

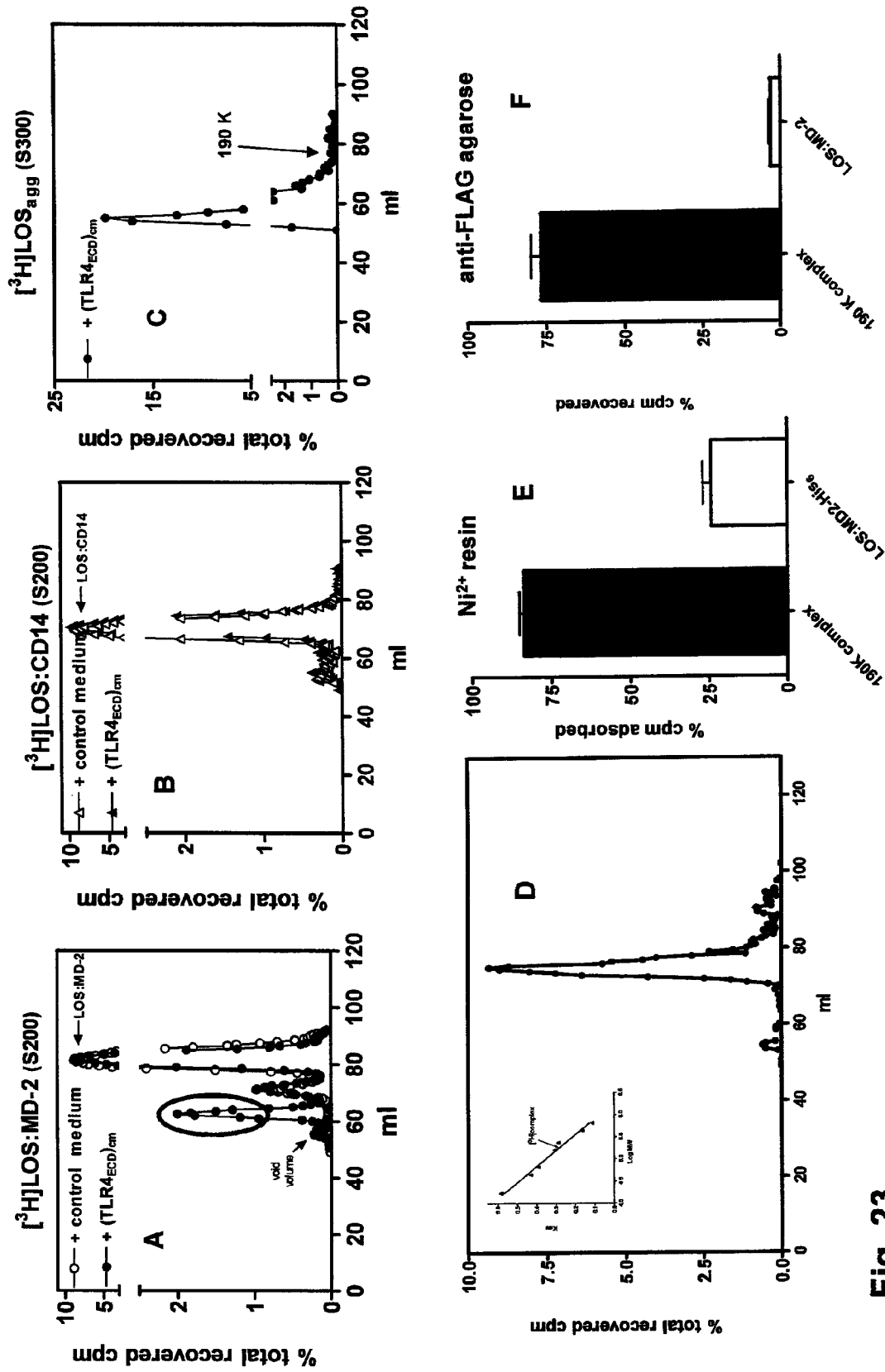


Fig. 23

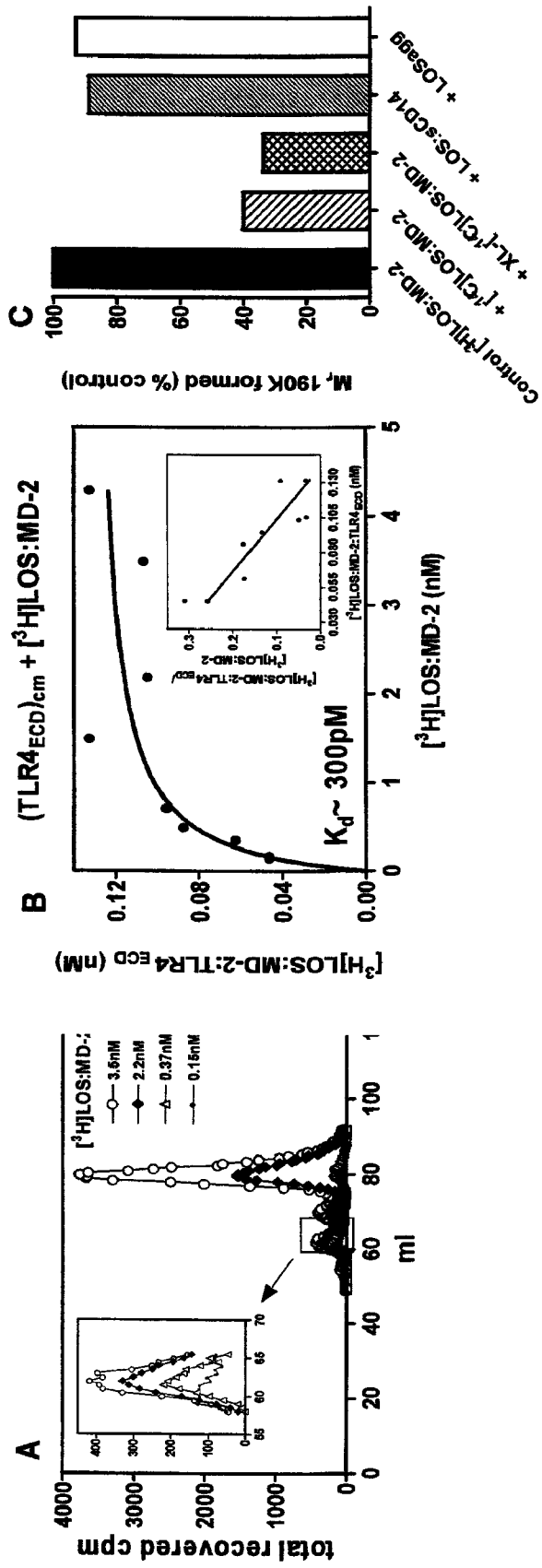


Fig. 24

**ISOLATED COMPLEXES OF COVALENTLY
CROSS-LINKED ENDOTOXIN AND
MODIFIED MD-2**

RELATED APPLICATION

This application claims priority under 35 U.S.C. 119(e) to provisional application U.S. Ser. No. 60/826,310, filed Sep. 20, 2006, which application is incorporated hereby by reference.

STATEMENT REGARDING FEDERALLY
SPONSORED RESEARCH

This invention was made with government support under grants AI059372 and AI044642 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The ability of an organism to withstand bacterial invasion depends upon sensitive and specific molecular systems. The molecules involved in these systems are designed to recognize specific bacterial products and trigger rapid responses to small numbers of invading bacteria.

Innate recognition systems include highly conserved "pattern recognition" host molecules that detect and respond to highly conserved and structurally unique microbial molecules. The best-studied example of such an innate system is the machinery engaged in recognition of endotoxins, which are unique surface glycolipids of gram-negative bacteria.

Potent pro-inflammatory cellular responses to endotoxin are mediated through activation of a member of the Toll-like receptor family of proteins, Toll-like receptor 4 (TLR4; Beutler et al., 2003; Means et al., 2000; and Ulevitch et al., 1999). An important feature of TLR4-dependent cell activation by endotoxin is its extraordinary sensitivity, permitting timely host responses to small numbers of invading gram-negative bacteria, essential for efficient host defense (Beutler et al., 2003; Means et al., 2000; and Ulevitch et al., 1999).

TLR4 contains a leucine-rich extracellular domain involved in ligand recognition, a transmembrane region, and an intracellular domain responsible for triggering signalling pathways that result in activation of genes of the innate immune defense system (Beutler et al., 2001; and Medzhitov et al., 1998). Maximal potency of TLR4-dependent cell activation by endotoxin requires four different extracellular and cell surface host proteins: lipopolysaccharide (LPS) binding protein (LBP), CD14, MD-2 and TLR4 (Beutler et al., 2003; Miyake et al., 2003; and Ulevitch, 2000).

TLR4 requires MD-2 for CD14-dependent cellular response to low concentrations of endotoxin, but neither the precise nature of the ligand that binds to TLR4 nor the role of MD-2 has been fully defined. MD-2, either endogenously expressed or exogenously added, associates with TLR4 on the cell surface (Viriyakosol et al., 2001; Schromm et al., 2001; Visintin et al., 2001; Re et al., 2002; Akashi et al., 2003; Visintin et al., 2003; and Re et al., 2003) and its endogenous expression is needed for optimal surface expression of TLR4. TLR4 responsiveness to endotoxin is disrupted by point mutations of MD-2 (Schromm et al., 2001; Kawasaki et al., 2003; Ohnishi et al., 2001; and Mullen et al., 2003) (e.g., C95Y, Lys128 and Lys132) despite surface expression of TLR4/MD-2 complexes.

SUMMARY OF THE INVENTION

The activation of inflammatory reactions mobilizes protective host defenses. However, excessive responses, or dysfunc-

tional regulation of these responses, can lead to severe, even life-threatening pathology. Thus, optimal physiological functioning of inflammatory responses requires careful positive and negative regulation. Accordingly, agents and methods that affect such regulation, for example by affecting TLR4-mediated cell activation, would have great clinical benefit and are needed.

The present invention provides a complex including endotoxin covalently bound to MD-2 by means of a cross-linker molecule. Surprisingly, these complexes, when devoid of any other host or microbial molecules, are potent and water soluble and do not require additional lipid carrier molecules (e.g., serum albumin) for water solubility. In certain embodiments, the cross-linker molecule can be tris-succinimidyl aminotriacetate (TSAT); bis(sulfosuccinimidyl) suberate (BS³); disuccinimidyl suberate (DSS); bis(2-[sulfosuccinimidyl]oxy[ethyl]sulfone) (BOSCOES); bis(2-[succinimidyl]oxy[ethyl]sulfone) (Sulfo-BOSCOES); ethylene glycol bis-(succinimidylsuccinate) (EGS); ethylene glycol bis-(sulfosuccinimidylsuccinate) (SULFO-EBS); and Dimethyl 3,3'-dithiobis-propionimidate (DTBP). In certain embodiments, the cross-linker molecule is trivalent (such as TSAT). In certain embodiments, the cross-linker molecule is bivalent (such as BS³, Sulfo-Boscoes, EGS, Sulfo-EBS, or DTBP). As used herein the term "bivalent" is used to mean that the molecule has a valence of two, or binds together two separate molecules. As used herein the term "trivalent" is used to mean that the molecule has a valence of three, or binds together three separate molecules, or three parts of a molecule (or two parts of one molecule to one part of another molecule). In certain embodiments, the complex consists essentially of one cross-linker molecule bound to one molecule of endotoxin and to one molecule of MD-2. In certain embodiments, the complex is soluble in water. In certain embodiments, the complex has a molecular weight of about 25,000.

In certain embodiments, the endotoxin is a wild-type endotoxin. In certain embodiments the endotoxin is a gram-negative bacterial endotoxin, such as *Neisseria*, *Escherichia*, *Pseudomonas*, *Haemophilus*, *Salmonella*, or *Francisella* bacterial endotoxin (e.g., endotoxin from *Neisseria meningitidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Salmonella typhimurium*, or *Francisella tularensis*). In certain embodiments, the endotoxin is a hepta-acylated endotoxin, a hexa-acylated endotoxin, a penta-acylated endotoxin or a tetra-acylated endotoxin. In certain embodiments, the endotoxin is under-acylated (e.g., a tetra-acylated endotoxin or a penta-acylated endotoxin).

The present invention provides a complex including endotoxin covalently bound to either wild-type or variant MD-2 by means of a cross-linker molecule. The variant MD-2 varies from wild-type MD-2 at one or more of amino acid residues 25, 37, 51, 55, 58, 68, 69, 90, 91, 95, 99, 102, 103, 105, 114, 121, 122, 125, 126, 127, 128, 129, 130, 131, 132, 133, and/or 148. The reference numbering scheme is that used in Kawasaki et al., *J. Immunol* 170:413-20 (2003). The variation may be an amino acid substitution, such as a conserved substitution. For example, the wild-type amino acid may be substituted with an alanine. In certain embodiments, the variant MD-2 varies as compared to a wild-type MD-2, wherein the modification is an amino acid substitution of one or two of amino acid residues 68, 69, 126, 127, 128, 129, 130, and/or 131.

The present invention also provides a method for making the complexes of the invention.

The present invention also provides compositions containing the complex including endotoxin covalently bound to

MD-2 by means of a cross-linker molecule, which may contain a pharmaceutically acceptable carrier.

The present inventors discovered that endotoxin:MD-2 complexes containing wild-type endotoxin produce TLR4-dependent cell stimulation, while complexes containing mutant forms of endotoxin (for example, under-acylated forms of endotoxin) inhibit TLR4-dependent cell stimulation. The present inventors have also discovered that MD-2 can have inhibitory as well as stimulatory effects on TLR4-dependent cell activation by endotoxin. In certain embodiments, the complex binds to TLR4. In certain embodiments, the complex inhibits TLR4-dependent activation of cells. The complex may inhibit TLR4-dependent activation of cells at a concentration of less than about 500 nM of the complex, or any value between 500 nM and 300 pM. In certain embodiments, the complex may inhibit TLR4-dependent activation of cells at a concentration of less than about 200 nM of the complex, less than 100 nM of the complex, less than 10 nM of the complex, less than 1 nM of the complex, or even less than 300 pM of the complex. In certain embodiments the complex inhibits TLR4-dependent activation of cells when the complex is present at a concentration between about 300 pM-100 nM.

The present invention also provides methods of using the complexes of the invention, e.g., methods to inhibit TLR4-dependent activation of cells by endotoxin in vitro or in vivo. Methods using complexes with mutant endotoxin are useful, e.g., to decrease undesirable endotoxin-mediated inflammation. In certain embodiments, the cells are those that express TLR4 but not MD-2 such as mucosal epithelial cells derived from the gastrointestinal or respiratory tracts. In one embodiment, the cell is an airway epithelial cell derived from the trachea or bronchi.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the expression and bioactivity of recombinant MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17). SDS/PAGE immunoblots were performed of control culture medium or medium from HiFive cells infected with recombinant baculovirus encoding wild-type (wt) or C95Y MD-2. MD-2 was detected using anti-(His)₄ (4×His tag disclosed as SEQ ID NO: 18) antibody. In FIG. 1A, HEK/TLR4 cells were incubated in HEPES-buffered HBSS+/0.1% albumin with ¹⁴C-LOS_{agg} (3 ng/ml) with or without LBP (30 ng/ml) and/or 60 μl of culture medium containing wt MD-2 (“MD-2”) (open bars); LOS_{agg} plus LBP and sCD14 (250 ng/ml) with or without wt (closed bars) or C95Y “MD-2” (striped bars), or ¹⁴C-LOS:sCD14 (2 ng LOS/ml) with or without wt or C95Y “MD-2” (filled bars). After overnight incubation, extracellular IL-8 was assayed by ELISA. In FIG. 1B, HEK/TLR4 cells were incubated with increasing amounts of wt (■) or C95Y (○) “MD-2” plus ¹⁴C-LOS:sCD14 (2 ng/ml) and the cell activation was measured. Results shown are from one experiment (duplicate samples) representative of four independent experiments.

FIG. 2 shows that a bioactive complex (M_r~25,000) containing MD-2 and ¹⁴C-LOS is formed by incubation of ¹⁴C-LOS:sCD14 with wt, but not C95Y, MD-2. In FIG. 2A, dialyzed control insect cell medium (0) or medium containing wt (◆) or C95Y (*) MD-2 was incubated for 30 minutes, 37° C. with ¹⁴C-LOS:sCD14 (1:1 vol/vol) in HBSS+/10 mM HEPES and chromatographed on Sephacryl S100. Column fractions were analyzed for ¹⁴C-LOS. Identical results were obtained in analytical (5 ng ¹⁴C-LOS per ml+200 μl culture medium) or more preparative runs (reagents concentrated 20×). In FIG. 2B, peak fractions (M_r~25,000) from treatment

of ¹⁴C-LOS:sCD14 with wt “MD-2” (FIG. 2A) were re-chromatographed on S100 in HBSS+/10 mM HEPES without HSA; recovery of ¹⁴C-LOS was >80%. In FIG. 2C, HEK (□) or HEK/TLR4 (■) were incubated overnight with the indicated amounts of LOS added as purified M_r~25,000 (LOS:MD-2) complex. Cell activation was measured by IL-8 accumulation. Results shown correspond to one experiment, in duplicate, representative of three similar experiments. FIG. 2D depicts adsorption and elution of bioactive M_r~25,000 complex to HisBind resin. Peak fractions of the purified complex (FIG. 2B; 10 ng ¹⁴C-LOS) were dialyzed against PBS and incubated with HisBind resin (0.125 ml) for one hour at 25° C. and processed as described in the Methods for Example 1. Non-adsorbed and adsorbed material eluted with 200 mM imidazole was precipitated with trichloroacetic acid to concentrate sample for SDS-PAGE/immunoblot analysis. Alternatively, adsorbed material was eluted with 2% SDS and counted by liquid scintillation spectroscopy. In FIG. 2D, adsorption of ¹⁴C-LOS:sCD14 was tested as a negative control. Overall recovery of ¹⁴C-LOS was greater than 90%. Results shown are the mean or representative of two closely similar experiments.

FIG. 3 depicts delivery of ³H-LOS:MD-2 but not ³H-LOS_{agg} or ³H-LOS:sCD14 to HEK/TLR4. HEK (□) or HEK/TLR4 (■) cells were incubated with ³H-LOS (0.75 ng/ml) in the form of LOS_{agg}, LOS:sCD14, or LOS:MD-2. After overnight incubation at 37° C., cells were washed and lysed as described in the Methods for Example 1. The amount of ³H-LOS associated with the cells was measured by liquid scintillation spectroscopy. Results are from one experiment in duplicate, representative of three similar experiments.

FIG. 4 depicts the effects of added MD-2 on activation of HEK/TLR4 by ³H-LOS:MD-2 and delivery of ³H-LOS:MD-2 to HEK/TLR4. In FIG. 4A, cells were incubated in HBSS+, 10 mM HEPES/0.1% albumin with ¹⁴C-LOS:MD-2 (0.3 ng/ml) and increasing amounts of wt (■), C95Y (*) MD-2 or negative control medium (□) as well as with wt MD-2 but no ¹⁴C-LOS:MD-2 (○). After overnight incubation, extracellular accumulation of IL-8 was measured. The concentrated and dialyzed conditioned media contained about 10 ng (wt or C95Y) MD-2 μl. Results are from one experiment in duplicate, which are representative of three similar experiments. In FIG. 4B, purified ¹⁴C-LOS:MD-2 (1 ng/ml) was pre-incubated with (●) or without (○) an amount of MD-2 that completely inhibited activation (40 μl of 20-fold concentrated and dialyzed conditioned media) for 30 minutes, 37° C. in HBSS+/10 mM HEPES before chromatography on Sephacryl S200. Column fractions were analyzed for ¹⁴C-LOS by liquid scintillation spectroscopy. In FIG. 4C, ³H-LOS:MD-2 (0.75 ng/ml; about 3000 cpm) with or without excess MD-2 as indicated in FIG. 4B was incubated with HEK/TLR4 cells overnight at 37° C. as described in the Methods for Example 1. After supernatants were removed, cells were washed and then lysed as described in the Methods for Example 1. The amount of radioactivity associated with the cells was determined by liquid scintillation spectroscopy. No radioactivity was associated with parental cells.

FIG. 5 depicts a possible mechanism of action of MD-2 in endotoxin-dependent activation of TLR4. TLR4 activation may involve either conformational changes in MD-2 that follow the interaction of MD-2 with endotoxin and TLR4 (A) or transfer of endotoxin from MD-2 to TLR4 (B).

FIG. 6 depicts endotoxin responsiveness of well-differentiated primary cultures of human airway epithelia. FIG. 6A shows that polarized epithelia were treated by apical application of NTHi LOS (100 ng/ml), soluble CD14 (sCD14; 250 ng/ml) and LPS binding protein (LBP; 100 ng/ml) or of IL-1β

(100 ng/ml) in a 50 μ l volume. Twenty-four hours later, epithelia were harvested and analyzed for human β -defensin-2 (HBD-2) mRNA expression by real time PCR. Results shown represent 2 epithelia/trial, n=3 different donors; *P<0.05. Matched, untreated epithelia served as controls (CTL). FIG. 6B shows the polarity of epithelial responses to IL-1 β and NTHi LOS. Reagents were applied to the apical, basolateral or both surfaces as indicated in the same concentrations as in FIG. 6A. IL-1 β induced HBD-2 expression from either surface, while NTHi LOS failed to induce responses from either side. Results represent replicate data from two different specimens, (four epithelial condition); *P<0.05.

FIG. 7 depicts results indicating the generation of a functional adenoviral vector expressing human MD-2. A replication incompetent adenoviral vector expressing MD-2 was used. To show that MD-2 protein produced by the vector is functional, HEK293 cells were transduced with a multiplicity of infection of 50 (MOI 50). Twenty-four hours later, the HEK293 cell culture supernatants were harvested and, in dilutions as indicated, applied to HEK cells with or without TLR4 in the presence of LOS:sCD14 (2 ng LOS/ml). TLR4-dependent cell activation was manifested as extracellular accumulation of IL-8, monitored by ELISA. The x-axis indicates the concentration (dilution) of cell culture supernatant applied. Results demonstrate that the adenoviral vector directs production of MD-2 that is secreted and functional. TLR4-cells showed no significant response to LOS-sCD14 medium containing MD-2.

FIG. 8 depicts the effects of MD-2 on endotoxin responsiveness in human airway epithelia. Effect of transduction of well-differentiated, polarized human airway epithelia with Ad-MD-2 on cellular responsiveness to NTHi LOS as measured by HBD-2 mRNA expression (FIGS. 8A and 8C) and by NF- κ B-luciferase activity (FIG. 8B). In the latter, cells were pretreated with an adenoviral vector expressing MD-2 48 hours before stimulation with LOS as described in the Materials of Example 2 below. Cells were treated with *N. meningitidis* LOS aggregates (prepared as described in the Materials of Example 2 below) plus LBP and sCD14 (FIGS. 8A and 8B) or with purified LOS:sCD14 (FIG. 8C). The fold increase in HBD-2 mRNA expression was quantified by real time PCR, and is indicated on the y-axis. Results were normalized to the control level of HBD-2 expression in untreated cells set at 1. Results shown are means \pm SE of three different human specimens; *P<0.05. NF- κ B-luciferase activity was measured 24 hours following endotoxin treatment (n=5 epithelia/condition, *P<0.05). In FIG. 8C, results represent means \pm SE for four different human specimens; *P<0.05. FIG. 8D shows the effect of soluble, recombinant MD-2 (rMD-2) on cellular responsiveness to LOS:sCD14. Well-differentiated human airway epithelia were treated with *N. meningitidis* LOS:sCD14 (5 ng LOS/ml), with or without the addition of cell culture supernatants containing rMD-2 as indicated on the x-axis. Twenty-four hours after endotoxin treatment, HBD-2 expression was quantified by real time PCR. Results shown represent findings from four different human airway specimens. *P<0.05.

FIG. 9 shows the responsiveness of airway epithelia to purified LOS:MD-2 complex. Well-differentiated airway epithelia, before and after adenoviral transduction to express MD-2, were treated with apically applied LOS:CD14 aggregates (open bar) or LOS:MD-2 aggregates (filled bar) and assayed for HBD-2 expression 24 hours later as described in the Materials for Example 2. Hatched bar represents results from cells pretreated with Ad-MD-2 twenty-four hours before application of LOS:MD-2 complex. Each bar repre-

sents means \pm SE for results from four different human epithelial specimens; *P<0.05 compared with LOS:CD14 condition.

FIG. 10A shows that MD-2 mRNA expression in human airway epithelia is induced by several stimuli. Agents used included heat-killed NTHi (strain 12), the NTHi outer membrane protein P6, TNF- α IFN- γ , and phorbol myristate acetate (PMA). Concentrations used are indicated in the Materials for Example 2 below. For comparison, MD-2 mRNA level form Mac is shown. The fold increase in MD-2 mRNA expression, relative to the level of MD-2 mRNA in control resting airway epithelia, was quantified using real time PCR. Shown are the results from three independent experiments on three different epithelial preparations. *P<0.05. FIG. 10B shows the effect of pretreatment of airway epithelia with heat-killed NTHi on LOS responsiveness. Epithelia were exposed to NTHi for 24 hours as described in FIG. 10A above, and were then treated with apically applied LOS:CD14 complex (hatched bar). Control cells were treated with LOS:CD14 alone (open bar) or NTHi alone (filled bar). Twenty-four hours later, HBD-2 expression was assayed using real-time PCR. Results shown are mean \pm SE for epithelia from five different specimens.

FIGS. 11A-11D shows proposed mechanisms by which expression of MD-2 in the airway can regulate the responsiveness of the airway epithelial cells to endotoxin. Endogenous and exogenous expression of MD-2 refers to airway epithelial cells. E_{(agg)(memb)}, purified endotoxin (LPS or LOS) aggregates or gram-negative bacterial outer membrane to which LBP first binds. Complexes of endotoxin (E) with sCD14 and soluble MD-2 (sMD-2) are, by contrast, monomeric.

FIG. 12 depicts Sephacryl™ S200 chromatography of ¹⁴C-msbB LOS aggregates (1 μ g) pre-incubated with 100 ng LBP and 24 μ g sCD14 for 15 minutes at 37° C. The arrow indicates elution of wt LOS-sCD14 complex.

FIG. 13 depicts sephacryl S200 chromatography of ¹⁴C-msbB LOS-sCD14 (160 ng) pre-incubated with ~5 μ g MD-2 for 15 minutes at 37° C. The arrow indicates elution of wt LOS-MD-2 complex.

FIG. 14 depicts cell activation as measured by accumulation of extracellular IL-8, monitored by ELISA. HEK/TLR4 cells were incubated overnight at 37° C. with increasing amounts of wt or mutant (msbB) LOS-MD-2, as indicated.

FIG. 15 depicts cell activation as measured by accumulation of extracellular IL-8, monitored by ELISA. HEK/TLR4 cells were incubated overnight at 37° C. with 0.1 ng/ml of wt LOS-MD-2 increasing amounts of mutant msbB LOS-MD-2, as indicated.

FIG. 16. Mutant MD-2 (K125A.F126A) forms LOS:MD-2 complex (FIG. 16A) that acts as weak agonist (FIG. 16B) and inhibits activation of HEK/TLR4 cells by wt LOS:MD-2 (FIG. 16C). Sephacryl™ S200 chromatography of LOS:sCD14 after incubation with culture medium from cells transfected with expression vector alone (*) or expression vectors directing expression and secretion of wt (closed circles) or mutant (open circles) MD-2 (FIG. 16A). Dose-dependent activation of HEK/TLR4 cells by purified wt (closed circles) and mutant (open circles) LOS:MD-2 (FIG. 16B). Dose-dependent inhibition by mutant LOS:MD-2 of cell activation induced by wt LOS:MD-2 (0.1 ng LOS/ml) (FIG. 16C). Cell activation was monitored by extracellular accumulation of IL-8 and measured by ELISA (FIG. 16B, FIG. 16C). Results shown are representative of four closely similar and independent experiments.

FIG. 17. Monomeric complex of wt LOS with F126A MD-2 mutant is a potent TLR4 antagonist. FIG. 17A shows

dose-dependent activation of HEK/TLR4 cells by wt and mutant LOS:MD-2 complexes. Purified LOS:MD-2 complexes were incubated with HEK293/TLR4 cells in HBSS+, HEPES, 0.1% human serum albumin for 16 h. Extracellular IL-8 recovered in harvested culture medium was measured by ELISA. FIG. 17B shows inhibition of wt LOS:MD-2 activity by LOS:MD-2 (F126A). HEK293/TLR4 cells were incubated for 16 h with increasing amounts of LOS:MD-2 (F126A)±20 pM wt LOS:MD-2 before assay of extracellular IL-8 by ELISA. Results shown are representative of 3 closely similar experiments.

FIG. 18. Molecular model of MD-2 molecule (Gangloff et al. 2004), and effects of site-specific mutations of murine or human MD-2 (Kawasaki et al. 2003).

FIG. 19. Potent pro-inflammatory activity of nasally administered wt LOS:MD-2 in mice. Mice (6/experimental group) were treated by nasal administration of 50 µl of PBS, purified LOS aggregates (30 or 300 EU=3 or 30 ng LOS) or purified LOS:MD-2 (30 or 300 EU=3 or 30 ng LOS in LOS:MD-2), as indicated. Four hours after administration, animals were sacrificed and sampled by broncho-alveolar lavage to monitor accumulation of total WBC and neutrophils (and cytokines; to be assayed). Results shown represent mean±SEM of values obtained.

FIGS. 20A-20E. Chemical cross (X)-linking of [¹⁴C]LOS:MD-2. Molecular models of endotoxin (FIG. 20A) and MD-2 (FIG. 20B), highlighting sites that contain reactive amino groups, —NH₂ and ethanolamine (EtN) in the lipid A backbone and inner core sugar backbone of endotoxin (LOS) and lysines (K122, K125, K128, K130, K132) in MD-2, if fatty acyl chains of lipid A are buried in the predicted hydrophobic cavity of MD-2. FIG. 20C shows [¹⁴C]LOS:MD-2 (100 ng LOS/ml) was incubated either alone (lane 1) or with 1 mM of TSAT (lane 2, molecular structure given in FIG. 20D) or BS³ (lane 3, molecular structure given in FIG. 20E) in PBS, pH 7.4 for 1 h at 25° C. The reaction was stopped by the addition of 1 M Tris buffer, pH 8.0. An aliquot of the reaction mixture was TCA precipitated, the pellet resuspended in SDS-PAGE sample buffer and samples were electrophoresed through a 4-25% gradient gel on the PhastGel™ system (GE Healthcare). [¹⁴C]LOS was detected by using a Typhoon™ (GE Healthcare) imaging system. The migration of free [¹⁴C]LOS and cross-linked (XL)-[¹⁴C]LOS:MD-2 are indicated. SDS extracts [¹⁴C]LOS from native [¹⁴C]LOS:MD-2 but not the cross-linked complex.

FIGS. 21A-21C. Flow chart of purification of cross (X)-linked [¹⁴C]LOS:MD-2 (FIG. 21A). Sephacryl™ S200 chromatography of native and cross-linked reaction mixtures of [¹⁴C]LOS:MD-2 (FIG. 21B). The samples were treated for 1 h at 25° C. with 0.3% (wt/vol) deoxycholate (DOC) to extract [¹⁴C]LOS from native (non-cross-linked) [¹⁴C]LOS:MD-2, diluted in PBS, pH 7.4 and re-purified by gel exclusion chromatography (1.6×70 cm) in PBS, pH 7.4 to permit extracted [¹⁴C]LOS to re-aggregate and be separated from cross-linked [¹⁴C]LOS:MD-2 (M_r~190,000). Fractions (1 ml) were collected at a flow rate of 0.5 ml/min Recovery of [¹⁴C]LOS was monitored by liquid scintillation spectroscopy. FIG. 21C shows SDS-PAGE/image analysis of [¹⁴C]LOS recovered in fraction 53 (void volume) and fraction 75 (M_r~25,000). Results shown are representative of two closely similar experiments.

FIGS. 22A and 22B. Comparison of dose-dependent effects of native and cross-linked [¹⁴C]LOS:MD-2 on HEK/TLR4 cells. Assay of TLR4 agonist activity: cells were incubated for 20 h at 37° C. with increasing concentrations of native or cross-linked endotoxin (E):MD-2 (wild-type (wt; hexaacylated) meningococcal LOS:wt MD-2) (FIG. 22A).

Assay of TLR4 antagonist activity: cells were incubated for 20 h at 37° C. with a mixture of 20 pM wt (non-cross-linked) LOS:MD-2 and increasing concentrations of cross-linked LOS:MD-2 (FIG. 22B). Cell activation was monitored by measuring extracellular accumulation of IL-8 by ELISA. Results shown represent the mean of two closely similar experiments.

FIGS. 23A-23F. Specific binding of [³H]LOS:MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17) to the predicted ectodomain of TLR4 (TLR4_{ECD}): Characterization of the [³H]LOS-containing product: Concentrated conditioned cell culture medium, harvested 48 h after transfection of HEK293T cells with pFLAG-CMV (control medium) or pFLAG-CMV-TLR⁴²⁴⁻⁶³⁴ (TLR4_{ECD})_{cm}, was diluted in PBS, pH 7.4, and incubated for 30 min at 37° C. with 1 nM [³H]LOS, as indicated (FIGS. 23A-23C). Reactants and products were resolved by Sephacryl S200 (FIGS. 23A, 23B) or S300 (FIG. 23C) chromatography and recovery of [³H]LOS monitored by liquid scintillation spectroscopy. The novel earlier eluting [³H]LOS-containing complex formed during incubation of [³H]LOS:MD-2 with (TLR4_{ECD})_{cm} (see encircled fractions, (FIG. 23A)) was subjected to re-chromatography on a Sephacryl™ S300 column (1.6×70 cm) (FIG. 23D) to determine the purity and apparent M_r of the novel complex by comparison to elution of protein standards of known molecular mass (see inset). The presence of MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17) and FLAG-TLR4_{ECD} in the M_r~190,000 [³H]LOS-containing complex was determined by co-capture of [³H]LOS by, respectively, Ni²⁺ Sepharose™ FF resin (GE Healthcare) (FIG. 23E) or anti-FLAG M2 agarose (FIG. 23F). In brief: 0.2 pmol of [³H]LOS-containing complex was incubated for 1 h at room temperature with either 50 µl of Ni²⁺ Sepharose™ FF resin (GE Healthcare) or 75 µl anti-FLAG M2 agarose (FIG. 23F) in 20 mM phosphate, 0.5 M NaCl, pH 7.4. After incubation, the beads were sedimented, washed, and adsorbed [³H]LOS measured by liquid scintillation spectroscopy. The size and content of the novel complex suggests a dimer of a 1:1:1 complex of ([³H]LOS:MD-2/TLR4_{ECD})₂. Results shown are representative of 3 or more separate experiments.

FIGS. 24A-24C. Saturable, high affinity binding of [³H]LOS:MD-2 to TLR_{ECD}: Competition by native and cross-linked LOS:MD-2. Concentrated conditioned medium containing TLR4_{ECD} (TLR4_{ECD})_{cm} was diluted in PBS to ~75 pM TLR4_{ECD} and incubated at 37° C. for 30 min with increasing concentrations of [³H]LOS:MD-2. Formation of the M_r~190,000 complex was monitored by Sephacryl™ S200 chromatography in PBS, pH 7.4. Elution profiles from selected doses of [³H]LOS:MD-2 are shown in FIG. 24A and molar amounts of the M_r~190,000 complex formed, calculated from specific radioactivity of [³H]LOS, as a function of [³H]LOS:MD-2 concentration is shown in FIG. 24B. [³H]LOS:MD-2 (0.4 nM; 25,000 cpm/pmol) was incubated with (TLR4_{ECD})_{cm} (~75 pM) either alone (“control”) or with excess weakly [¹⁴C]-labeled (625 cpm/pmol) LOS aggregates, LOS:sCD14 (10 nM) or native or cross-linked LOS:MD-2 (2.4 nM) (FIG. 24C). Results shown are representative of 2 or more independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have discovered that MD-2 interacts directly with endotoxin-CD14 complexes to generate endotoxin-MD-2 complexes that produce TLR4-dependent cell stimulation or inhibition. This stimulation or inhibition can be produced at concentrations consistent with the ability of the innate immune system to detect and respond to minute

amounts of endotoxin. Thus, endotoxin bound to MD-2, rather than endotoxin itself, is a ligand for triggering TLR4 receptor activation or inhibition.

The present invention provides a complex including endotoxin covalently bound to MD-2 by means of a cross-linker molecule. Surprisingly, these complexes, when devoid of any other host or microbial molecules, are potent and water soluble and do not require additional lipid carrier molecules (e.g., serum albumin) for water solubility.

The MD-2 and/or E:MD-2 complex may be purified. In the context of the present invention, an "isolated" or "purified" molecule or complex is a molecule or complex that exists apart from its native environment and is therefore not a product of nature. An isolated molecule or complex may exist in a purified form or may exist in a non-native environment such as, for example, a transgenic host cell. For example, an "isolated" or "purified" protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

E:MD-2 Complexes and Compositions

Disclosed herein is the formation and isolation of a novel, bioactive, monomeric endotoxin:MD-2 complex. The complex can be generated with about physiologic (pM) concentrations of endotoxin and soluble MD-2. This complex, at pg/ml concentrations, activates cells in a TLR4-dependent fashion without the inclusion of other host or bacterial factors. The surprising and unexpected success in achieving formation of a bioactive endotoxin:MD-2 complex at such low concentrations of endotoxin and MD-2 reflects the importance of presenting endotoxin to MD-2 after endotoxin has been first modified by LBP and CD14.

As interactions of CD14 with endotoxin are greatly enhanced by prior interaction of endotoxin with LBP (Giannini et al., 2002; Iovine et al., 2002; Hailman et al., 1994; Yu et al., 1996; Tapping et al., 1997; Thomas et al., 2002; and Giardina et al., 2001), these findings indicate that MD-2: endotoxin interactions leading to the generation of the bioactive endotoxin:MD-2 complex are greatly enhanced by presentation of endotoxin as a monomeric complex with CD14. This may reflect, for example, a greater reactivity of MD-2 for disaggregated vs. aggregated forms of endotoxin, or the need for an additional protein-protein interaction between CD14 and MD-2. Whatever the precise molecular basis of the high affinity and reactivity of endotoxin:CD14 complexes with MD-2, these surprising findings indicate a direct role of MD-2 in endotoxin recognition and delivery of endotoxin to host cells containing TLR4, not requiring prior association of MD-2 with TLR4, as has been previously presumed (Viriyakosol et al., 2001; Visinin et al., 2001; Visintin et al., 2003; and Mullen et al., 2003). These findings also indicate that the key role of LBP and CD14 in enhancing cell responses to endotoxin is to transform aggregates of endotoxin to monomeric endotoxin:CD14 complexes that are preferentially reactive with MD-2. Conversely, the remarkably potent activity of the purified endotoxin: MD-2 complex toward HEK/TLR4 cells indicates that CD14 is not needed as part of a more complex hetero-oligomeric receptor, as was previously suggested (Miyake, 2003; Kawasaki et al., 2003; Muroi et al., 2002; Latz et al., 2002; and da Silva Correia et al., 2001).

The reaction pathway described herein in which endotoxin molecules in purified aggregates (or membranes) containing thousands to millions of endotoxin molecules/particle are extracted and transferred to first CD14 and then MD-2 provides a unique physico-chemical mechanism to attain the potency that is needed for response. The ability to generate a

homogeneous protein-endotoxin complex that, alone, triggers TLR4-dependent cell activation, interacts with host cells in an almost exclusively TLR4-dependent fashion and that can be metabolically labeled to sufficient specific radioactivity to monitor interactions at pM concentrations makes it possible for the first time to measure host cell-endotoxin interactions that are directly relevant to TLR4-dependent cell activation.

Many endotoxin-responsive cells contain membrane-associated CD14 and MD-2 (associated with TLR4) (Means et al., 2000; Miyake et al., 2003; Takeda et al., 2003). However, as described herein, resting airway epithelial cells, like HEK/TLR4 cells, express TLR4 without MD-2 and respond to endotoxin only if LBP, sCD14 and soluble MD-2 are added. Each of these proteins are likely to be present in biological fluids at the concentrations needed to drive endotoxin-dependent TLR4 activation, especially in view of the very low extracellular MD-2 concentrations demonstrated in this study to be sufficient. Thus, the reaction pathway defined is relevant at the cell surface when TLR4/MD-2 complexes are endogenously present and also when only TLR4 is present at the cell surface and MD-2, which has been produced and secreted by neighboring cells, is present in the extracellular medium.

The findings presented herein thus indicate that MD-2 can engage both endotoxin and TLR4, and that simultaneous interaction of MD-2 with endotoxin and within TLR4 is important for TLR4-dependent cell activation by endotoxin. It is thus predicted that binding sites within MD-2 for TLR4 and endotoxin are topologically as well as structurally distinct, permitting engagement of endotoxin:MD-2 complexes with TLR4, as well as interaction and transfer of endotoxin from endotoxin:CD14 complexes to MD-2 already associated with TLR4 (FIG. 5). The complete lack of reactivity of the C95Y MD-2 mutant with endotoxin:sCD14 explains the complete absence of activity in this mutant protein (FIG. 2B) despite a partial retention of reactivity with TLR4.

In contrast to ¹⁴C-LOS:sCD14, the "stability/solubility" of LOS:MD-2 in aqueous buffer and its bioactivity does not require albumin. That albumin is no longer required once the endotoxin:MD-2 complex is formed indicates that a hydrophobic site in MD-2, possibly a deep hydrophobic site, accommodates and shields the hydrophobic lipid A region of the bound endotoxin, making subsequent transfer to TLR4 less likely. Thus, binding of endotoxin to MD-2 may induce conformational changes in MD-2 that lead to TLR4 activation (FIG. 5).

FIG. 5 depicts mechanisms of action of MD-2 in endotoxin-dependent activation of TLR4. TLR4 activation may involve (A) conformational changes in MD-2 that follow the interaction of MD-2 with endotoxin and TLR4 and/or (B) transfer of endotoxin from MD-2 to TLR4.

The association of endotoxin with MD-2 forms the complexes of the invention. The endotoxin molecules and the MD-2 molecules may be wild-type (wt) molecules, or they may be mutant molecules. The complex may have a molecular weight of about 25,000. The complex may consist essentially of one molecule of endotoxin bound to one molecule of MD-2. The complex may be soluble in water. The complexes may be soluble in water to a greater extent than is an endotoxin molecule not bound to MD-2. The complex may bind to TLR4 and may produce TLR4-dependent activation of cells, e.g., at a concentration of about 1 nM or less of the complex the complex may produce a half maximal activation of cells, e.g., at a concentration of about 30 pM or less of the complex the complex may produce a half maximal activation of cells.

The present invention also provides a composition including a complex of the invention, optionally including a phar-

maceutically acceptable carrier. The composition can be used, e.g., to promote innate immune responses and as an immunological adjuvants.

Endotoxins

In some embodiments of the invention, the endotoxin molecules are wildtype (wt) endotoxin molecules, e.g., endotoxin derived from gram-negative bacteria. The endotoxin may be a wild-type endotoxin derived from any of a broad array of gram-negative bacterial species. The list of possible gram-negative bacteria includes many species of clinical importance such as *Neisseria meningitidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Salmonella typhimurium*, and *Francisella tularensis*.

The endotoxin may be a wild-type endotoxin, i.e., the form of endotoxin naturally found in the wildtype gram-negative bacteria. Alternatively, the endotoxin may be a variant endotoxin, i.e., the endotoxin varies from the wildtype form in some way.

In some embodiments of the invention, the endotoxin molecules are mutant endotoxin molecules. In some embodiments of the invention, the mutant endotoxin molecules are endotoxin molecules that are capable of binding to MD-2 and these complexes to TLR4 without producing the same level of TLR4-dependent cell activation (i.e., the mutant endotoxin complexes produce less activation) as produced by TLR4 dependent cell activation by complexes containing wild-type endotoxin molecules. These mutant endotoxin molecules may be under-acylated (i.e., the endotoxin lacks at least one acyl group as compared to the wildtype form of the molecule). Examples of such under-acylated endotoxins are in PCT Publication No. WO 97/19688. These under-acylated endotoxin molecules may be produced via enzymatic release of non-hydroxylated fatty acids from endotoxin, or they may be produced using bacteria having genes disrupted that encode an acyltransferase (e.g., *htrB*, *msbB*) needed for biosynthetic incorporation of non-hydroxylated fatty acids into endotoxin. The complex containing the under-acylated endotoxin may be capable of producing less TLR4-dependent activation of cells as compared to a complex including an endotoxin that is hexa-acylated.

The endotoxin molecules may be hepta-acylated, hexa-acylated, penta-acylated or tetra-acylated, or a combination thereof.

Cross-Linking Molecules

The endotoxin and MD-2 molecules are covalently linked using a chemical cross-linking agent. Many different cross-linking agents can be used. In certain embodiments the cross-linking agent is about 400-1000 daltons or about 3-12 angstroms in length. The cross-linkers useful in the present invention must be at least bivalent so that they can covalently join two molecules, the endotoxin molecule to the MD-2 molecule. In certain embodiments, the cross-linker can be tris-succinimidyl aminotriacetate (TSAT); bis(sulfosuccinimidyl) suberate (BS³); disuccinimidyl suberate (DSS); bis(2-[sulfosuccinimidyl]oxyethylsulfone) (BOSCOES); bis(2-[succinimidyl]oxyethylsulfone) (Sulfo-BOSCOES); ethylene glycol bis-(succinimidylsuccinate) (EGS); ethylene glycol bis-(sulfosuccinimidylsuccinate) (Sulfo-EBS); or Dimethyl 3,3'-dithiobispropionimidate (DTBP). In certain embodiments, the cross-linker is trivalent (such as TSAT). In certain embodiments, the cross-linker is bivalent such as BS³, Sulfo-Boscoes, EGS, Sulfo-EBS, or DTBP. In certain embodiments of the present invention, a single molecule of endotoxin is covalently bound to a single MD-2 molecule by means of a single cross-linker molecule. The single cross-linker molecule can be, for example, bivalent or trivalent.

Methods for attaching cross-linkers are well known in the art (cf. Hermanson, 1995 *Bioconjugate Techniques*, Academic Press, Inc. New York, pp. 728; Wong, 1991 *Chemistry of Protein Conjugation and Cross-linking*. CRC Press, pp. 340; Brinkley, 1992 A brief survey of methods for preparing protein conjugates with dyes, haptens and cross-linking reagents *Bioconjugate Chem.* 3:2-13).

MD-2 Molecules

The MD-2 molecule may also be a wild-type MD-2 or a variant MD-2 molecule. The MD-2 may be a recombinant MD-2.

As used herein, the MD-2 proteins include variants or biologically active fragments of the protein. A "variant" of the protein is a protein that is not completely identical to a native protein. A variant protein can be obtained by altering the amino acid sequence by insertion, deletion or substitution of one or more amino acid. The amino acid sequence of the protein is modified, for example by substitution, to create a polypeptide having substantially the same or improved qualities as compared to the native polypeptide. The substitution may be a conserved substitution. A "conserved substitution" is a substitution of an amino acid with another amino acid having a similar side chain. A conserved substitution would be a substitution with an amino acid that makes the smallest change possible in the charge of the amino acid or size of the side chain of the amino acid (alternatively, in the size, charge or kind of chemical group within the side chain) such that the overall polypeptide retains its spatial conformation but has altered biological activity. For example, common conserved changes might be Asp to Glu, Asn or Gln; H is to Lys or Arg or Phe; Asn to Gln, Asp or Glu and Ser to Cys, Thr or Gly. Alanine is commonly used to substitute for other amino acids in mutagenesis studies. The 20 essential amino acids can be grouped as follows: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine having non-polar side chains; glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine having uncharged polar side chains; aspartate and glutamate having acidic side chains; and lysine, arginine, and histidine having basic side chains (Stryer (1981); Lehninger (1975)).

It is known that variant polypeptides can be obtained based on substituting certain amino acids for other amino acids in the polypeptide structure in order to modify or improve biological activity. For example, through substitution of alternative amino acids, small conformational changes may be conferred upon a polypeptide that results in increased bioactivity.

One can use the hydropathic index of amino acids in conferring interactive biological function on a polypeptide, wherein it is found that certain amino acids may be substituted for other amino acids having similar hydropathic indices and still retain a similar biological activity.

The amino acid sequence of the variant MD-2 protein corresponds essentially to the native protein amino acid sequence. As used herein "corresponds essentially to" refers to a polypeptide sequence that will elicit a biological response substantially the same as the response generated by native protein. Such a response may be at least 60% of the level generated by native protein, and may be any integer between 60% to 200% (e.g., 61%, 62%, 63%, . . . 198%, 199% or 200%). In certain embodiments, the level is at least 80%, 85%, 90% or 95% of the level generated by wildtype (native) protein. For example, variants of the wildtype endotoxin will elicit a biological response (i.e., TLR4-dependent cell activation) substantially the same as the response generated by the native endotoxin.

A variant of the invention may include amino acid residues not present in the corresponding native protein, or may

include deletions relative to the corresponding native protein. A variant may also be a truncated fragment as compared to the corresponding native protein, i.e., only a portion of a full-length protein. Protein variants also include peptides having at least one D-amino acid.

The MD-2 of the present invention may be expressed from isolated nucleic acid (DNA or RNA) sequences encoding the protein. Amino acid changes from the native to the variant protein may be achieved by changing the codons of the corresponding nucleic acid sequence. Recombinant is defined as a peptide or nucleic acid produced by the processes of genetic engineering. It should be noted that it is well-known in the art that, due to the redundancy in the genetic code, individual nucleotides can be readily exchanged in a codon, and still result in an identical amino acid sequence.

The starting material (such as an MD-2 gene) used to make the complexes of the present invention may be substantially identical to wild-type genes, or may be variants of the wild-type gene. Further, the polypeptide encoded by the starting material may be substantially identical to that encoded by the wild-type gene, or may be a variant of the wild-type gene.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence," (b) "comparison window," (c) "sequence identity," (d) "percentage of sequence identity," and (e) "substantial identity." (a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" makes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence in the comparison window may include additions or deletions (i.e., gaps) compared to the reference sequence (which does not include additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Preferred, non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988); the local homology algorithm of Smith et al. (1981); the homology alignment algorithm of Needleman and Wunsch (1970); the search-for-similarity-method of Pearson and Lipman (1988); the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993).

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wis., USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins et al. (1988); Higgins et al. (1989); Corpet et al. (1988); Huang

et al. (1992); and Pearson et al. (1994). The ALIGN program is based on the algorithm of Myers and Miller, *supra*. The BLAST programs of Altschul et al. (1990); Altschul et al. (1997), are based on the algorithm of Karlin and Altschul *supra*.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length *W* in the query sequence, which either match or satisfy some positive-valued threshold score *T* when aligned with a word of the same length in a database sequence. *T* is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters *M* (reward score for a pair of matching residues; always >0) and *N* (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity *X* from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached.

In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (*P(N)*), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a test nucleic acid sequence is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid sequence to the reference nucleic acid sequence is less than about 0.1, less than about 0.01, or even less than about 0.001.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997). Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al., *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (*W*) of 11, an expectation (*E*) of 10, a cutoff of 100, *M*=5, *N*=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (*W*) of 3, an expectation (*E*) of 10, and the BLOSUM62 scoring matrix. Alignment may also be performed manually by inspection.

For purposes of the present invention, comparison of nucleotide sequences for determination of percent sequence identity to the promoter sequences disclosed herein is preferably made using the BlastN program (version 1.4.7 or later) with its default parameters or any equivalent program. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes

reference to a specified percentage of residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window, as measured by sequence comparison algorithms or by visual inspection. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may include additions or deletions (i.e., gaps) as compared to the reference sequence (which does not include additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

(e)(i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide includes a sequence that has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, or 94%, 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 70%, at least 80%, 90%, or even at least 95%.

Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions (see below). Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. However, stringent conditions encompass temperatures in the range of about 1° C. to about 20° C., depending upon the desired degree of stringency as otherwise qualified herein. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides they encode are substantially identical. This may occur, e.g., when a copy of a nucleic

acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is when the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

(e)(ii) The term "substantial identity" in the context of a peptide indicates that a peptide includes a sequence with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, or 94%, or even 95%, 96%, 97%, 98% or 99%, sequence identity to the reference sequence over a specified comparison window. Optimal alignment may be conducted using the homology alignment algorithm of Needleman and Wunsch (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution.

For sequence comparison, typically one sequence acts as a reference sequence to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

As noted above, another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions. The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA. "Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target nucleic acid sequence.

"Stringent hybridization conditions" and "stringent hybridization wash conditions" in the context of nucleic acid hybridization experiments such as Southern and Northern hybridizations are sequence dependent, and are different under different environmental parameters. Longer sequences hybridize specifically at higher temperatures. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984); $T_m = 81.5^\circ \text{C.} + 16.6 (\log M) + 0.41 (\% \text{ GC}) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. T_m is reduced by about 1° C. for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the T_m can be decreased 10° C. Generally, stringent conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However,

severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4° C. lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10° C. lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20° C. lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T of less than 45° C. (aqueous solution) or 32° C. (formamide solution), the SSC concentration can be increased so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993). Generally, highly stringent hybridization and wash conditions are selected to be about 5° C. lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH.

An example of highly stringent wash conditions is 0.15 M NaCl at 72° C. for about 15 minutes. An example of stringent wash conditions is a 0.2×SSC wash at 65° C. for 15 minutes (see Sambrook et al. (2001) for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1×SSC at 45° C. for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6×SSC at 40° C. for 15 minutes. For short probes (e.g., about 10 to 50 nucleotides), stringent conditions typically involve salt concentrations of less than about 1.5 M, more preferably about 0.01 to 1.0 M, Na ion concentration (or other salts) at pH 7.0 to 8.3, and the temperature is typically at least about 30° C. and at least about 60° C. for long probes (e.g., >50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of 2× (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the proteins that they encode are substantially identical. This occurs, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent conditions for hybridization of complementary nucleic acids that have more than 100 complementary residues on a filter in a Southern or Northern blot is 50% formamide, e.g., hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65° C. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1M NaCl, 1% SDS (sodium dodecyl sulphate) at 37° C., and a wash in 1× to 2×SSC (20×SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37° C., and a wash in 0.5× to 1×SSC at 55 to 60° C.

Methods of Modulation TLR4-Mediated Cell Activation and for Treating Conditions Associated with Endotoxin-Mediated Cell Activation

TLR4-dependent cell activation refers to the cascade of events produced when TLR4 is activated, e.g., by endotoxin, to produce responses, e.g., pro-inflammatory responses. The art worker can measure TLR4-dependent cell activation, e.g., by assaying the level of IL-8 produced by the cells.

The present invention also provides a method for modulating TLR4-mediated cell activation by endotoxin, including administering to the cell MD-2. Because the complexes are both potent and water soluble, they are useful in the treatment of conditions associated with TLR4-dependent cell activation. The MD-2 can be administered prior to exposure of the cell to endotoxin. The MD-2 can be administered while the cell is exposed to endotoxin. In some embodiments of the invention, administration of a stoichiometric excess of MD-2 relative to TLR4 inhibits the endotoxin-mediated cell activation. In some embodiments of the invention, the MD-2 is administered to achieve a concentration of about 10-100 ng/ml. In some embodiments of the invention, administration of MD-2 to cells, e.g., cells having a constitutively low level of MD-2 expression, increases endotoxin-mediated cell activation. These cells may be, for example, airway epithelial cells or pulmonary macrophages.

The present invention also provides methods for treating conditions associated with endotoxin-mediated cell activation. The conditions include sepsis, trauma, liver disease, inflammatory bowel disease, cystic fibrosis, asthma, complications in renal dialysis, autoimmune diseases, cancer chemotherapy sequelae, and intracellular gram-negative bacterial infections, e.g., infection caused by *Francisella tularensis*. The conditions can be treated by administration of a complex of the invention. Treatment, as used herein, includes both prophylactic treatments and therapeutic treatments.

Endotoxin Responsiveness of Human Airway Epithelia

The surfaces of the conducting airways and alveoli of the lung are a large interface between the host and the environment. Despite ongoing daily exposure to microbes and their components by inhalation, the intrapulmonary airways and airspaces normally maintain a sterile state without significant inflammation (Reynolds, 1997). This remarkable condition reflects the success of the concerted activities of the innate and adaptive immune systems.

Inducible antimicrobial peptides, including the cationic β -defensins, represent an important component of the innate immune system (Zasloff, 2002; Schutte et al., 2002; and Ganz, 2002). In humans, the expression of four β -defensins has been reported in pulmonary epithelia (McCray et al., 1997; Schroder et al., 1999; Bals et al., 1998; Jia et al., 2001; Harder et al., 2001; and Garcia et al., 2001), and a recent genome-wide search uncovered evidence of a much larger family of β -defensin genes encoded in five chromosomal clusters (Schutte et al., 2002). Recent data from several groups suggest that the regulation of inducible β -defensins expression in epithelia may parallel many aspects of antimicrobial peptide expression in *Drosophila*, including sensing of the extracellular environment by the Toll-like receptor (TLR) family of pattern recognition receptors and intracellular signaling through NF- κ B and other pathways.

Human β -defensins-2 (HBD-2) is an inducible cationic antimicrobial peptide expressed at many mucosal surfaces, including the skin, cornea, gut, gingiva and airway epithelium (Bals et al., 1998; Harder et al., 1997; Mathews et al., 1999; O'Neil et al., 1999; Liu et al., 1998; and McNamara et al., 1999). In the airways, HBD-2 mRNA is expressed at low levels in resting cells but is markedly induced by pro-inflammatory stimuli including IL-1 β , TNF- α , and *Pseudomonas aeruginosa* (Singh et al., 1998; and Harder et al., 2000). Furthermore, the 5' flanking sequence of the HBD-2 gene contains several cis-acting elements that may mediate transcription in response to inflammatory stimuli, including NF-

κB, IFN-γ, AP-1, and NF-IL-6 response elements (Liu et al., 1998; Harder et al., 2000; and Tsutsumi-Ishii et al., 2003).

Sensitive cellular response to many endotoxins requires the concerted action of at least four host extracellular and cellular proteins: LPS binding protein (LBP), CD 14, MD-2 and TLR-4. The expression of inducible antimicrobial peptides such as human defensin-β (HBD-2) by epithelia is a component of the innate pulmonary defense. In well-differentiated primary cultures of human airway epithelia, TLR4 but little or no MD-2 is expressed, and these cells are relatively unresponsive to added endotoxin, even in the presence of LBP and CD14. However, the responsiveness of these cells to endotoxin is markedly amplified by either the endogenous expression or the exogenous addition of MD-2, indicating that the constitutively low levels of MD-2 expression in these cells at “rest” are important in maintaining their hyporesponsiveness to endotoxin. Changes in MD-2 expression in the airway epithelium and/or neighboring cells can be achieved by exposure of these cells to specific bacterial and host products and may thereby regulate airway responsiveness to endotoxin.

Under resting conditions, human airway epithelia are hyporesponsive to endotoxin stimulation. The present inventors tested LOS from two different gram-negative bacterial species and several different presentations to LOS, including LOS:sCD14, which is active at picogram/milliliters toward highly endotoxin-responsive cells such as monocytes, macrophages, and endothelial cells. The inventors focused on responses of airway epithelia to LOS, rather than LPS, because most gram-negative bacteria that colonize the upper airway produce LOS or LPS without O-antigen. Although airway epithelial cells are relatively unresponsive to endotoxin, these cells are not generally hyporesponsive to all stimuli, as shown by their robust responses to proinflammatory cytokines including IL-1.

Hypo-responsiveness to endotoxin is a common characteristic of epithelial cells lining mucosal surfaces that are repeatedly exposed to gram-negative bacteria or cell-free (sterile) forms of endotoxin. The molecular basis of endotoxin hyporesponsiveness is unknown. As sensitive responses to endotoxin generally depend on TLR4-dependent signaling (Shimazu et al., 1999), hypo-responsiveness likely represents the functional deficiency of one or more elements of pathway(s) leading to and resulting from TLR4 activation. TLR4 is a membrane protein containing repeats of a leucine rich motif in the extracellular portion of the protein and a cytoplasmic domain homologous to the intracellular domain of the human IL-1 receptor (Medzhitov et al., 1997). The IL-1 responsiveness of airway epithelia indicates that the overlapping intracellular signaling pathways for activated TLR and IL-1 receptors (Hoffmann et al., 1999; Janeway et al., 2002; and Beutler et al., 2003) are present and functionally intact in human airway epithelia, including those important in NF-κB-regulated HBD-2 expression. Even though airway epithelia express TLR4, the cells responded poorly to LOS:sCD14. Therefore it is likely that there is a defect in TLR4-dependent recognition and/or response to endotoxin.

MD-2, also termed lymphocyte antigen 96 (LY96), was first identified by Shimazu and colleagues as a molecule that conferred TLR4-dependent responses to minute amounts of endotoxin (Shimazu et al., 1999; and Yang et al., 2000). Cells that are TLR4+/MD-2⁻ are virtually unresponsive to endotoxin (Shimazu et al., 1999; and Nagai et al., 2002). As described herein, human airway epithelia exhibit endotoxin responses consistent with a TLR4+/MD-2⁻ phenotype. The complementation of this deficiency of MD-2 via endogenous expression or extracellular addition was sufficient to convert

human airway epithelia from endotoxin hypo-responsive to highly endotoxin responsive without changing basal NF-κB-regulated HBD-2 expression of these cells or that stimulated by treatment with an unrelated agonist (e.g., IL-1β). These data indicate that the hypo-responsiveness of “resting” airway epithelia is due specifically to the absence of MD-2 expression.

It was previously observed that TLRs 1-6 and CD14 were expressed in differentiated human airway epithelia. Although it was reported that *P. aeruginosa* LPS activated CD14-dependent NF-κB signaling, the increase in HBD-2 mRNA that was observed in response to *P. aeruginosa* LPS was no greater (~3-fold) than what the present inventors found with added LOS in the absence of MD-2 complementation; i.e., very modest when contrasted to the induction of HBD-2 expression caused by IL-1 (3 or by endotoxin in the presence of MD-2).

Similar to the finding in airway epithelia, cell lines derived from intestinal epithelia respond robustly to IL-1 (3 stimulation but not to purified, protein-free LPS with NF-κB signaling. However, published data on intestinal epithelia indicate both diminished TLR4 expression as well as absent MD-2. Accordingly, complementation of both TLR4 and MD-2 in intestinal epithelia is needed for significant activation of NF-κB and IL-8 reporter genes in response to endotoxin. These data suggest that airway and intestinal epithelia, both derivatives of the endodermal foregut, have evolved pattern recognition receptor profiles that minimize stimulation by endotoxin that is frequently encountered at these mucosal surfaces. The ability of the airway to mobilize protective TLR4-dependent responses without the need for MD-2 in response to specific products of gram-positive bacterial airway pathogens (e.g., pneumococci) is consistent with the apparently greater retention of TLR4 expression by airway vs. intestinal epithelia.

On the basis of current understanding of MD-2 actions, mobilization of MD-2 in the airway could have at least two roles: (1) combining, when endogenously expressed, with endogenously expressed TLR4 to increase surface expression of TLR2 (MD-2), and (2) participating directly in endotoxin recognition by extracting endotoxin from monomeric endotoxin:CD14 complexes either before or after interaction of MD-2 to the TLR4. TLR4 alone has no apparent ability to engage the endotoxin:(s) CD14 complex. The ability of soluble, extracellular MD-2 plus endotoxin:sCD14 and preformed endotoxin:MD-2 complex to activate resting human airway epithelia implies that sufficient TLR4 is produced by these cells to respond sensitively to endotoxin when endotoxin is presented as a complex with MD-2. No mechanism for TLR-independent interaction of endotoxin: MD-2 complex has been described. Therefore, the inventors believe that the ability of pico-molar amounts of LOS:MD-2 to activate resting human airway epithelia suggests that there is sufficient apical surface expression of TLR4 (even without endogenous MD-2 to interact directly with extracellular endotoxin:MD-2 and produce potent cell activation.

Results presented herein indicate that MD-2 plays a direct role in endotoxin recognition by TLR4. Endotoxin is transferred from an endotoxin:(s)CD14 complex to MD-2 to form an endotoxin:MD-2 complex that appears to be the ligand for endotoxin-dependent TLR4 activation. TLR4 alone has no apparent ability to productively engage the endotoxin:(s) CD14 complex. In addition, endogenous expression of MD-2 can increase surface expression of TLR4, as a TLR4/MD-2 complex, indicating a chaperone-like function for MD-2 as well. The ability of exogenously added MD-2 to increase cellular responsiveness to LOS: sCD14 and the ability of

purified LOS-MD-2 to directly activate “resting” airway epithelia indicates some apical surface expression of TLR4 even without endogenous expression of MD-2. This would be biologically important because it would permit TLR4 from one cell to engage MD-2 or endotoxin:MD-2 derived from MD-2 secreted by a neighboring cell.

Thus, these results indicate that the regulation of MD-2 expression is a key determinant of airway epithelial responses to endotoxin. Under resting conditions, low MD-2 expression in airway epithelia renders cells poorly responsive to endotoxin, whereas up-regulation of MD-2 alone can greatly enhance cellular responses to endotoxin. A variety of stimuli can induce MD-2 mRNA expression in airway epithelia, perhaps by more than one receptor-mediated pathway. MD-2 can be secreted by epithelia or mononuclear cells and the application of secreted MD-2 enhances TLR4 signaling in MD-2 deficient cells. There may be stimuli (e.g., cytokines, bacterial products) that induce MD-2 expression in airway epithelia to levels sufficient to enhance endotoxin responsiveness. In intestinal epithelia, expression of MD-2 is regulated by cytokine signals including TNF- α and interferon- γ (Abreu et al., 2001; and Visintin et al., 2001). Stimulated pulmonary macrophages might also secrete sufficient MD-2 to enhance TLR4 signaling in epithelia. In either case, production of MD-2 by epithelia or exogenous provision of MD-2 from neighboring cells would complement the airway cells for enhanced TLR4 signaling in response to endotoxin. However, because TLR4-dependent cell activation by endotoxin requires simultaneous interaction of MD-2 with endotoxin and TLR4 (FIG. 11), excessive production of MD-2 could blunt cellular responsiveness to endotoxin by promoting conversion of extracellular endotoxin:sCD14 complex to endotoxin:MD-2 while presaturating TLR4 with MD-2, leaving no cellular targets (i.e., free TLR4) for the extracellular endotoxin:MD-2 complex. Thus, the regulation of MD-2 expression in the airways (e.g., airway epithelia and/or pulmonary macrophages) may permit both up- and down-regulation of endotoxin responsiveness in the airway.

One advantage of such a hierarchy of responses is that it would help to minimize the frequency of epithelial-induced inflammatory signals from endotoxin. Ambient air contains bacteria and endotoxin (Mueller-Annelling et al., 2004) and the aerosolized concentrations of endotoxin can increase dramatically in some agricultural and industrial environments from ≤ 10 EU/m³ to $>1,000$ EU/m³ (Douwes et al., 2003). Under normal conditions, the low expression of MD-2 in epithelia can serve to dampen endotoxin responsiveness to common environmental exposures and thereby avoid unwanted states of chronic inflammation in the face of frequent encounters with environmental endotoxin and other bacterial cell wall components. Conversely, up-regulation of MD-2 expression can be important to enhance host defense responses to invading gram-negative bacteria. However, in certain disease states, such as cystic fibrosis or asthma, enhanced expression of MD-2 could lead to exaggerated endotoxin responsiveness with pathologic consequences.

Formulations and Administration

Complexes containing wild-type endotoxin produce TLR4-dependent cell stimulation, while complexes containing mutant forms of endotoxin inhibit TLR4-dependent cell stimulation. Methods of using the complexes, e.g., methods to increase or inhibit TLR4-dependent activation of cells by endotoxin in vitro or in vivo are provided. Methods using complexes with mutant endotoxin are useful, e.g., to decrease undesirable endotoxin-mediated inflammation. Methods

using complexes with wild-type endotoxin are useful, e.g., to promote innate immune responses and to serve as an immunological adjuvant.

Complexes of the invention, or MD-2 alone, including their salts, can be administered to a patient. Administration in accordance with the present invention may be in a single dose, in multiple doses, and/or in a continuous or intermittent manner, depending, for example, upon the recipient’s physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration may be essentially continuous over a preselected period of time or may be in a series of spaced doses. The amount administered will vary depending on various factors including, but not limited to, the condition to be treated and the weight, physical condition, health, and age of the patient. A clinician employing animal models or other test systems that are available in the art can determine such factors.

To prepare the complexes, the complexes are produced as described herein or otherwise obtained and purified as necessary or desired.

One or more suitable unit dosage forms including the complex can be administered by a variety of routes including topical, oral, parenteral (including subcutaneous, intravenous, intramuscular and intraperitoneal), rectal, dermal, transdermal, intrathoracic, intrapulmonary and intranasal (respiratory) routes.

The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods known to the pharmaceutical arts. Such methods include the step of mixing the complex with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system. By “pharmaceutically acceptable” it is meant a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious or unsuitably harmful to the recipient thereof. The therapeutic compounds may also be formulated for sustained release, for example, using microencapsulation (see WO 94/07529, and U.S. Pat. No. 4,962,091).

The complex may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampoules, pre-filled syringes, and small volume infusion containers, or in multi-dose containers. Preservatives can be added to help maintain the shelf life of the dosage form. The complex and other ingredients may form suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the complex and other ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

These formulations can contain pharmaceutically acceptable carriers and vehicles that are available in the art. It is possible, for example, to prepare solutions using one or more organic solvent(s) that is/are acceptable from the physiological standpoint, chosen, in addition to water, from solvents such as acetone, ethanol, isopropyl alcohol, glycol ethers such as the products sold under the name “Dowanol,” polyglycols and polyethylene glycols, C₁-C₄ alkyl esters of short-chain acids, ethyl or isopropyl lactate, fatty acid triglycerides such as the products marketed under the name “Miglyol,” isopropyl myristate, animal, mineral and vegetable oils and polysiloxanes.

It is possible to add other ingredients such as antioxidants, surfactants, preservatives, film-forming, keratolytic or comedolytic agents, perfumes, flavorings and colorings. Antioxidants such as t-butylhydroquinone, butylated hydroxyanisole, butylated hydroxytoluene and α -tocopherol and its derivatives can be added.

The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are available in the art. Examples of such substances include normal saline solutions such as physiologically buffered saline solutions and water. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions at a pH of about 7.0-8.0.

The complex can also be administered via the respiratory tract. Thus, the present invention also provides aerosol pharmaceutical formulations and dosage forms for use in the methods of the invention. In general, such dosage forms include an amount of complex effective to treat or prevent the clinical symptoms of a specific condition. Any attenuation, for example a statistically significant attenuation, of one or more symptoms of a condition that has been treated pursuant to the methods of the present invention is considered to be a treatment of such condition and is within the scope of the invention.

For administration by inhalation, the composition may take the form of a dry powder, for example, a powder mix of the complex and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form in, for example, capsules or cartridges, or, e.g., gelatin or blister packs from which the powder may be administered with the aid of an inhalator, insufflator, or a metered-dose inhaler (see, for example, the pressurized metered dose inhaler (MDI) and the dry powder inhaler disclosed in Newman (1984).

Intranasal formulations may include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

The complex may also be administered in an aqueous solution, for example, when administered in an aerosol or inhaled form. Thus, other aerosol pharmaceutical formulations may include, for example, a physiologically acceptable buffered saline solution. Dry aerosol in the form of finely divided solid compound that is not dissolved or suspended in a liquid is also useful in the practice of the present invention.

For administration to the respiratory tract, for example, the upper (nasal) or lower respiratory tract, by inhalation, the complex can be conveniently delivered from a nebulizer or a pressurized pack or other convenient means of delivering an aerosol spray. Pressurized packs may include a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Nebulizers include, but are not limited to, those described in U.S. Pat. Nos. 4,624,251; 3,703,173; 3,561,444; and 4,635,627. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, Mass.),

Schering Corp. (Kenilworth, N.J.) and American Pharmoseal Co., (Valencia, Calif.). For intra-nasal administration, the therapeutic agent may also be administered via nose drops, a liquid spray, such as via a plastic bottle atomizer or metered-dose inhaler. Typical of atomizers are the Mistometer (Winthrop) and the Medihaler (Riker). The complex may also be delivered via an ultrasonic delivery system. In some embodiments of the invention, the complex may be delivered via an endotracheal tube. In some embodiments of the invention, the complex may be delivered via a face mask.

Furthermore, the complex may also be used in combination with other therapeutic agents, for example, pain relievers, anti-inflammatory agents, antihistamines, and the like, whether for the conditions described or some other condition.

The present invention further pertains to a packaged pharmaceutical composition such as a kit or other container. The kit or container holds a therapeutically effective amount of a pharmaceutical composition of the complex and instructions for using the pharmaceutical composition for treating a condition.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1

Isolation of an Endotoxin:MD-2 Complex

Reported herein is the purification of a stable, monomeric, bioactive endotoxin-MD-2 complex generated by treatment of endotoxin:sCD14 with recombinant MD-2. Efficient generation of this complex occurred with pM amounts of endotoxin and ng/ml amounts of MD-2 and, under these conditions, required the presentation of endotoxin to MD-2 as a monomeric endotoxin:CD14 complex. Higher concentrations of the monomeric endotoxin:MD-2 complex can be generated by co-incubation of higher concentrations of soluble MD-2 and monomeric endotoxin:CD14 complex. TLR4-dependent delivery of endotoxin to human embryonic kidney (HEK) cells and cell activation at pM concentrations of endotoxin occurred with purified endotoxin:MD-2 complex, but not purified endotoxin aggregates LBP and/or sCD14. The presence of excess MD-2 inhibited delivery of endotoxin:MD-2 to HEK/TLR4 cells and cell activation. These findings demonstrate that TLR4-dependent activation of host cells by pM concentrations of endotoxin occurs by sequential interaction and transfer of endotoxin to LBP, CD14 and MD-2 and simultaneous engagement of endotoxin and TLR4 by MD-2.

Recombinant MD-2 Secreted by Infected Insect Cells

To further define the mechanism by which 14 C-LOS:sCD14 promotes cell activation and the role of MD-2, the inventors generated conditioned insect cell culture medium containing soluble, polyhistidine-tagged recombinant wild-type (wt) or C95Y mutant MD-2 according to the method of Viriyakosol et al. (2001). A human embryonic kidney cell line (HEK293) that stably expresses TLR4 (HEK/TLR4), but lacks both CD14 and MD-2 (Yang et al., 2000), was used to evaluate the effect of MD-2 on the ability of lipooligosaccharide (LOS) to interact with TLR4 and promote activation.

Conditioned medium from insect cells inoculated with baculovirus encoding either wild-type (wt) or mutant C95Y MD-2, but not conditioned control medium, contained a polyhistidine-tagged protein that migrated with a size appropriate to that reported for MD-2 ($M_r \sim 20,000$; (Shimazu et al., 1999)). In the absence of added conditioned medium, HEK/TLR4 cells were not activated by 14 C-LOS aggregates with or

without LBP and sCD14 or by the isolated ^{14}C -LOS:sCD14 complex (FIG. 1A). However, addition of dialyzed conditioned medium from cells expressing wt MD-2 with ^{14}C -LOS_{agg}+LBP and sCD14 or with purified ^{14}C -LOS:sCD14 alone resulted in robust activation of HEK/TLR4 (FIG. 1A). Little or no activation of these cells occurred when wt MD-2 was added with LOS_{agg} with or without LBP, but without sCD14. Parental HEK cells (TLR4⁻) were not activated by endotoxin under any of the conditions tested. Thus, activation of HEK293 cells by LOS required the concerted action of LBP, sCD14 (to produce LOS:sCD14), MD-2, and TLR4. The effects of the conditioned medium containing wt MD-2 were not seen with control conditioned medium or medium containing C95Y MD-2 (FIG. 1A) even when added at 100-fold greater amounts (FIG. 1B). Maximum cell activation was produced with as little as 30 ng wt MD-2 per ml added.

Formation and Function of a Novel Endotoxin:MD-2 Complex

There is a close correlation between the bioactivity of endotoxin and changes in the physical state of endotoxin induced by reversible protein associations. Because incubation of wt MD-2 with ^{14}C -LOS:sCD14 is necessary for activation of HEK/TLR4 cells (FIGS. 1A and 1B), the result of incubation of wt MD-2 with ^{14}C -LOS:sCD14 at concentrations of wt MD-2 and LOS comparable to that used in the bioassays (FIG. 1) was examined by gel filtration. Treatment of ^{14}C -LOS:sCD14 with wt MD-2 efficiently generated a new ^{14}C -LOS-containing complex that eluted as $M_r \sim 25,000$ on Sephacryl S100 (FIG. 2A). In contrast, treatment of ^{14}C -LOS:sCD14 with the non-functional C95Y MD-2 produced no change in the chromatographic behavior of ^{14}C -LOS:sCD14 (FIG. 2A). Re-chromatography of the peak fraction (s) from preparative generation of this new ^{14}C -LOS-containing complex (FIG. 2B) yielded a single, symmetrical peak (recovery >90% with or without albumin, FIG. 2B). This ^{14}C -LOS-containing complex is fully resolved from albumin and any residual ^{14}C -LOS:sCD14 or sCD14 released from LOS:sCD14 during formation of the complex judged by gel filtration chromatography and immunoassay for CD14 and LOS:sCD14. The isolated $M_r \sim 25,000$ complex activated HEK/TLR4 cells in a potent dose, and TLR4-dependent manner (FIG. 2C); half-maximal activation occurred at approximately 150 pg of ^{14}C -LOS per ml (30 pM). Cell activation did not require addition of sCD14 or albumin.

The apparent size of this active complex, as judged by gel-sieving chromatography, was consistent with a monomeric complex of LOS:MD-2. To determine if the ^{14}C -LOS in this active complex was linked to MD-2, the ability of nickel charged agarose resin (HisBind) to co-capture poly-histidine-tagged MD-2 and ^{14}C -LOS was examined. Both MD-2 and ^{14}C -LOS adsorbed to the HisBind resin and were eluted with 200 mM imidazole (FIG. 2D). The low adsorption of ^{14}C -LOS:sCD14 confirmed that the binding to the HisBind resin of ^{14}C -LOS in the bioactive $M_r \sim 25,000$ complex was specific and reflected its association with MD-2. Thus, treatment of ^{14}C -LOS:sCD14 with soluble MD-2 generated an apparently monomeric ^{14}C -LOS:MD-2 complex that activated HEK/TLR4 cells in a potent (pg/ml) dose-dependent and TLR4-dependent manner that was independent of CD14. Also generated was a ^3H -LPS:MD-2 complex from ^3H -LPS purified from *E. coli* LCD25 (Munford et al., 1992) with chromatographic and functional properties virtually identical to ^{14}C -LOS:MD-2.

Efficient Formation of Bioactive Endotoxin:MD-2 Complex Requires Monomeric Endotoxin-CD14 Complex

The demonstration that ^{14}C -LOS:sCD14 could activate HEK/TLR4 cells by first transferring ^{14}C -LOS:sCD14 to

MD-2 indicated that it was this step that was facilitated by presentation of endotoxin as a monomeric complex with CD14. Various presentations of ^{14}C -LOS:sCD14 (i.e., ^{14}C -LOS_{agg} with or without LBP, with or without sCD14) were compared for their ability to react with MD-2 to form the LOS:MD-2 complex (assessed by gel filtration chromatography) and subsequently activate HEK/TLR4 cells. Only ^{14}C -LOS:sCD14 (either purified or generated during incubation of ^{14}C -LOS_{agg} with LBP and sCD14) was able to react with MD-2 to produce ^{14}C -LOS:MD-2 and activate HEK/TLR4 cells (Table 1). These findings directly demonstrate the role of CD14 (i.e., endotoxin-CD14) in the delivery of endotoxin to MD-2 and demonstrate CD14 is not part of the complex that directly activates TLR4.

TABLE 1

Ability of various forms of [^{14}C]-LOS +/- proteins to form LOS-MD-2 and to activate HEK/TLR4.		
Materials	LOS-MD-2	Activation
LOS _{agg} ± LBP	---	---
LOS _{agg} + LBP, MD-2	---	---
LOS _{agg} , LBP, sCD14	---	---
LOS _{agg} , LBP, sCD14, MD-2	+++	+++
LOS-sCD14	---	---
LOS-sCD14 + MD-2	+++	+++
LOS-sCD14 + Conditioned Culture media (No MD-2)	---	---

Purified [^{14}C]-LOS_{agg} or [^{14}C]-LOS-sCD14 (3 ng LOS/ml)+/-indicated proteins (30 ng/ml LBP, 250 ng/ml sCD14, 60 μl culture medium) were incubated at 37° C. for 30 min. After this incubation, samples were analyzed by gel filtration chromatography to monitor formation of [^{14}C]-LOS-MD-2 and by incubation with HEK/TLR4 cells to measure cell activation.

Molecular Requirements for MD-2 Dependent Delivery of Endotoxin

Table 1 also indicates that endotoxin must be presented in the form of a monomeric endotoxin-MD-2 complex to activate HEK/TLR4 cells. This could reflect a unique ability of MD-2 to deliver endotoxin to TLR4. To test this hypothesis, cell association of purified LOS_{agg}, LOS-sCD14 or LOS-MD-2 complexes to parental and HEK/TLR4 cells were compared. Initial experiments with [^{14}C]-LOS did not reveal significant cell association of radiolabeled LOS under any condition. These negative results could simply reflect the limited amount of surface TLR4 available and needed to engage LOS-MD-2 for cell activation. To address this, LOS was isolated after metabolic labelling with [^3H]-acetate to achieve nearly 10-fold higher specific radioactivity (~4000 cpm/ng) and generated [^3H]-LOS_{agg} and protein: [^3H]-LOS complexes. Using the [^3H]-LOS, specific TLR4-dependent cell association of [^3H]-LOS-MD-2 only, with virtually no TLR4-independent cell association of LOS-MD-2, was readily detected (FIG. 3). In addition, no cell association of either [^3H]-LOS_{agg} or [^3H]-LOS-sCD14 to HEK cells±TLR4 was detected (FIG. 3).

These findings indicate a bifunctional role for MD-2, coupling endotoxin recognition to TLR4 activation. If simultaneous engagement of endotoxin and TLR4 by MD-2 is required for TLR4-dependent cell activation by endotoxin, the presence of a stoichiometric excess of MD-2 relative to TLR4 should inhibit cell activation by endotoxin. To test this hypothesis, the effect of adding varied amounts of conditioned insect cell culture medium containing wt, C95Y, or no MD-2 was examined. Addition of medium containing wt

MD-2, but not control medium, produced a dose-dependent inhibition of the activation of HEK/TLR4 by 4C-LOS:MD-2 (FIG. 4A). Medium containing C95Y MD-2 had an intermediate inhibitory effect consistent with the (partial) retention of TLR4 binding by this mutant MD-2 species. Inhibitory effects of added MD-2 had no direct effect on LOS:MD-2 (FIG. 4B, no change in chromatographic behavior), but blocked TLR4-dependent cell association of 3H-LOS:MD-2 (FIG. 4C). This finding is consistent with a need for simultaneous engagement of endotoxin and TLR4 by individual molecules of MD-2 for TLR4-dependent cell activation. Thus, depending on levels of expression, MD-2, line LBP, can have inhibitory as well as stimulatory effects on TLR4-dependent cell activation by endotoxin.

Materials and Methods

LBP and sCD14 were provided by Xoma (US) LLC. (Berkeley, Calif.). Both parental HEK293 and cells stably transfected with TLR4 (HEK/TLR4) were provided by Dr. Jesse Chow, Eisai Research Institute, (Andover, Mass.). Chromatography matrices and electrophoresis supplies were purchased from Amersham Biosciences (Piscataway, N.J.). Human serum albumin was obtained as an endotoxin-free, 25% stock solution (Baxter Healthcare Corp., Glendale, Calif.). [¹⁴C]-LOS or [³H]-LOS was isolated from an acetate auxotroph of *Neisseria meningitidis* Serogroup B after metabolic labeling and isolated as previously described (28). [¹⁴C or ³H]-LOS_{agg} (apparent M_r>20 million) and [¹⁴C or ³H]-LOS-CD14 (M_r~60,000) were purified as previously described (Thomas et al. 2002). [³H]-LPS from *E. coli* LCD25 was purchased from List Biologicals (Campbell, Calif.) as processed as described previously (Iovine et al., 2002).

Preparation of Recombinant MD-2

MD-2 cDNA was isolated, linearized, and inserted, using NcoI and XhoI-sensitive restriction sites, into the baculovirus transfection vector pBAC11 (Novagen) that provides a six residue polyhistidine tag at the carboxyl terminal end of MD-2 and 5' flanking signal sequence (gp64) to promote secretion of the expressed protein. DNA encoding each desired product was sequenced in both directions to confirm fidelity of the product. Production and amplification of recombinant viruses were undertaken in collaboration with the Diabetes and Endocrinology Research Center at the Veterans' Administration Medical Center, Iowa City, Iowa. Sf9 cells were transfected with linear baculovirus DNA and the pBAC11 vector using Bacfectin, according to a procedure described by Clontech. For production of recombinant protein, HiFive cells (Invitrogen) were incubated in serum-free medium and inoculated at an appropriate virus titer. Supernatants were collected and dialyzed either against HEPES-buffered (10 mM, pH 7.4) Hanks' balanced salts solution with divalent cations (HBSS⁺), pH 7.4 or 50 mM phosphate, 150 mM NaCl, pH 7.4 (PBS). To absorb the expressed polyhistidine tagged protein, nickel charged agarose resin (HisBind, Novagen, Madison, Wis.) was incubated batchwise with culture medium pre-dialyzed against PBS containing 5 mM imidazole. After extensive washing with this same buffer, adsorbed material was eluted with 200 mM imidazole. Flow-through and eluate fractions were analyzed by immunoblot as described below. The presence of [¹⁴C-LOS] was evaluated by liquid scintillation spectroscopy.

Immunoblotting

To detect polyhistidine labeled wt and C95Y MD-2, an anti-polyhistidine antibody (Tetra-His antibody, Qiagen, Valencia, Calif.) was used. Samples were electrophoresed using an Amersham Biosciences PhastGel System (10-15% gradient acrylamide gel) and transferred to nitrocellulose by

semi-dry transfer. The nitrocellulose was washed with Tris-buffered saline (TBS), pH 7.5, containing 0.05% Tween-20 and 0.2% TritonX-100 (TBSTT), blocked to reduce non-specific background with 3% BSA in TBSTT for 1 hr at 25° C., and incubated with the anti-His₄ antibody in TBSTT overnight. After washing with TBSTT, the blot was incubated with donkey anti-mouse IgG conjugated to horseradish peroxidase (BioRad) for 1 hr at 25° C. in TBS containing 3% goat serum and washed with TBSTT exhaustively. Blots were developed using the Pierce SuperSignal substrate system.

HEK Cell Activation Assay

HEK cells+/-TLR4 have been extensively characterized and were cultured as has been described (Yang et al., 2000). For cell activation assays, cells were grown to confluency in 48 well plates. Cell monolayers were washed with warm PBS 2x and incubated overnight at 37° C., 5% CO₂, and 95% humidity in HBSS⁺, 0.1% HSA with the supplements indicated in the legends to FIGS. 1-3 and Table 1. Activation of HEK cells was assessed by measuring accumulation of extracellular IL-8 by ELISA as previously described (Denning et al., 1998).

Chromatography

Columns of Sephacryl HR S200 (1.6 cmx30 cm) or S100 (1.0 cmx60 cm) were pre-equilibrated in 10 mM HEPES, HBSS+/-0.1% HSA. Aliquots containing [¹⁴C]-LOS_{agg}+/-LBP, +/-sCD14, +/-dialyzed conditioned insect cell medium or [¹⁴C]-LOS-sCD14+/-dialyzed conditioned insect cell medium or [¹⁴C]-LOS-MD-2 were incubated at 37° C., 30 min before gel filtration chromatography. Fractions (1 ml) were collected (flow rate: 0.5 ml/min) at room temperature using an Amersham Biosciences AKTA FPLC. Samples for chromatography contained from 2 ng to 200 ng [¹⁴C]-LOS_{agg}, [¹⁴C]-LOS-sCD14 or [¹⁴C]-LOS-MD-2 in 1 ml of column buffer+/-0.1% HSA. Aliquots of the collected fractions were analyzed by liquid scintillation spectroscopy using a Beckman LS liquid scintillation counter to detect [¹⁴C]-LOS. Recoveries of [¹⁴C]-LOS were >70%+/-albumin. All solutions used were pyrogen-free and sterile-filtered. After chromatography, selected fractions to be used in bioassays were pooled and passed through sterile syringe filters (0.22 μm) with greater than 90% recovery of radiolabeled material in the sterile filtrate. Fractions were stored under sterile conditions at 4° C. for more than 3 months with no detectable changes in chromatographic or functional properties. Columns were calibrated with BioRad gel filtration standards that included thyroglobulin (V₀), γ-globulin, ovalbumin, myoglobin, and vitamin B12 (V_i) and human serum albumin. Experiments utilizing [³H]-LOS and [³H]-LPS were carried out by the same procedure.

Cell Association of Various Forms of Endotoxin

HEK or HEK/TLR4 cells were grown to confluency in 6 well plates, washed twice with warm PBS, and [³H]-LOS aggregates or [³H]-LOS-protein complexes+/-indicated supplements were incubated overnight at 37° C., 5% CO₂, and 95% humidity in DMEM, 0.1% HSA with the supplements indicated in the legends to FIGS. 3 and 4. After the incubation, supernatants (extracellular media) were collected, cells were washed twice with cold PBS, and cells were lysed and solubilized using RNeasy lysis buffer (Qiagen). The amount of radioactivity associated with the cells was determined by liquid scintillation spectroscopy. Total recovery of radioactivity was >90%.

Example 2

MD-2 Expression in Human Airway Epithelia

The results presented herein demonstrate that in well-differentiated primary cultures of human airway epithelia TLR4,

but little or no MD-2, is expressed. These cells are relatively unresponsive to added endotoxin even in the presence of LBP and CD14. However, the responsiveness of these cells to endotoxin is markedly amplified by either the endogenous expression or the exogenous addition of MD-2, indicating that the constitutively low levels of MD-2 expression in these cells at "rest" is important in maintaining their hypo-responsiveness to endotoxin. Changes in MD-2 expression in the airway epithelium and/or neighboring cells can be achieved by exposure of these cells to specific bacterial and host products and can thereby regulate airway responsiveness to endotoxin.

Human Airway Epithelia are Hyporesponsive to Applied Endotoxin

Nontypeable *Haemophilus influenzae* (NTHi) is a common commensal, and sometimes a pathogen, of the respiratory tract (Lerman et al., 1979; Smith et al., 1989; Bandi et al., 2001). The experiments described herein explored whether endotoxin (LOS) from NTHi increased HBD-2 expression after application to the apical surface of human airway epithelia. As shown in FIGS. 6A and 6B, following the apical application of LOS in the presence of sCD14 and LBP, there was little or no change in the HBD-2 mRNA abundance. In contrast, IL-1 β stimulated a large increase in HBD-2 expression (>10,000-fold).

It was possible that the striking lack of responsiveness of airway epithelia to endotoxin alone reflected a polar distribution of receptors for endotoxin on the basolateral surface of airway epithelia. To address this possibility, NTHi LOS was applied to either the apical or basolateral surface of airway epithelia and HBD-2 expression quantified by real-time PCR. As shown in FIG. 6B, neither the apical or basolateral application of LOS in the presence of sCD14 and LBP caused significant induction of HBD-2 mRNA expression. In contrast, IL-1 β application to either or both sides of the epithelium again induced robust HBD-2 expression. These results indicate that human airway epithelia are relatively hypo-responsive to endotoxin.

MD-2 Expression in Human Airway Epithelia Enhances Endotoxin Responsiveness

Pattern recognition receptors from the family of Toll-like receptors (TLRs) play a central role in the recognition of bacterial products, including endotoxin from gram-negative bacteria (Medzhitov et al., 2000). TLR4 is a key receptor for recognition and signaling in response to endotoxin (Medzhitov et al., 1997) and optimal responses require presentation of the bacterial product in the presence of LBP, CD14, and MD-2 (Shimazu et al., 1999; Gioannini et al., 2003; and Abreu et al., 2001). Human airway epithelia were screened for the expression of TLR4, CD14 and MD-2 mRNAs using RT-PCR. Alveolar macrophages served as a positive control. FIG. 7 demonstrates that airway epithelia express the mRNAs for TLR4 and CD14. However, while macrophages demonstrated an MD-2 signal, no significant MD-2 transcripts were detected in airway epithelia following 35 cycles of PCR.

The apparent absence of MD-2 expression in primary cultures of human airway epithelia suggests that the failure of endotoxin to induce HBD-2 expression in these cells reflects a lack of available MD-2 and a resultant inability to form an optimal complex for TLR4-dependent signaling. To address this hypothesis, the deficiency of MD-2 in airway epithelia was circumvented by using an adenoviral vector containing the human MD-2 cDNA. To demonstrate that the Ad-MD-2 construct directed expression of functional MD-2 protein, the vector was first used to transduce parental HEK293 cells. Conditioned medium recovered from the transduced cells were then assayed for the presence of active MD-2 by mea-

suring activation of HEK293 cells \pm TLR4 by added LOS-sCD14 (the bioactive product of LBP/sCD14 treatment of LOS; (Giardina et al., 2001; Gioannini et al., 2002; and Gioannini et al., 2003)). As shown in FIG. 8, conditioned medium from Ad-MD-2 transduced cells produced a dose-dependent augmentation of IL-8 release by HEK/TLR4⁺ cells but not the parental (TLR4⁻) cells, consistent with the functional expression of MD-2 by the vector.

Transduction of the human airway epithelial cells with Ad-MD-2, but not with the control Ad-vector, markedly increased the cellular levels of MD-2 mRNA. It is important to note that the Ad-MD-2 complemented epithelia exhibited markedly enhanced endotoxin responsiveness following apical application of either purified LOS+LBP and sCD14 (FIG. 8A) or purified LOS: sCD14 (FIG. 8C).

LOS-induced increases in HBD-2 mRNA were paralleled by increased secretion of HBD-2 peptide and increased NF-KB luciferase activity in MD-2 complemented cells (FIG. 9B). In contrast, basolateral application of LOS to Ad-MD-2 complemented epithelia failed to elicit significant NF-KB signaling or induction of HBD-2 expression (n=2). These results indicate that the expression of MD-2 in airway epithelia confers endotoxin (LOS) responsiveness. Conversely, in the absence of MD-2, surface epithelia are relatively unresponsive to apical or basolateral endotoxin stimulation.

Extracellular Complementation with Recombinant MD-2 Protein Enhances Endotoxin Signaling in Human Airway Epithelia

Genetically manipulated cells such as the HEK/TLR4⁺ cells have been used to show that endogenous (co-) expression of MD-2 or addition of secreted MD-2 to TLR4⁺/MD-2⁻ cells confers increased endotoxin responsiveness. The primary cultures of human airway epithelia provide a more natural setting to test the effect of exogenous addition of recombinant MD-2 (rMD-2) on cellular responsiveness to endotoxin (i.e., LOS-sCD14). As shown in FIG. 9D, addition of conditioned insect cell culture medium containing rMD-2 increased the response of the human airway epithelial cultures to LOS-sCD14 by >100-fold. Control conditioned medium, by contrast, had no effect.

The findings presented above indicate that MD-2 expression is the principal limiting factor for responsiveness of human airway epithelia to endotoxin. The need for MD-2 could reflect its role either in TLR-4 trafficking, posttranslational modifications and surface expression, and/or in endotoxin recognition and delivery to TLR-4. To test the latter possibility more directly, the inventors examined the responsiveness of airway epithelia to purified LOS: MD-2 complex. FIG. 9 show that the apical application of 2 ng/ml (400 pM) of LOS: MD-2 produced significant activation of resting epithelia, but not of cells induced by adenoviral transduction, to express MD-2 along with TLR-4. This contrasts with effects of LOS: sCD14 that potently activated cells transduced with Ad-MD-2, but no resting cells (FIG. 8C). Because cell activation by LOS: MD-2 requires expression by cells of TLR-4 without MD-2, these findings confirm that the well-differentiated primary cultures of airway epithelia express and produce TLR-4 without MD-2.

Expression of MD-2 in Airway Epithelia is Regulated by Pro-Inflammatory Stimuli

The demonstration that expression (or addition) of MD-2 could markedly increase the responsiveness of the airway epithelia to endotoxin prompted a search for more natural conditions in which endogenous MD-2 expression might be up-regulated. The effects of specific host or bacterial products on cellular MD-2 levels were examined. As shown in FIG. 10, real-time PCR demonstrated marked increases in steady state

MD-2 mRNA levels following exposure of the apical surface of the cultured airway epithelium to heat killed NTHi, the NTHi outer membrane protein P6 or the combination of TNF- α and interferon gamma. The levels of MD-2 mRNA attained under these conditions were similar to that induced by phorbol myristate acetate but still significantly less than MD-2 mRNA levels in human alveolar macrophages. These findings demonstrate that, in response to specific stimuli, levels of MD-2 transcript can be up-regulated in human airway epithelia.

FIG. 10 depicts results indicating that MD-2 mRNA expression in human airway epithelia is inducible in response to several stimuli. Fold increase in MD-2 mRNA expression was quantified using real time PCR. FIG. 10 represents the results from three independent experiments on three different epithelial preparations. * indicates $p < 0.05$.

Materials and Methods

Reagents

Phorbol myristate acetate (PMA), human recombinant IL-1 β , TNF- α and INF- γ were obtained from Sigma (St Louis, Mo.). Soluble CD14 (sCD14) and LPS binding protein (LBP) were provided by Xoma (US) LLC (Berkeley, Calif.). Lipooligosaccharide (LOS) from non-typeable *Haemophilus influenzae* was isolated by a mini-phenol-water extraction procedure, as previously described (Inzana et al., 1997). LOS was also isolated from *Neisseria meningitidis* and used as purified aggregates (LOS agg) and as monomeric LOS-CD14 complexes as previously described (Giardina et al., 2001; and Gioannini et al., 2002). P6 from non-typeable *Haemophilus influenzae* was a generous gift from Dr. Timothy F. Murphy, SUNY, Buffalo. NTHi strain 12 (Frick et al., 2000) was a kind gift of Dr. Dwight Look.

Cell Culture

Primary cultures of human airway epithelia were prepared from trachea and bronchi by enzymatic dispersion using established methods (Karp et al., 2002). Briefly, epithelial cells were dissociated and seeded onto collagen-coated, semi-permeable membranes with a 0.4 μm pore size (Millicell-HA; surface area 0.6 cm^2 ; Millipore Corp., Bedford, Mass.). Human airway epithelial cultures were maintained in Ultrosor G (USG) media at 37° C., 5% CO₂. Millicell inserts were placed into 24-well plastic cell culture plates (Costar, Cambridge, Mass.). Twenty-four hours after seeding, the mucosal medium was removed and the cells were allowed to grow at the air-liquid interface as reported previously (Karp et al., 2002). Only unpassaged, well-differentiated cultures (>2 weeks old) were used in these studies. The presence of tight junctions was confirmed by transepithelial resistance measurements using a volt-ohm meter (World Precision Instruments, Sarasota, Fla.; resistance >500 $\Omega\text{-cm}^2$). For each experimental protocol, the interventions were performed on the indicated number of primary culture specimens from different donors. For each individual experiment, a matched, unstimulated sample from the same specimens served as the control.

Isolation of Macrophages from Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) was obtained from normal volunteers as previously reported (McCray et al., 1997). BALF was filtered through two layers of gauze and spun down at 3500 rpm at 4° C. for 5 minutes. The cell pellets were resuspended in 10 ml sterile saline and quantified using a Coulter counter.

Adenoviral Vector Constructs

A commercially available NF- κB -Luc plasmid (Clontech Laboratories Inc., Palo Alto, Calif.) was used as a template to generate a recombinant adenovirus vector (Ad-NF- κB -Luc).

The fragment containing the firefly luciferase gene driven by four tandem copies of the NF- κB consensus sequence fused to a TATA-like promoter from Herpes simplex virus thymidine kinase gene was released by KpnI and XbaI double digestion. The fragment was inserted into a promoterless adenoviral shuttle plasmid (pAd5mcsplA) and Ad-NF- κB -Luc virus was generated by homologous recombination as previously described and stored in 10 mM Tris with 20% glycerol at -80° C. (Anderson et al., 2000). The particle titer of adenoviral stock was determined by A260 reading. The functional titer of the adenoviral stock was determined by plaque titering on 293 cells and expression assays for the encoded protein.

An adenoviral vector expressing human MD-2 was generated. Total RNA was isolated from human alveolar macrophages using TRI Reagent (Molecular Research Center, Inc., Cincinnati, Ohio). RNA was reverse transcribed to generate cDNA using superscript II reverse transcriptase (Life Technologies, Rockville, Md.) following the manufacturer's protocol. PCR was employed to amplify a cDNA including the ORF of human MD-2 (Genbank AB018549). The primers consisted of forward-5'-CTTGTCGACATTTGTAAAGCTTTGGAGATATTGAA-3' (SEQ ID NO: 1) and reverse-5'-ATTGAATTCTAATTTGAATTAGGTTGGTGTAGGA-3' (SEQ ID NO:2) (Shimazu et al., 1999). The reaction was performed at 95° C. for 5 min, followed by 35 cycles at 94° C. for 1 min, 60° C. for 1 min and 72° C. for 1 min. The chain reaction was elongated at 72° C. for 10 min. The fidelity of the PCR product was confirmed by DNA sequencing. The MD-2 cDNA was digested by SalI at the 5' end and EcoRI at the 3' end and then inserted into an adenoviral shuttle plasmid (pAd5 cmcplA), containing the CMV promoter and Ad-MD2 virus was generated by homologous recombination. The titering and storage of the Ad-MD2 virus were identical to those described above for Ad-NF- κB -Luc. An adenoviral vector expressing the human TLR-4 cDNA was also used in these studies. The methods for the construction of this vector were published previously (Arbour et al., 2000).

Messenger RNA Analysis

Semiquantitative RT-PCR

RT-PCR was used to detect expression of TLR4, CD14 and MD-2 mRNA in human airway epithelia and alveolar macrophages. 1 μg of total RNA from each sample was reverse transcribed using random hexamer primers with SuperScript (GibcoBRL). First strand cDNA was amplified by PCR. The primer set for TLR4 consisted of forward-5'-TGAGCAGTCGTGCTGGTATC-3' (SEQ ID NO:3); reverse-5'-CAGGGCTTTTCTGAGTCGTC-3' (SEQ ID NO:4) and amplified a product of 166 bp. The primer set for CD14 consisted of forward-5'-CTGCAACTTCTCCGAACCTC-3' (SEQ ID NO:5) and reverse-5'-CCAGTAGCTGAGCAGGAACC-3' (SEQ ID NO:6) and produced a cDNA fragment of 215 bp. The primer set for MD-2 included forward-5'-TGTAAGCTTTGGAGATATTGAA-3' (SEQ ID NO:7) and reverse-5'-TTTGAATTAGGTTGGTGTAGGA-3' (SEQ ID NO:8) and amplified a product of 508 bp. As a control for amplification, GAPDH was amplified in each reaction using the following primers-forward-5'-GTCAGTGGTGGACCTGACC-3' (SEQ ID NO:9); reverse-5'-AGGGGTCTACATGGCAACTG-3' (SEQ ID NO: 10). Each reaction contained approximately 1.25 pM of the primers, 3 mM Mg²⁺. After an initial denaturing step (95° C. for 3 min), 35 cycles of denaturing (94° C. for 30 sec), annealing (60° C. for 30 sec), and extension (72° C. for 30 sec), followed by 5 min at 72° C. for

elongation were conducted. PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide.

Real-Time Quantitative PCR for Detecting HBD-2 and MD-2

Real-time PCR was employed to detect human β -defensin-2 and MD-2 and to quantify changes in expression. Real-time quantitative PCR was performed using a sequence detector (ABI PRISM 7700, Applied Biosystems, Foster City, Calif.) and Taqman technology (Roche Molecular Diagnostic Systems) following the manufacturer's protocols (Bustin, 2000). The primers and probes were designed using the Primer Express program (Applied Biosystems). For MD-2, the primers were forward 5'-CAACAATATCATTCTCTCTCAAGGG-3' (SEQ ID NO:11), reverse 5'-GCATTCTCTCTGGGCTCCC-3' (SEQ ID NO:12), and probe 5'-AAAATTTTCTAAGGGAAAATACAAATGTGTTGTTGAAGC-3' (SEQ ID NO: 13). For HBD-2, the forward primer was 5'-CCTGTTACCTGCCTTAA-GAGTGGA-3' (SEQ ID NO:14), the reverse primer 5'-ACCACAGGTGCCAATTTGTTA-3' (SEQ ID NO:15), and the probe was 5'-CCATATGTCATCCAGTCTTTTGCCTAGAAGG-3' (SEQ ID NO: 16). Both probes contain a fluorescent reporter (6-Carboxyfluorescein [FAM]) at the 5' end and a fluorescent quencher (6-Carboxytetramethylrhodamine [TAMRA]) at the 3' end. As an internal control for normalization, human GAPDH real-time quantitative PCR was conducted in every reaction. The primers and probes were purchased from Roche Molecular Diagnostic Systems. The PCR fragments were amplified for 40 cycles (15 sec at 95° C. and 1 min at 60° C.).

Transduction of Human Airway Epithelia with Adenoviral Expression Vectors

Primary cultures of well-differentiated human airway epithelia were prepared as described herein. In vector transduction experiments, the cells were transduced with either Ad-NF- κ B-Luc alone or with both Ad-NF- κ B-Luc and Ad-MD-2 two days before performing experimental interventions. The vectors were formulated in 5 mM EGTA and applied to the apical surface (50 MOI) of the cells for 2 hours in a 100 μ l volume as described previously to access receptors (Wang et al., 2000). The vector solution was removed, and the cells were transferred to another multiwell culture dish with fresh culture medium. On the experimental day, LOS \pm specific endotoxin-binding proteins as indicated in the individual figure legends were applied in 50 μ l to the apical or basolateral side of the cells as noted. Control groups received PBS (negative control) or IL-1, (100 ng/ml, applied apically and basolaterally as positive control). 18-24 hours later, the cells were disrupted in the 1 \times lysis buffer provided with the luciferase assay kit (Promega) to measure luciferase activity. In addition, some samples were prepared to isolate total RNA.

HEK Cell Activation Assay

HEK cells \pm TLR4 were obtained from Dr. Jesse Chow (Eisai Research Institute, Andover, Mass.) and were cultured as described (Yang et al., 2000; and Giannini et al., 2003). For cell activation assays, cells were grown to confluency in 48 well plates. Epithelia were washed with warm PBS 2 \times and incubated overnight at 37° C., 5% CO₂, and 95% humidity in Hanks' balanced salts solution, 0.1% human serum albumin with the supplements indicated in the legends to FIG. 8. Activation of HEK cells was assessed by measuring accumulation of extracellular IL-8 by ELISA as previously described (Denning et al., 1998).

Production of Recombinant MD-2 Protein

Recombinant MD-2 protein (rMD-2) was produced in baculovirus for application to airway epithelia. The human

MD-2 cDNA was first sub-cloned into pGEM-T easy vector for transformation of *E. coli* JM109 and amplification. The DNA was then isolated, linearized, and inserted into pBAC11 (using NcoI and XhoI-sensitive restriction sites) for transfection into insect cells. A vector encoding a six-histidine (6 \times His tag disclosed as SEQ ID NO: 17) ("poly-HIS") extension of the C-terminus was used. The DNA encoding MD-2 was sequenced in both directions to confirm the fidelity of the product. Sf9 cells were used for production and multiplication of virus containing pBAC11 plasmids. To maximize recombinant protein production, High Five (Invitrogen) cells were inoculated with recombinant virus in serum-free medium, incubated 24-48 hr and culture medium then collected for analysis. The presence of recombinant MD-2-(HIS)₆ (6 \times His tag disclosed as SEQ ID NO: 17) was determined by SDS-PAGE and immuno-blots of the culture medium, using an anti-His4 (4 \times His tag disclosed as SEQ ID NO: 18) mAb (Qiagen). The culture medium was dialyzed against sterile Hanks' balanced salts solution buffered with 10 mM HEPES, pH 7.4 and supplemented with 0.1% human serum albumin before use in bioassays.

Stimulation of Airway Epithelia with Pro-Inflammatory Products

Several agents were applied to human airway epithelia to investigate the regulation of MD-2 expression. PMA, TNF- α , and INF- γ were applied to the apical and basolateral surfaces at 100 ng/ml. Bacterial products including the NTHi membrane protein P6 (5 μ g/ml) and heat killed NTHi (Strain 12, ~100 bacteria per epithelial cell, Frick et al., 2000) were applied to the apical surface. Following an 18-24 hr stimulation, the cells were lysed and total RNA was extracted. Real-time PCR was conducted as described herein.

The present studies indicate that, under resting conditions, human airway epithelia are hypo-responsive to endotoxin stimulation. This observation was confirmed using LOS from two different gram-negative bacterial species and several different presentations of LOS, including LOS-sCD14 that is active at pg/ml concentrations toward highly endotoxin responsive cells such as monocytes, macrophages and endothelial cells (Giardina et al., 2001; Giannini et al., 2002; and Iovine et al., 2002). However, airway epithelial cells are not generally hypo-responsive to all stimuli as shown by their robust responses to pro-inflammatory cytokines including IL-1 β .

Example 3

Activities of Endotoxin:MD-2 Complexes

Endotoxin species with potent pro-inflammatory activity are typically hexa-acylated. That is, the lipid A region contains 6 covalently linked fatty acids, including 4 mol/mol of 3-OH fatty acids linked directly to the di-N-acetylglucosamine backbone of lipid A and 2 mol/mol non-hydroxylated fatty acids (NFA) that are linked to two of the four 3-OH fatty acids via an ester bond with the 3-OH group. The presence of the NFA is important for potent bioactivity of endotoxin. Either enzymatic release of the 2 NFA or disruption of the genes (*htrB*, *msbB*) encoding the two acyltransferases needed for their biosynthetic incorporation yields a tetra-acylated endotoxin derivative that lacks pro-inflammatory agonist activity and, instead, antagonizes wild-type endotoxin activity. Removal of just one of the two NFA (i.e. *msbB* mutant; penta-acylated endotoxin species) also typically exhibits markedly reduced agonist and partial antagonist activity toward human endotoxin-responsive cells.

Under-acylated endotoxin reacts normally with LBP, CD14 and MD-2 to produce an endotoxin-MD-2 complex that engages TLR4 without producing the changes in TLR4 needed for receptor and cell activation. The presence of an excess of under-acylated endotoxin-MD-2 complex (e.g., tetra- or penta-acylated endotoxin) competitively inhibits the activity of a wt endotoxin-MD-2 complex.

An msbB mutant of *Neisseria meningitidis* serogroup B (NMB) was used to examine the effects of changes in acylation on TLR4 engagement and activation (see WO 97/19688). The parent strain was used for isolation of wild-type (wt; hexa-acylated) endotoxin (lipo-oligosaccharide; LOS). As described hereinabove in the previous Examples, metabolic labeling of the bacteria with ^3H - or ^{14}C -acetate was used to radiolabel the fatty acids of LOS during bacterial growth and de novo synthesis. Using the same methods described hereinabove in the previous Examples, production of predominantly hexa-acylated LOS by the wt strain (mol ratios of C12:0, 3-OH-12:0 and 3-OH-14:0=1.0/1.0/1.0) and of predominantly penta-acylated LOS by the msbB mutant strain (mol ratios=0.5/1.0/0.9) and reaction of each LOS species with LBP, sCD14 and MD-2 to form an endotoxin-MD-2 complex (see FIGS. 11 and 12 for formation and isolation of msbB LOS-sCD14 and msbB LOS-MD-2 complexes) was verified. The bioactivity of the wt and mutant endotoxin-MD-2 complexes was tested against HEK/TLR4 cells.

As shown in FIG. 14, the activities of the endotoxin:MD-2 complexes differ markedly between the complex containing wt (hexa-acylated) LOS and that containing the mutant msbB (penta-acylated) LOS, with the complex containing the mutant LOS causing less cell activation. Moreover, addition of increasing amounts of the mutant msbB LOS:MD-2 complex significantly reduced cell activation by the wt LOS:MD-2 complex. Limited cell activation seen at higher doses of the msbB LOS:MD-2 complex closely resembles levels of cell activation produced by addition of these amounts of the msbB LOS:MD-2 complex alone (compare FIGS. 14 and 15) indicating that the mutant endotoxin:MD-2 complexes can efficiently compete with the wt endotoxin:MD-2 complex for interaction with cellular TLR4, and thus blunt endotoxin-induced cell activation.

Example 4

Characterization of MD-2 Mutants

The present inventors identified and characterized an MD-2 mutant that forms a monomeric complex with endotoxin (E) that acts as a TLR4 antagonist. Potent TLR4-dependent cell activation by E depends on transfer of E from LBP to CD14 to MD-2. MD-2 has the dual function of binding to the extracellular domain of TLR4 and to E. By site-directed mutagenesis, structural requirements of MD-2 for TLR4 binding and cellular E responsiveness have been determined but not, directly, those for E binding. Using ^3H -E:sCD14 and gel filtration chromatography, the inventors have tested wild-type (wt) and several MD-2 mutants (K122A, K128A, K132A, F121A.K122A, K125A.F126A, Y131A.K132A, K128A.K130A, K55A.K58A, R68A.R69A, R90A.R91A, F121A.K122A.Y131A.K132A, D99A, N26A, N114A, Y102A.S103A, C25A, C37A, C51A, C95A, C105A, C133A, C148A) secreted by transiently transfected HEK293T cells for their ability to react with ^3H -E:sCD14 (M_r ~60,000) to form monomeric ^3H -E:MD-2 (M_r ~30,000). Seven MD-2 mutants, including K55A.K58A, R68A.R69A, K128A.K130A, K125A.F126A, K122A and C133A (but not the other six single Cys to Ala mutants) retained wt or near wt

ability to produce E:MD-2. The numbering scheme for the mutants is based on Kawasaki et al., *J. Immunol.* 170:413 (2003). The MD-2 mutant K125A.F126A also formed E:MD-2, bound TLR4, but the mutant complex induced only ~10% as much activation (IL-8) of HEK/TLR4 cells as did wt E:MD-2 (FIG. 16). In 100× molar excess, the mutant complex inhibited cell activation by wt E:MD-2, consistent with binding of E:MD-2^{K125A.F126A} to TLR4 without efficient receptor activation. MD-2^{K125A.F126A} alone (not complexed to E) had much less inhibitory effect, reflecting greater stability of E:MD-2 than free MD-2. Our findings show that a structural requirement of MD-2, distinct from E and TLR4 binding, is needed for activation of TLR4 by E:MD-2. This MD-2 mutant provides a useful tool for unraveling the mechanism of TLR4 activation by E and dampening TLR4 activation by E.

The inventors have also constructed the single site-specific mutants F126A and K125A mutants to determine whether K125 and/or F126 are functionally important. Each of these mutants reacted normally with E:sCD14 to form a monomeric E:MD-2 complex (data not shown). E:MD-2 (K125A) was at least as active as E:MD-2 (wt) in activation of HEK/TLR4 cells, as judged by induced synthesis and secretion of IL-8 (data not shown). In contrast, E:MD-2 (F126A) produced virtually no activation of HEK/TLR4 (FIG. 17A). Moreover, addition of the E:MD-2 (F126A), in molar excess, produced a dose-dependent inhibition of cell activation by the wt complex (FIG. 17B), consistent with binding of the mutant E:MD-2 complex to TLR4, but without inducing efficient activation of TLR4. The ability of a mutant of MD-2 to confer potent TLR4 antagonist properties on an endotoxin species that is normally a potent TLR4 agonist shows that it is the properties of the E:MD-2 complex, and not that of endotoxin per se, that defines TLR4 agonist and antagonist properties. The ability to convert potent TLR4 agonists to antagonists by discrete modifications of endotoxin and/or MD-2 made it possible to test the ability of nasally administered endotoxin:MD-2 complexes (Example 6 below), depending on endotoxin or MD-2 structure, to stimulate or blunt TLR4-dependent airway inflammation.

Comparison of the inventors' findings to those previously published suggests that several other single site mutations of residues neighboring F126 have similar properties to the F126A mutant. That is (as noted in FIG. 18), substitution of P127, K128, G129, H130 or Y131 with Ala in mouse MD-2 yielded an MD-2 variant that still formed a heterodimer with TLR4 on the cell surface but showed a marked defect in cellular responsiveness to endotoxin. The asterisks on the model denote other sites where mutation of wild-type residues with alanine gave a phenotype similar to that of F126A. Thus, of all the residues in MD-2 modified by site-specific alanine mutagenesis, less than a dozen mutants had a phenotype similar to that of F126, suggesting specific but not unique roles of each of these residues in MD-2.

Example 5

Preparation and Purification of Endotoxin:MD-2 Complex

The inventors used two different procedures for preparative production and purification of endotoxin:MD-2 complex. The first procedure used recombinant MD-2 expressed in insect cells, where culture conditions are somewhat more favorable for retention of secreted MD-2 in functional form before exposure to monomeric endotoxin:sCD14 complex. The second procedure used, more analytical procedures,

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MD-2 expressed from mammalian cells. In the mammalian cells, formation and recovery of monomeric endotoxin:MD-2 complex is greatly enhanced by addition of endotoxin:sCD14 complex to culture medium to react with MD-2 as it is being secreted.

In the first procedure, the harvested insect cell culture medium containing secreted soluble MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17) was incubated with preformed monomeric E:sCD14 for 30 min at 37° C. Albumin (>0.3% (wt/vol) was added to maintain the solubility of E as it is being transferred from CD14 to MD-2. This incubation can be carried out in small (<1 ml) to very large volumes (at least 1 Liter), producing in the latter case up to 1 mg monomeric E:MD-2 complex. The complex is purified from the culture medium by gel sieving chromatography and, if necessary, metal chelation (e.g., HisLink) chromatography after concentration of the culture medium containing the monomeric E:MD-2 complex by ultrafiltration.

In the second procedure, cultured mammalian cells (e.g., HEK293T cells) were transiently transfected with an expression plasmid (e.g., pEFBOS) containing cDNA encoding MD-2, the medium washed after 16-24 h and fresh serum-free medium supplemented with ³H-LOS:sCD14 (5-10 ng LOS/ml) and 0.03% albumin. The medium was harvested after 24-48 h, concentrated by ultrafiltration and ³H-LOS:MD-2 purified as described above.

The present methods of making E/MD-2 complex take advantage of the much more favorable physico-chemical properties of the complex as compared to that of either endotoxin or MD-2 alone. The expression and secretion of MD-2 in insect cells is advantageous because culturing is done at lower temperature (e.g., 27° C.) than that used for mammalian cell cultures (37° C.). Consequently, the secreted MD-2 suffers less temperature-dependent loss of activity. This problem has been bypassed by spiking monomeric endotoxin: sCD14 into the culture medium at the time of peak MD-2 secretion, taking advantage of the very fast transfer of E from sCD14 to MD-2 and the much greater stability of E:MD-2 in comparison to MD-2 alone. The inventors have recovered the E:MD-2 complex from the culture medium after 24 to 48 h at 37° C. and it still has full bioactivity. Agonist versus antagonist properties of endotoxin variants (e.g., differing in degree of acylation) are mirrored by the properties of the respective E:MD-2 complexes.

Example 6

In Vivo Testing of E:MD-2 Complex

The present inventors have also performed in vivo testing showing potent pro-inflammatory activity of nasally instilled E:MD-2. The inventors performed experiments in mice to assess the ability of nasally administered purified wt E:MD-2 complex as compared to the same E added as purified endotoxin aggregates to induce airway inflammation. As shown in FIG. 19, the monomeric E:MD-2 complex was at least ten times more potent than the E aggregates (without MD-2), as judged by induction of airway accumulation of neutrophils. This supports the observation that E:MD-2 complex has special properties not associated with E or MD-2 alone. The potency of the instilled E:human MD-2 complex was consistent with prior in vitro observations indicating that potent endotoxin-induced TLR4 activation or inactivation (antagonism) is possible with combinations of murine TLR4 with either murine or human MD-2. These findings also confirm that, as predicted by the properties of primary cultures of these cells, conducting airway epithelia express on their lumi-

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nal surface TLR4 without MD-2, rendering these cells directly responsive to instilled wt E:MD-2. This experiment also demonstrated that E:MD-2 could effectively be administered by airway instillation.

Example 7

Covalent Cross-Linking of Endotoxin to MD-2 in a Monomeric Endotoxin:MD-2 Complex

Because the predicted ectodomain of different TLRs confer responsiveness to different microbial products, it has been generally presumed that the ectodomain of TLRs are directly involved in microbial ligand recognition. If so, in the case of TLR4 activation by endotoxin, the role of MD-2, like that of LBP and CD14 before it, could be to facilitate delivery of endotoxin to TLR4 by virtue of its capacity to bind to endotoxin and to TLR4 simultaneously. However, in contrast to the properties of LBP-treated endotoxin aggregates and monomeric endotoxin:CD14 complex, the remarkable stability and water solubility of the monomeric endotoxin:MD-2 complex suggests that fatty acyl chains of endotoxin are more fully buried within predicted deep and tapered hydrophobic cavity in MD-2 (FIG. 20B). Thus, endotoxin:MD-2, rather than endotoxin transferred from MD-2, may be the ligand for TLR4 that specifies receptor activation. If so, covalent tethering of bound endotoxin to MD-2 by chemical cross-linking should yield an even more stable endotoxin:MD-2 complex that is still a potent TLR4 agonist. Molecular modeling suggests that ethanolamine residues in or near the lipid A region of endotoxin from *Neisseria meningitidis* (lipooligosaccharide, LOS) are in close proximity to several lysine residues in MD-2 (FIG. 20A). The inventors therefore tested several different compounds that have the ability to cross-link neighboring —NH₂ groups.

For example, the inventors treated monomeric complex of ¹⁴C-LOS:MD-2 with the amine reactive cross-linkers TSAT or BS³ (FIGS. 20D and 20E). SDS-PAGE analysis of LOS:MD-2 with or without cross-linker indicated that in the absence of TSAT or BS³, LOS migrated near the dye front, whereas cross-linked ¹⁴C-LOS traveled with M_r~25,000, consistent with a product in which LOS was covalently attached to MD-2 (FIG. 20C). Approximately 30-50% of ¹⁴C-LOS was cross-linked to MD-2. Remaining non-cross-linked ¹⁴C-LOS was removed by treatment of the cross-linked reaction mixture with 0.3% deoxycholate to release non-covalently bound LOS from MD-2, followed by gel filtration (in phosphate-buffered saline, pH7.4, without deoxycholate) to separate cross-linked ¹⁴C-LOS:MD-2 (cross-linked (XL)-¹⁴C-LOS:MD-2) from LOS that re-aggregates (FIGS. 21A and 21B) and free MD-2.

In contrast to the native LOS:MD-2 complex (i.e., non-chemically cross-linked LOS:MD-2), the isolated XL-¹⁴C-LOS:MD-2 was unable to activate TLR4 and, instead, acted as an antagonist, inhibiting activation of HEK293/TLR4 cells by native LOS:MD-2 (FIGS. 22A and 22B). Using a novel assay that demonstrates specific, saturable and high affinity (K_D<300 pM) binding of [³H]LOS:MD-2 (25,000 cpm/pmol) to recombinant soluble TLR4 extracellular domain (amino acids 24-634; TLR4_{ECD}) (FIGS. 23A-23F and FIGS. 24A-24B), the inventors have also shown that weakly labeled (625 cpm/pmol) XL-¹⁴C-LOS:MD-2 competed as well as native ¹⁴C-LOS:MD-2 with binding of [³H]LOS:MD-2 to TLR4_{ECD} (FIG. 24C) providing further evidence that the cross-linked complex can interact with TLR4 but not activate the receptor, consistent with its action as a TLR4 antagonist. The stability and potency of XL-¹⁴C-LOS:MD-2 suggests a

novel approach to design TLR4 antagonists. At high specific radioactivity, the cross-linked complexes TLR4_{ECD} can also be used for screening and identification of novel (natural or synthetic) TLR4 agonists or antagonists, by measuring the ability of the test compounds to compete with the XL-LOS: MD-2 for binding to TLR4_{ECD}.

Example 8

Structural Properties of E:MD-2 Determine Agonist or Antagonist Effects on TLR4

Materials LBP and sCD14 were gifts from Xoma (Berkeley, Calif.) and Amgen Corp. (Thousand Oaks, Calif.) respectively. Insect derived soluble MD-2 containing a hexapolyhistidine tag on the C-terminal end was prepared as previously described in Gioannini et al. (2004). Human serum albumin (HSA) was obtained as an E-free, 25% stock solution (Baxter Health Care, Glendale, Calif.). [³H]LOS (25,000 cpm/pmol) from an acetate auxotroph of *Neisseria meningitidis* serogroup B (NMB) was metabolically labeled and isolated as described Giardina et al. (2001). Chromatography matrices (Sephacryl™ HR S200 and S300, Ni FF Sepharose®) were purchased from GE Healthcare (Piscataway, N.J.).

Production of recombinant proteins—HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (~80% confluency in 6-well plates or T75 flask for preparative amounts of material) were transfected with 4 μg (6-well plate) or 20 μg (T75 flask) of DNA using PolyFect reagent (Qiagen®). After 12-16 h, plates were rinsed in PBS and 1 ml per well for 6-well plates or 10 ml for T-75 flask of serum-free medium (293 SFM, Invitrogen™)+0.4% HSA+/-[³H]LOS:sCD14 (1 nM; see below) were added. Media containing expressed proteins were collected 24-48 h later. Conditioned medium containing secreted TLR4_{ECD}+/-MD-2 wt or indicated mutants maintained activity to react with [³H]LOS:MD-2 or [³H]LOS:sCD14 for at least 6 months when stored at 4° C. Expression vectors containing DNA of interest for production of FLAG-TLR4_{ECD}, amino acids 24-634, (pFLAG-CMV-TLR4) and MD-2-FLAG-His (pEF-BOS) as well as MD-2 containing the indicated mutations have been previously described and characterized (Re et al. 2002) or have been generated according to that protocol.

Preparative amounts of wt and F126A MD-2 were generated from infections of High Five™ (BTI-Tn-4) insect cells with baculovirus containing the gene for human MD-2^{F126A} inserted into pBAC11. Wt human MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17) was generated in insect cells as described previously (Gioannini et al. 2004). In order to generate F126A MD-2 in insect cells, pEF-BOS-MD-2^{F126A}-FLAG-His DNA was linearized, and inserted, using NcoI and XhoI-sensitive restriction sites, into the baculovirus transfection vector pBAC11 (Novagen) that contains a 5' flanking signal sequence to promote secretion of the expressed protein. DNA encoding MD-2^{F126A} was sequenced in both directions to confirm fidelity of the product.

Preparation of [³H]LOS:protein complexes—[³H]LOS_{agg}, [³H]LOS:sCD14, and [³H]LOS:MD-2 complexes were prepared as previously described (Gioannini et al. 2005; Gioannini et al. 2004; Gioannini et al. 2002). Briefly, [³H]LOS_{agg} (M_r>20×10⁶) were obtained after hot phenol extraction of [³H]LOS, followed by ethanol precipitation of [³H]LOS_{agg} and ultracentrifugation. Monomeric [³H]LOS:CD14 complexes (M_r~60,000) were prepared by incubating [³H]LOS_{agg} for 30 min at 37° C. with sub-stoichiometric LBP

(molar ratio 100:1 LOS:LBP) and 1-1.5× molar excess of sCD14 to LOS followed by gel exclusion chromatography (Sephacryl™ S200, 1.6 cm×70 cm column) in PBS, pH 7.4, 0.03% HSA to isolate monomeric [³H]LOS:sCD14 complex. [³H]LOS:MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17) (M_r~25,000) was generated by treatment of [³H]LOS:sCD14 (10 μg preparative or 10 ng analytical) by incubation for 30 min at 37° C. with High Five™ insect cell medium containing MD-2-His₆ (6×His tag disclosed as SEQ ID NO: 17) (25 ml preparative or 0.025 ml analytical). Preparative samples were concentrated to 2 ml before application to 1.6 cm×70 cm Sephacryl™ S200 column for isolation of [³H]LOS:MD-2.

Radiochemical purity of [³H]LOS_{agg} was confirmed by Sephacryl™ S500 (Gioannini et al. 2002) and that of [³H]LOS:sCD14, and [³H]LOS:MD-2 by Sephacryl™ S200 chromatography (Gioannini et al. 2004, Gioannini et al. 2001).

Reaction of secreted MD-2, TLR4_{ECD} and MD-2/TLR4_{ECD} with [³H]LOS:protein complexes—Conditioned serum-free medium of transfected HEK293T cells was harvested after 24 h in culture and used for subsequent incubation with [³H]LOS:sCD14. Alternatively, the culture medium was “spiked” with [³H]LOS:sCD14 (1 nM) at the time of addition of the medium to the transfected cells to permit reaction of MD-2 with [³H]LOS:sCD14 upon secretion. Media harvested without [³H]LOS:sCD14 are denoted as HEK/(secreted recombinant protein)_{CM} whereas media spiked with [³H]LOS:sCD14 during cell culture are represented as (HEK/recombinant protein(s) secreted+³H]LOS:sCD14)_{CM}. Media harvested without [³H]LOS:sCD14 were incubated with 1 nM [³H]LOS:sCD14 for 30 min (or 24 h) at 37° C. and then analyzed by gel sieving chromatography. Media spiked with [³H]LOS:sCD14 during cell culture were analyzed directly after harvesting the medium by gel sieving chromatography. Harvested media could be stored at 4° C. without any detectable change in either reactivity with freshly added [³H]LOS:sCD14 or chromatographic profile of [³H]-labeled compounds in “spiked” media. Reaction products were analyzed by Sephacryl™ HR S200 or S300 (1.6×70 cm) chromatography in PBS, pH 7.4, 0.03% HSA. Fractions (1.0 or 0.5 ml) were collected at a flow rate 0.5 or 0.3 ml/min at room temperature using AKTA Purifier or Explorer 100 FPLC (GE Healthcare). Radioactivity in collected fractions was analyzed by liquid scintillation spectroscopy (Beckman LS liquid scintillation counter). Recoveries of [³H]LOS were >70% in all cases. All solutions used were pyrogen-free and sterile filtered. After chromatography, selected fractions were sterile-filtered (0.22 μm) and kept at 4° C. for 3-6 months with no detectable changes in chromatographic or functional properties.

To measure the apparent K_d of [³H]LOS:MD-2^{F126A} interactions with TLR4_{ECD}, [³H]LOS:MD-2^{F126A} was incubated with concentrated (8-10×) conditioned medium containing TLR4_{ECD} in a final volume of 0.5 or 1 ml in PBS, pH 7.4, for 30 min at 37° C. The same conditioned medium (containing secreted TLR4_{ECD}) was used with all concentrations of [³H]LOS:MD-2^{F126A} tested for Scatchard analysis. Scatchard analysis was done using GraphPad Prism 4.

Immunoblotting—To detect polyhistidine labeled wt and mutant MD-2, an anti-polyhistidine antibody (Tetra-His antibody, Qiagen®, Valencia, Calif.) was used. Samples were electrophoresed (BioRad minigel system) through a 4-15% gradient acrylamide gel (Tris/HEPES/SDS buffer) and transferred to nitrocellulose. The nitrocellulose was washed with Tris-buffered saline (TBS), pH 7.5, containing 0.05% Tween-20 and 0.2% TritonX-100 (TBSTT), blocked to reduce non-

specific background with 3% BSA in TBSTT for 1 hr at 25° C., and incubated with the anti-His₄ antibody in TBSTT overnight. After washing with TBSTT, the blot was incubated with donkey anti-mouse IgG conjugated to horseradish peroxidase (BioRad) for 1 hr at 25° C. in TBS containing 3% goat serum and washed with TBSTT exhaustively. Blots were developed using the Pierce SuperSignal substrate system.

HEK293 cell activation assay-HEK293/TLR4 cell lines have been extensively characterized and were cultured as has been previously described (Yang et al. 2000) or according to recommendations of Invivogen, Inc. For cell activation assays by LOS:sCD14, cells were grown to confluency in a 6-well plate and then transfected with vector (4 μg DNA) containing wt or mutant MD-2 as described above. After transfection, serum-free medium was added for 24 h at 37° C. in 5% CO₂, and 95% humidity. The supernatants were removed; cells were dislodged and seeded in a 96-well plate (1×10⁵ cells/well) in triplicate. The cells were then stimulated with 200 pM LOS:sCD14 for 3 h in DMEM, 0.1% HSA. The short incubation time (3 h) was used to preclude significant secretion of MD-2 that would compete with cell surface MD-2/TLR4 for reaction with LOS:sCD14. Supernatants were removed and evaluated for extracellular accumulation of IL-8 by ELISA.

For cell activation by LOS:MD-2 containing either wt or F126A MD-2, HEK293 cells +/-TLR4 were seeded in a 96-well plate in triplicate (1×10⁵ cells/well) and stimulated with increasing concentrations of LOS:MD-2 complex in DMEM, 0.1% HSA for 20 h. Activation of HEK293 cells was assessed by measuring accumulation of extracellular IL-8 by ELISA (BD Clontech, Inc., Palo Alto, Calif.).

Results

Presence of monomeric E:sCD14 during secretion of MD-2 from HEK293T cells results in high yields of bioactive E:MD-2. —The inventors have previously demonstrated that bioactive monomeric endotoxin (LOS or LPS):MD-2 can be efficiently generated by incubation of monomeric endotoxin:sCD14 with conditioned medium harvested from insect cells expressing and secreting recombinant human MD-2 (Gioannini et al. 2004). However, initial efforts to reproduce these findings with harvested conditioned medium from HEK293T cells transiently expressing human MD-2 were unsuccessful, despite secretion and extracellular accumulation of MD-2. Kennedy and coworkers (Kennedy et al. 2004 had previously shown that recombinant human MD-2 secreted into serum-free culture medium lost activity (i.e., ability to support serum/LPS-dependent activation of HEK/TLR4 cells) in a time (24 h) and temperature (37° C.)-dependent manner unless serum (LBP and sCD14) and LPS were also present in the medium. These findings are consistent with the much greater stability at 37° C. of monomeric LPS:MD-2 (as compared to MD-2 alone) that could be formed rapidly by serum (LBP and sCD14)-dependent conversion of LPS aggregates to LPS:sCD14 and reaction of LPS:sCD14 with secreted MD-2. In an attempt to produce monomeric endotoxin:MD-2 from MD-2 secreted by transiently transfected HEK293T cells, the medium was supplemented (“spiked”) with [³H] LOS:sCD14 (1 nM). This change in experimental design resulted in virtually quantitative conversion of [³H]LOS:sCD14 to monomeric [³H]LOS:MD-2 in medium from cells expressing and secreting MD-2 but not in medium of control cells. In the absence of MD-2, some of the added [³H]LOS:sCD14 aggregates during incubation overnight and elutes in the void volume (V₀). The recovered [³H]LOS:MD-2 produced dose-dependent activation of HEK/TLR4 cells but not of parental cells lacking TLR4. The potency of [³H]LOS:MD-2 derived from recombinant MD-2 produced by

HEK293T cells or by insect (High Five™) cells was essentially the same, confirming the stability of mammalian cell-derived recombinant MD-2 when complexed, in monomeric form, with endotoxin.

MD-2 mutants that bind TLR4 but do not support TLR4-dependent cell activation by endotoxin: Comparison of reactivity with [PH]LOS:sCD14. —The same experimental design was used to re-examine the functional properties of three human MD-2 double mutants (F121A.K122A, K125A.F126A, and Y131A.K132A). These mutants interact with TLR4, as judged by co-precipitation and/or FACS-based analyses, but did not support robust cellular endotoxin responsiveness. These results suggest either a defect in LPS binding (i.e., reactivity with endotoxin:CD14) and/or activation of TLR4 by bound endotoxin:MD-2. Gel filtration chromatography analysis of medium from cells “spiked” with [³H]LOS:sCD14 and producing the mutant MD-2s indicated only the K125A.F126A MD-2 reacted with [³H]LOS:sCD14 to produce monomeric 3H-LOS:MD-2. Even though MD-2K121A.K122A and MD-2^{Y131A.K132A} were secreted in amounts comparable to MD-2^{K125A.F126A}, neither medium containing MD-2^{F121A.K122A} nor MD-2^{Y131A.K132A} produced MD-2-dependent alteration of [³H]LOS:sCD14, implying no interaction of these two MD-2 mutant species with E:sCD14.

Reaction of human MD-2^{F121A.K122A} and MD-2^{Y131A.K132A} mutants with LOS:sCD14 depends on co-expression of TLR4 ectodomain. —In many cells expressing MD-2, TLR4 is also expressed resulting in formation of MD-2/TLR4 heterodimer complex that reacts with monomeric E:CD14 to trigger cell activation. The functional stability (i.e., reactivity with E:CD14) of wild-type human MD-2 in serum-free medium at 37° C. is markedly enhanced by co-expression of the predicted ectodomain of TLR4 (residues 24-634), raising the possibility that for certain mutant MD-2 species co-expression of the TLR4 ectodomain might be necessary to maintain MD-2 in a form reactive with E:sCD14. To test this possibility, the mutants of MD-2 that did not react with [³H]LOS:sCD14 (i.e., MD-2^{F121A.K122A} and MD-2^{Y131A.K132A}) and, for comparison, wild-type MD-2, were co-expressed and secreted with the TLR4 ectodomain into a culture medium “spiked” with [³H]LOS:sCD14. After 24 h, the culture medium was harvested and analyzed by gel filtration chromatography. A Sephacryl™ S200 gel filtration system that resolves [³H]LOS:sCD14 (M_r~60,000) from the two possible radiolabeled products formed, [³H]LOS:MD-2 (M_r~25,000) and ([³H]LOS:MD-2/TLR4_{ECD})₂ (M_r~190,000) was used to differentiate the products of transfer of [³H]LOS from [³H]LOS:sCD14 to MD-2 and/or MD-2/TLR4_{ECD}, respectively. TLR4_{ECD} expressed without MD-2 does not react with [³H]LOS:sCD14. Co-expression of wild-type MD-2 and TLR4_{ECD} yielded both [³H]LOS:MD-2 and ([³H]LOS:MD-2/TLR4_{ECD})₂. Co-expression of TLR4_{ECD} with either MD-2^{F121A.K122A} or MD-2^{Y131A.K132A} did not yield [³H]LOS:MD-2, as expected, but did yield ([³H]LOS:MD-2/TLR4_{ECD})₂. Thus, the association of both MD-2^{F121A.K122A} and MD-2^{Y131A.K132A} with the ectodomain of TLR4 permitted reaction of [³H]LOS:sCD14 with a pre-associated MD-2/TLR4_{ECD} complex.

The region encompassing residues 121-132 in MD-2 forms a rim surrounding the opening to a deep hydrophobic cavity in which the acyl chains of endotoxin are buried when bound to MD-2. This region in human MD-2 contains several lysines (K122, K125, K128, K130 and K132) that have been implicated in E-MD-2 interactions. To test whether the TLR4-dependence of the reactivity of MD-2^{F121A.K122A} and MD-2^{Y131A.K132A} reflected the effects of substitution of lysine 122 or 132, the single mutants MD-2^{K122A} and MD-2^{K132A}

were expressed and tested. Strikingly, both MD-2^{K122A} and MD-2^{K132A}, albeit MD-2^{K132A} < MD-2^{K122A}, retained the ability to react with [³H]LOS:sCD14 in a TLR4-independent manner as manifest by the generation of [³H]LOS:MD-2 when these mutants were expressed and secreted into culture medium containing [³H]LOS:sCD14 with or without TLR4_{ECD}.

Because LOS:sCD14 is the substrate for both MD-2 and MD-2/TLR4_{ECD}, differences in TLR4-independent reactivity of different MD-2 species could affect the yield of [³H]LOS:MD-2/TLR4_{ECD} by differentially affecting the availability of LOS:sCD14 to react with MD-2/TLR4_{ECD}. To eliminate the potentially complicating factor of formation of LOS:MD-2, co-expression of the various wt and mutant MD-2 species was repeated with TLR4_{ECD} in the absence of [³H]LOS:sCD14. The conditioned media (containing secreted MD-2 and MD-2/TLR4_{ECD}) were harvested after 24 h and then incubated for 30 min at 37° C. with freshly added [³H]LOS:sCD14. MD-2/TLR4_{ECD}, but not MD-2 alone, retained reactivity with [³H]LOS:sCD14 under these conditions and thus ([³H]LOS:MD-2/TLR4_{ECD})₂ but not [³H]LOS:MD-2 was formed. Each of the mutant MD-2 species tested reacted with [³H]LOS:sCD14 to form ([³H]LOS:MD-2/TLR4_{ECD})₂, though the yield was reduced ~50% in the double vs. the single mutants.

Expression of the same MD-2 mutants in HEK293 cells stably expressing TLR4 yielded similar amounts of surface MD-2/TLR4 as judged by FACS analysis of surface MD-2, but only cells expressing wild-type or MD-2^{K122A} or MD-2^{K132A} were activated by LOS:sCD14. Thus, despite the ability of MD-2^{F126A.K122A} and MD-2^{Y131A.K132A} to extract LOS from [³H]LOS:sCD14 when co-expressed with TLR4_{ECD}, these mutants could not apparently confer TLR4 activation after the binding of LOS to MD-2/TLR4.

Monomeric complex of LOS:MD-2^{F126A} is a potent TLR4 antagonist. —The ability of a mutant MD-2 to bind both TLR4 and endotoxin without conferring TLR4 activation suggests that a complex of endotoxin with this MD-2 mutant acts as an effective TLR4 antagonist. Unfortunately, the inability of MD-2^{F126A.K122A} and MD-2^{Y131A.K132A} to react with LOS:sCD14 unless these MD-2 mutants were pre-associated with TLR4 precluded testing this prediction with these mutants. However, the ability of MD-2^{K125A.F126A} to react efficiently in the absence of TLR4 with [³H]LOS:sCD14 and to bind TLR4 without supporting robust TLR4-dependent cell activation by endotoxin suggested that the LOS:MD-2^{K125A.F126A} mutant complex acts as a TLR4 antagonist rather than a TLR4 agonist. To test this hypothesis, the dose-dependent effects of the purified LOS:MD-2^{K125A.F126A} on HEK/TLR4 cells was examined, both alone and in the presence of wild-type LOS:MD-2. In comparison to wild-type LOS:MD-2, LOS:MD-2^{K125A.F126A} produced much more limited activation of HEK/TLR4 cells and, in molar excess, reduced cell activation triggered by wild-type LOS:MD-2 to levels closely similar to that produced by the mutant complex alone. Engagement of TLR4 by LOS:MD-2^{K125A.F126A} triggers only limited receptor activation and, in excess, inhibits activation of TLR4 by the wild-type complex.

To further define the importance of lysine 125 and/or phenylalanine 126 in MD-2 function, single site mutants of human MD-2 were produced and tested. As expected, both MD-2^{K125A} and MD-2^{F126A} reacted readily with [³H]LOS:sCD14 to form monomeric [³H]LOS:MD-2. LOS:MD-2^{K125A} was as potent as wild-type LOS:MD-2 in activation of HEK/TLR4 cells, but LOS:MD-2^{F126A} produced essentially no activation of HEK/TLR4 cells. Instead, in molar excess, LOS:MD-2^{F126A} produced virtually complete dose-depen-

dent inhibition of cell activation by wild-type LOS:MD-2. Thus, the single amino acid alteration, F126A, in human MD-2 was sufficient to convert a potent TLR4 agonist (wild type LOS:MD-2) to a potent TLR4 antagonist.

High affinity interaction of LOS MD-2^{F126A} with the ectodomain of TLR4. —The potency of LOS:MD-2^{F126A} as an antagonist of TLR4-dependent cell activation strongly suggested that this mutant complex retained ~normal binding properties toward the ectodomain of TLR4 where interactions with MD-2 and MD-2 complexed to endotoxin take place. To test this hypothesis more directly, the inventors took advantage of the very high specific radioactivity of the purified LOS:MD-2 complexes (~25,000 cpm/pmol) and gel filtration chromatography to measure specific, saturable, high affinity interactions between [³H]LOS:MD-2^{F126A} and TLR4_{ECD}. Incubation of [³H]LOS:MD-2^{F126A} with harvested culture medium containing secreted TLR4_{ECD} yielded a supra-molecular complex containing LOS, MD-2 and TLR4_{ECD} of M_r ~190,000, representing ([³H]LOS:MD-2/TLR4_{ECD})₂ that is identical to the product of the reaction of [³H]LOS:sCD14 with wild-type MD-2/TLR4_{ECD}. This reaction was specific, saturable and of high affinity with an apparent K_d of ~200 pM, similar to that between wild-type LOS:MD-2 and TLR4_{ECD}. Thus, interactions of TLR4_{ECD} with wild-type LOS:MD-2 and LOS:MD-2^{F126A} were virtually identical, as judged both by the product formed and apparent K_d, consistent with the ability of LOS:MD-2^{F126A} to act as a potent TLR4 antagonist. Discussion

The application of novel experimental approaches to address the identification of specific structural determinants in MD-2 for activation of TLR4 by bound E:MD-2 and the role of TLR4 in the transfer of endotoxin from CD14 to MD-2 has been described herein. These approaches have led to several new insights concerning the structure and function of human MD-2. First, the findings indicate that the structural requirements in human MD-2 for transfer of E from CD14 to MD-2 are more stringent when MD-2 is presented as a soluble extracellular protein in the absence of TLR4 than when pre-associated with the ectodomain of TLR4. Several mutants of MD-2 are described (i.e., F121A.K122A, Y131A.K132A, and K132A) that retain much greater reactivity with LOS:sCD14 when co-expressed with the TLR4 ectodomain. The functional relevance of the interactions of co-expressed MD-2 and TLR4_{ECD} with LOS:sCD14 was demonstrated most clearly in the K132A mutant. Despite little or no transfer of [³H]LOS from [³H]LOS:sCD14 to MD-2^{K132A}, MD-2^{K132A} co-expressed with full-length TLR4 supported potent cell activation by LOS:sCD14, presumably reflecting the observed reactivity of cell surface MD-2^{K132A}/TLR4 with LOS:sCD14.

Second, several aromatic residues situated either just inside the opening of the hydrophobic cavity in MD-2 (i.e., Phe¹²¹ and Tyr¹³¹) or extending outward from the rim of this cavity (Phe 26), are important for activation of TLR4 by bound E:MD-2. This role was demonstrated most clearly for Phe¹²⁶ but also strongly suggested for Phe¹²¹ and Tyr¹³¹, where substitution with alanine nearly ablated cell activation by endotoxin despite maintenance of MD-2 binding to TLR4 and reactivity of MD-2/TLR4_{ECD} with LOS:sCD14. The conservation of Phe¹²¹, Phe¹²⁶ and Tyr¹³¹ in almost all of the mammalian MD-2 species reported to date is consistent with a crucial role for each of these three aromatic residues in cellular responsiveness to endotoxin.

Third, simply by substituting phenylalanine 126 with alanine, the inventors could produce an E:MD-2 complex that was a potent TLR4 antagonist, despite the fact that the bound endotoxin was normally a potent activator of TLR4. Previous

studies have demonstrated contrasting agonist vs. antagonist effects of tetraacylated lipid A in mouse and human cells, respectively. These contrasting species-dependent effects are due, substantially, to discrete structural differences between murine and human MD-2, indicating that activation or blocking of activation of TLR4 can be determined not only by the structural properties of endotoxin/lipid A bound by MD-2 but also by the structure of MD-2 itself. The properties we have described for human MD-2^{F126A} support this view and demonstrate for the first time that a single amino acid substitution in MD-2 is sufficient to convert a potent TLR4 agonist (hexaacylated LOS:MD-2^{WT}) to a potent TLR4 antagonist (hexaacylated LOS:MD-2^{F126A}). This finding underscores the remarkably discrete structural variables that distinguish TLR4 agonists from TLR4 antagonists and demonstrates that what specifies TLR4 agonist properties is not the structural properties of endotoxin alone but rather that of the monomeric endotoxin:MD-2 complex.

The functional instability of secreted MD-2 in the absence of the TLR4 ectodomain adds an additional layer of complexity to interpretations of the effects of changes in MD-2 structure on MD-2 function. By spiking the cell culture medium with [³H]LOS:sCD14 (25,000 cpm/pmol), the inventors could measure transfer of [³H]LOS from [³H]LOS:sCD14 to secreted MD-2 before inactivation of MD-2 and thus assay directly the reactivity of ng amounts of wild-type and mutant MD-2 with LOS:sCD14 in the absence of TLR4. In the case of wild-type MD-2 and certain mutant MD-2 species (e.g., K122A, K125A, F126A), the ratio of the products ([³H]LOS:MD-2/([³H]LOS:MD-2/TLR4_{ECD})₂) formed roughly corresponded to the ratio of expression and secretion of MD-2 and TLR4_{ECD}—i.e., ratio of MD-2 to MD-2/TLR4_{ECD}—as determined by immunoblot, suggesting that for these secreted MD-2 species the reactivity of MD-2 with LOS:sCD14 is not significantly altered by prior engagement of MD-2 with TLR4_{ECD}. In contrast, for MD-2^{F121A,K122A}, MD-2^{Y131A,K132A} and MD-2^{K132A} interaction with TLR4_{ECD} was apparently crucial for reactivity of these MD-2 species with LOS:sCD14. MD-2, when secreted in molar excess of TLR4, can form an array of oligomers as well as persist as a monomer. It is generally believed that the functionally reactive form of MD-2, both with respect to endotoxin and TLR4 binding, is monomeric MD-2 and that the state of the resting MD-2/TLR4 heterodimer (i.e., before binding of endotoxin) may also be monomeric (Visintin et al. 2003). Thus, it is possible that, in the case of MD-2^{F121A,K122A}, MD-2^{Y131A,K132A} and MD-2^{K132A}, the functional half-life of monomeric MD-2, unless co-expressed with TLR4_{ECD}, is too short to have a chance to react with extracellular LOS:sCD14. The orientation of the side chains of Phe¹²¹ and Tyr¹³¹, as revealed in the crystal structure of human MD-2, suggest that these aromatic side chains may be needed to increase the stability of monomeric MD-2, in the absence of TLR4, by partially shielding the opening to the hydrophobic cavity.

In contrast, the normal (wild-type) reactivity of MD-2^{F121A} with endotoxin:CD14±TLR4 suggests that Phe¹²⁶ has no essential role in maintaining MD-2 in monomeric form in the absence of endotoxin and TLR4. Instead, the orientation of the side chain of Phe¹²⁶ suggests a more likely role in intermolecular protein-protein interactions, such as between MD-2 and MD-2 or MD-2 and TLR4, that may be induced when MD-2 is simultaneously engaged with activating endotoxin species and TLR4. One possibility is that occupation of the hydrophobic cavity of MD-2 by the multiple acyl chains of lipid A of endotoxin species that are normally potent TLR4 agonists induces displacement of side chains within the

hydrophobic cavity (e.g., Phe¹²¹ and Tyr¹³¹) and, secondarily, Phe¹²⁶ leading to the downstream protein-protein interactions needed for signal/transduction. The fact that LOS:MD-2 wt/TLR4_{ECD} and LOS:MD-2^{F126A}/TLR4_{ECD} are structurally indistinguishable by gel sieving and immunocapture analyses suggest more subtle conformational differences and/or a role of other (intra-membrane, cytosolic) regions of TLR4 or other cellular proteins in determining the agonist vs. antagonist action of wt and variant E:MD-2 complexes. The remarkably stable and soluble properties of monomeric LOS:MD-2^{WT} and LOS:MD-2^{F126A}, and of LOS:MD-2^{WT or F126A}/TLR4_{ECD}, in contrast to MD-2 alone, make these complexes valuable reagents for better defining the structural basis of TLR4 activation by bound E:MD-2.

While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

As used herein, “a” or “an” means at least one, unless clearly indicated otherwise.

All publications, patents and patent applications cited herein are herein incorporated by reference.

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What is claimed is:

1. A composition comprising an aqueous solution and a water-soluble complex, wherein the complex is a monomeric form of cross-linked MD-2:endotoxin consisting of one molecule of an endotoxin, one molecule of a cross-linker of 400-1000 daltons or 3-12 angstroms and one molecule of MD-2, wherein the cross-linker molecule is covalently bound to the endotoxin and wherein the cross-linker is covalently bound to the MD-2, and wherein the complex is a TLR4 antagonist.

2. The composition of claim 1, wherein the cross-linker molecule is trivalent or bivalent.

3. The composition of claim 1, wherein the cross-linker molecule is tris-succinimidyl aminotriacetate (TSAT); bis

(sulfosuccinimidyl)suberate (BS³); disuccinimidyl suberate (DSS); bis(2-[sulfosuccinimidyoxy]ethylsulfone) (Sulfo-BSOCOES); bis(2-[succinimidyoxy]ethylsulfone) (BSOCOES); ethylene glycol bis-(succinimidylsuccinate) (EGS); ethylene glycol bis-(sulfosuccinimidylsuccinate) (SULFO-EBS); or Dimethyl 3,3'-dithiobis-propionimidate (DTBP).

4. The composition of claim 1, wherein the endotoxin is a wild-type endotoxin.

5. The composition of claim 1, wherein the endotoxin is a gram-negative bacterial endotoxin.

6. The composition of claim 5, wherein the gram-negative bacterium is a *Neisseria*, *Escherichia*, *Pseudomonas*, *Haemophilus*, *Salmonella*, or *Francisella* bacterium.

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7. The composition of claim 1, wherein the endotoxin is a hepta-acylated endotoxin, a hexa-acylated endotoxin, a penta-acylated endotoxin, or a tetra-acylated endotoxin.

8. The composition of claim 1, wherein the endotoxin is an under-acylated endotoxin.

9. The composition of claim 1, wherein the complex has a molecular weight of 25,000 Daltons.

10. The composition of claim 1, wherein the MD-2 is a variant MD-2.

11. The composition of claim 10, wherein the variant MD-2 comprises a modification from wild-type MD-2, wherein the modification is an amino acid substitution of one or more of amino acid residues 25, 37, 51, 55, 58, 68, 69, 90, 91, 95, 99, 102, 103, 105, 114, 121, 122, 125, 126, 127, 128, 129, 130, 131, 132, 133, and/or 148.

12. The composition of claim 11, wherein the variant MD-2 comprises a modification from wild-type MD-2, wherein the modification is an amino acid substitution of one or two of amino acid residues 68, 69, 126, 127, 128, 129, 130, and/or 131.

13. The composition of claim 11, wherein the substitution is an alanine.

14. The composition of claim 1, wherein the aqueous solution is a pharmaceutically acceptable carrier.

15. A method of inhibiting TLR4 activation comprising contacting a cell with the composition of claim 1, wherein the cell expresses TLR4 but not MD-2.

16. The method of claim 15, wherein the cell is a mucosal epithelial cell derived from the gastrointestinal or respiratory tracts.

17. The method of claim 15, wherein the cell is an airway epithelial cell derived from the trachea or bronchi.

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18. The method of claim 15, wherein the complex inhibits TLR4-dependent activation of cells at a concentration of less than 500 nM of the complex.

19. The method of claim 18, wherein the complex inhibits TLR4-dependent activation of cells at a concentration of less than 1 nM of the complex.

20. The method of claim 19, wherein the complex inhibits TLR4-dependent activation of cells at a concentration of less than 300 pM of the complex.

21. The method of claim 15, wherein the complex binds to TLR4.

22. The complex of claim 15, wherein the complex inhibits TLR4-dependent activation of cells.

23. A method for treating a condition associated with endotoxin-mediated cell activation comprising administering the composition of claim 1.

24. The method of claim 23, wherein the condition is sepsis, trauma, liver disease, inflammatory bowel disease, cystic fibrosis, asthma, a complication in renal dialysis, an autoimmune disease, cancer chemotherapy sequelae, or an intracellular gram-negative bacterial infection.

25. A composition consisting of an aqueous solution and a water-soluble complex, wherein the complex consists of one molecule of an endotoxin, one molecule of a cross-linker of 400-1000 daltons or 3-12 angstroms and one molecule of MD-2, wherein the cross-linker molecule is covalently bound to the endotoxin and wherein the cross-linker is covalently bound to the MD-2, and wherein the complex is a TLR4 antagonist.

* * * * *