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Free Thiol Group of MD-2 as the Target for Inhibition of the Lipopolysaccharide-induced Cell Activation*

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Mateja Manček-Keber[‡1], **Helena Gradišar**[‡1], **Melania Iñigo Pestaña**[§], **Guillermo Martinez de Tejada**[§2], **and Roman Jerala**[‡¶3]

From the [‡]*Department of Biotechnology, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia, the* [¶]*Faculty of Chemistry and Chemical Technology, University of Ljubljana, 1000 Ljubljana, Slovenia, and the* [§]*Department of Microbiology and Parasitology, University of Navarra, 31080 Pamplona, Spain*

MD-2 is a part of the Toll-like 4 signaling complex with an indispensable role in activation of the lipopolysaccharide (LPS) signaling pathway and thus a suitable target for the therapeutic inhibition of TLR4 signaling. Elucidation of MD-2 structure provides a foundation for rational design of inhibitors that bind to MD-2 and inhibit LPS signaling. Since the hydrophobic binding pocket of MD-2 provides little specificity for inhibitors, we have investigated targeting the solvent-accessible cysteine residue within the hydrophobic binding pocket of MD-2. Compounds with affinity for the hydrophobic pocket that contain a thiol-reactive group, which mediates covalent bond formation with the free cysteine residue of MD-2, were tested. Fluorescent compounds 2-(4′-(iodoacetamido)anilino)naphthalene-6-sulfonic acid and *N*-pyrene maleimide formed a covalent bond with MD-2 through Cys^{133} and inhibited LPS signaling. Cell activation was also inhibited by thiol-reactive compounds JTT-705 originally targeted against cholesterol ester transfer protein and antirheumatic compound auranofin. Oral intake of JTT-705 significantly inhibited endotoxin-triggered tumor necrosis factor α production in mice. The thiol group of MD-2 also represents the target of environmental or endogenous thiol-reactive compounds that are produced in inflammation.

TLR4 is a receptor for lipopolysaccharide (LPS)[4] a major constituent of outer membrane of Gram-negative bacteria. MD-2 is the final LPS-binding protein in the recognition cascade before TLR4 transmits the signal across the cell membrane to activate the inflammatory response. MD-2 binds to the ectodomain of TLR4 and binds LPS either alone or in complex with TLR4 (1, 2). Mice deficient in MD-2 survive endotoxic shock (3). MD-2 has been indispensable in almost all investigated conditions of TLR4-triggered inflammation; therefore, it could represent the "Achilles' heel" of the inflammatory response to LPS and a target for a pharmacological intervention in endotoxemia as well as other conditions involving cell activation mediated by TLR4 (4, 5). The existence of a single free cysteine residue among the seven cysteine residues has been predicted from MD-2 mutagenesis (6, 7) and molecular modeling proposed that Cys^{133} lies in the hydrophobic pocket (8, 9). The hydrophobic binding site of MD-2 was also mapped by an apolar probe, bis-ANS, which does not contain acyl chains as most LPS antagonists yet preserves the characteristic structural motif of lipid A, consisting of a hydrophobic region and a pair of separated negatively charged groups (10). Crystal structures of MD-2 with bound eritoran or lipid IVa confirmed the location of Cys^{133} in the hydrophobic pocket in close vicinity of bound lipid A derivatives (11, 12). The free cysteine residue inside the binding pocket can thus be a target for irreversible inhibition of MD-2 activity. An inhibitory mechanism based on a covalent modification of a free cysteine residue in the active or binding site of a protein has been demonstrated for other proteins, such as in cysteine proteases, where the cysteine residue participates in the catalytic triad (13), in cholesteryl ester transfer protein (CETP) (14), IκB kinase (15), thioredoxin reductase (16), and sortase (17).

In our study, we investigated the possibility of targeting free cysteine residue of MD-2 for the inhibition of LPS signaling. We determined covalent binding into the hydrophobic pocket of MD-2 for fluorescent compounds 2-(4′-(iodoacetamido)anilino) naphthalene-6-sulfonic acid (IAANS) and *N*-pyrene maleimide. Drugs JTT-705 and auranofin, already in use for alternative indications, were also shown to bind to MD-2 and decrease LPS signaling. The identity of Cys^{133} as the residue responsible for this interaction was demonstrated by point mutagenesis. Our results confirm that the proposed mechanism of inhibition of MD-2 can have potential therapeutic value but may also have a physiological role.

MATERIALS AND METHODS

Reagents—LPS (from *Salmonella abortus equi* HL83) was prepared by a phenol extraction procedure and was kindly provided by Dr. Brandenburg (Forschungszentrum Borstel, Germany). Another type of LPS used in animal experiments, the smooth LPS of *Pseudomonas aeruginosa* PAO1 was obtained from the aqueous phase of a water-phenol extract and purified by treatment with chaotropic agents and detergents according to published procedures (18, 19). To ensure appropriate homogenization, purified LPS was subjected to three consecu-

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[2] Supported by Ministerio de Sanidad y Consumo, Spain, Grant FIS-PI050768.
[3] To whom correspondence should be addressed: Dept. of Biotechnology, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia. Tel.: 386-1-476-0335; Fax: 386-1-476-0300; E-mail: roman.jerala@ki.si.
[4] The abbreviations used are: LPS, lipopolysaccharide; CETP, cholesteryl ester transfer protein; IAANS, 2-(4′-(iodoacetamido)anilino) naphthalene-6-sulfonic acid; HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay; PMB, polymyxin B; TNF, tumor necrosis factor; WT, wild type; bis-ANS, 1,1′-bis(anilino)-4,4′bis(naphthalene)-8,8′-disulfonate.

tive cycles of heating (15 min at 56 °C) and cooling (5 min on ice) before use. The following compounds were tested: IAANS and *N*-(1-pyrene) maleimide from Molecular Probes (The Netherlands), JTT-705 from Cayman chemical (Ann Arbor, MI), and auranofin from Alexis Biochemicals (San Diego, CA). Iodoacetamide was purchased from Sigma. Chicken anti-human MD-2 polyclonal antibodies were prepared against recombinant human MD-2 by GenTel (Madison, WI), monoclonal mouse anti-human MD-2 9B4 antibodies were ordered from eBioscience (San Diego, CA), and secondary goat anti-mouse IgG antibodies conjugated with horseradish peroxidase were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Lines—Human embryonic kidney 293 and 293T (HEK293 and HEK293T) cells were cultured as an adherent monolayer at 37 °C, 5% CO_2, and normal humidity in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker, Walkersville, MD). Mouse macrophages RAW264.7 were cultured as suspension cells in RPMI 1640 (Invitrogen) supplemented with 12% (v/v) fetal bovine serum (BioWhittaker).

Site-directed Mutagenesis—A specific point mutation was introduced into plasmid pET14b human MD-2 using site-directed mutagenesis. A site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used, and the reaction was performed according to the manufacturer's instructions. The sequences of primers used to change the amino acid residue Cys^{133} to Phe were 5-AAATTTTCTAAGGGAAAATACAAATTTGTTGTTGAAGCTATTTCTGGGAG-3 and 5-CTCCCAGAAATAGCTTCAACAACAAATTTGTATTTTCCCTTAGAAAATTT-3. The complete sequence of MD-2 mutant was verified by DNA sequencing.

Preparation of Recombinant MD-2 WT and MD-2C133F—Recombinant MD-2 was produced in *Escherichia coli* as described previously (8), using solubilization of inclusion bodies followed by their purification and refolding on reversed phase column chromatography. Biological activity of MD-2 was tested on HEK293 cells transfected with TLR4.

Fluorescence Measurements—Fluorescence measurements were performed with an LS55 spectrofluorimeter (PerkinElmer Life Sciences) with a double monochromator. All measurements were done in 10 mM Tris-HCl buffer, pH 7.5, at 25 °C in a 5 × 5-mm quartz cell. A slit width of 5 nm was used for both excitation and emission. MD-2 WT or MD-2C133F (1 μM) was added to 5 μM compound solution. Binding of fluorescent IAANS and *N*-pyrene maleimide to MD-2 was measured using an excitation wavelength of 326 and 338 nm, respectively. Displacement of fluorescent probe bis-ANS was used to determine binding of nonfluorescent auranofin to MD-2. 1 μM bis-ANS and 1 μM MD-2 were mixed and incubated to reach stable fluorescence at an excitation wavelength of 380 nm. Then auranofin was added in 2 μM increments, and decrease of the initial emission fluorescence was followed.

Determination of Covalent Interaction between MD-2 and Synthetic Compound N-Pyrene Maleimide—Distribution of *N*-pyrene maleimide between the aqueous and organic phase was determined by extraction with chloroform. 1 volume of *N*-pyrene maleimide (40 μM) in 10 mM Tris-HCl buffer, pH 7.5, was extracted with 2 volumes of chloroform. The extraction was repeated twice. Afterward, the content of *N*-pyrene maleimide in aqueous and organic phase was determined by fluorescence measurement at an excitation wavelength of 338 nm. MD-2 (10 μM) was added to the aqueous solution of *N*-pyrene maleimide and incubated for 2 h at 25 °C. As a control, either MD-2 WT, which was preincubated with 50 μM of iodoacetamide to block free cysteine residues, or a mutant MD-2C133F was used in the reaction. The requirement for the native conformation and formation of a covalent bond between *N*-pyrene maleimide and MD-2 was analyzed by the addition of guanidine HCl (6 M) to the mixture of MD-2 with *N*-pyrene maleimide after previous preincubation and followed by chloroform extraction as described above. The extraction removed unbound and noncovalently bound compound. The complex was then precipitated with 5 volumes of ice-cold acetone for 1 h at −20 °C and centrifuged. Acetone was removed, and the pellet was washed with acetone. The pellet was then resolved in 6 M guanidine HCl, and the fluorescence spectra of samples were measured.

ELISA—An ELISA was used to determine binding of selected compounds to MD-2. Polyclonal chicken anti-human MD-2 antibodies in 50 mM Na_2CO_3, pH 9.6, were used to coat a 96-well microtiter plate. The plate was incubated overnight at 4 °C and then rinsed with phosphate-buffered saline. Excess binding sites were blocked with 1% bovine serum albumin (Sigma) in phosphate-buffered saline buffer. 1 μM MD-2 WT in 10 mM Tris-HCl buffer, pH 7.5, was preincubated with IAANS, *N*-pyrene maleimide, JTT-705, or auranofin in the range from 0 to 20 μM for 2 h at 25 °C. As a negative control, compounds were preincubated in buffer. 200 μl/well of preincubated solutions were added into a precoated plate and were incubated for 2 h at 25 °C. After rinsing, MD-2 bound to coated polyclonal chicken anti-MD-2 antibodies was detected by primary mouse anti-human MD-2 9B4 monoclonal antibodies, followed by secondary goat anti-mouse IgG antibodies conjugated with horseradish peroxidase. Both steps were carried out in phosphate-buffered saline for 1.5 h at 37 °C. Horseradish peroxidase was detected using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Sigma). When color was developed, the reaction was stopped with 1% SDS, and the absorbance at 420 nm was measured. The compounds were investigated also for binding to the mutant MD-2C133F by the same procedure.

Measurements of Macrophage Response to LPS and Its Inhibition—RAW264.7 cells were seeded at 2 × 10^5 cells/96-well in RPMI containing 12% fetal bovine serum and 100 μM PTIO. Individual compound was added and preincubated for 1 h. Cells were then stimulated with 100 ng/ml LPS overnight. NO was detected in supernatants using Griess reagent (Sigma). A standard curve was prepared using sodium nitrite, and absorbance measurements were converted to concentrations.

Luciferase Reporter Assay—To determine the effect of JTT-705 and auranofin on the activity of recombinant hMD-2, 2 μM hMD-2 WT or hMD-2C133F was mixed with 0, 40, 100, 200, or 300 μM concentrations of compound in 2 mM Tris-HCl buffer, pH 7.5, to allow thiolate reactivity of a cysteine residue and incubated for 1 h at room temperature. HEK293 cells were transiently transfected with hTLR4 expression vector as well as NF-κB-dependent luciferase and constitutive *Renilla* reporter plas-

mids. 6 h later, transfection media were changed, and 5 μl of MD-2·JTT-705 complex was added per well to reach 100 nM final concentration of MD-2. After 1 h of incubation, cells were stimulated with 100 ng/ml LPS. After 16 h, cells were lysed and analyzed for reporter gene activities using a dual luciferase reporter assay system on a Mithras LB940 luminometer. Data from luciferase activity were normalized using *Renilla* luciferase readings. Inhibition of the MyD88-dependent signaling pathway was determined by transfecting HEK293T cells with 3 ng of MyD88 expression vector and NF-κB-dependent luciferase reporter plasmids. 6 h later, transfection medium was changed followed by the addition of JTT-705 or auranofin and incubated for 16 h. Cells were lysed and analyzed for luciferase activity as described above.

Animal Experiment—Mouse feed pellets containing JTT-705 were made by first mixing 50 mg of the compound with 300 g of powdered mouse feed (Harlan) using an electric food blender. Then 75 ml of a 5% solution of cornstarch was added to the mixture, and the suspension was thoroughly homogenized in the food mixer, divided into pellets, and dehydrated in a glassware air dryer. Female C57BL/6J mice weighing 13–16 g were purchased from Harlan Spain (Harlan Interfauna Iberica S.A., Barcelona, Spain) and randomly distributed in experimental groups ($n = 8$). Animals provided with JTT-705-containing pellets ate their feed at the same rate (4 g/day, approximately) as animals fed regular mouse feed, and all mice had a similar weight at the end of the feeding period. 6 days after the beginning of the treatment, animals received an intraperitoneal injection containing a mixture of 50 ng of LPS and 18 mg of D-galactosamine resuspended in 200 μl of endotoxin-free saline following the method of Galanos *et al.* (20). In previous experiments, this dose of LPS-galactosamine killed 90–100% of the animals 48 h postinoculation. As a positive control of protection against endotoxic shock, mice from one of the groups fed regular mouse pellets received 150 μl of pyrogen-free saline containing 150 μg of polymyxin B (PMB) immediately after LPS challenge. To facilitate the therapeutic action of PMB, mice were gently massaged at the site of inoculation for a few seconds. Animal mortality was monitored at 6 and 12 h postinoculation and at daily intervals for 5 days. In separate experiments, serum levels of TNFα were determined in the animal groups ($n = 4$) at 60 min postinoculation coinciding with the cytokine peak production as established in previous experiments. For that purpose, mice were anesthetized, a sample of blood was obtained by cardiac puncture, and serum levels of TNFα were quantified using the Quantikine TNFα immunoassay kit (R&D Systems, Minneapolis, MI) following the manufacturer's instructions. All of the animal protocols used in this study were approved by the University of Navarra Animal Research Committee (protocol number 035/05).

Molecular Docking—The coordinates of human MD-2 (Protein Data Bank code 2E59) and models of compounds created by geometry minimization using Chemscape were used for docking calculations with AutoDock. Protein was kept rigid, whereas in the ligands 1–4 bonds were flexible, producing 100 docked structures.

RESULTS

Selection of Compounds with Potential Inhibitory Effect on MD-2—Crystal structure of MD-2 in complex with the tetraacylated antagonists lipid IVa and eritoran clearly identified the binding site for lipid A derivatives (11, 12). Most contacts between the protein and a ligand are achieved through hydrophobic interactions between hydrophobic residues of MD-2 and fatty acid chains of lipid A. Polar interactions are involved in a relatively low number of contacts. A single free cysteine residue (Cys^{133}) is conserved in all MD-2 orthologues but not in the MD-1 family, which does not bind LPS or activate LPS signaling (10). Cys^{133} lies inside the hydrophobic binding pocket and is exposed to solvent (Fig. 1*A*). Its replacement with hydrophobic residues retains the functional activity of MD-2 (6, 10). Due to the structural considerations described above, we anticipated that compounds comprising both the cysteine-reactive group and large hydrophobic moiety could increase the selectivity for targeting MD-2 and thus more effectively inhibit the LPS signaling pathway. We predicted that this type of interaction with MD-2 should prevent LPS binding. To test this hypothesis, we selected four compounds with the above mentioned requirements (Fig. 1*B*). Two of the compounds, IAANS and *N*-pyrene maleimide, were selected among the intrinsically fluorescent thiol-reactive compounds, whose binding to MD-2 could be monitored by fluorescence. Both fluorophores contain a bulky lipophilic moiety, which contributes to the binding energy and leads the thiol-reactive compound toward its target. Additionally, IAANS contains the ANS group, for which we previously showed high affinity for MD-2 (10). Two further compounds that comply with the set of desired structural motifs were selected among the drugs that are in clinical use or in clinical studies for different indications and whose literature data suggested immunosuppression of LPS stimulation (21, 22). Thioester JTT-705 has been developed as an inhibitor of CETP (14). CETP contains a free cysteine residue within a pocket where cholesteryl ester binds and therefore in many ways possesses structural properties resembling those of the MD-2 pocket. The second tested compound was auranofin, which is used as an anti-rheumatic drug. Auranofin is a gold-containing compound with sulfur-linked organic ligands. Au(I) has thiol reactivity, and proteins with free cysteine residues represent its potential targets (23).

Binding of Thiol-reactive Compounds to MD-2—IAANS and *N*-pyrene maleimide are fluorescent probes, and their fluorescence increases in nonpolar environment, such as organic solvent or upon binding to protein hydrophobic sites. Fluorescence of both IAANS (Fig. 2*A*) and *N*-pyrene maleimide (Fig. 2*B*) increased significantly by the addition of WT MD-2. Significantly lower fluorescence increase was measured by the addition of MD-2C133F. The remaining interaction is probably due to the noncovalent reversible binding of the probe to MD-2 mutant. Binding of auranofin could not be determined through direct fluorescence, since the compound does not posses intrinsic fluorescence. Therefore, we used a competition displacement assay with bis-ANS as before to determine competition with LPS (10). The gradual addition of auranofin to the bis-ANS·MD-2 complex displaced bis-ANS from MD-2, causing a

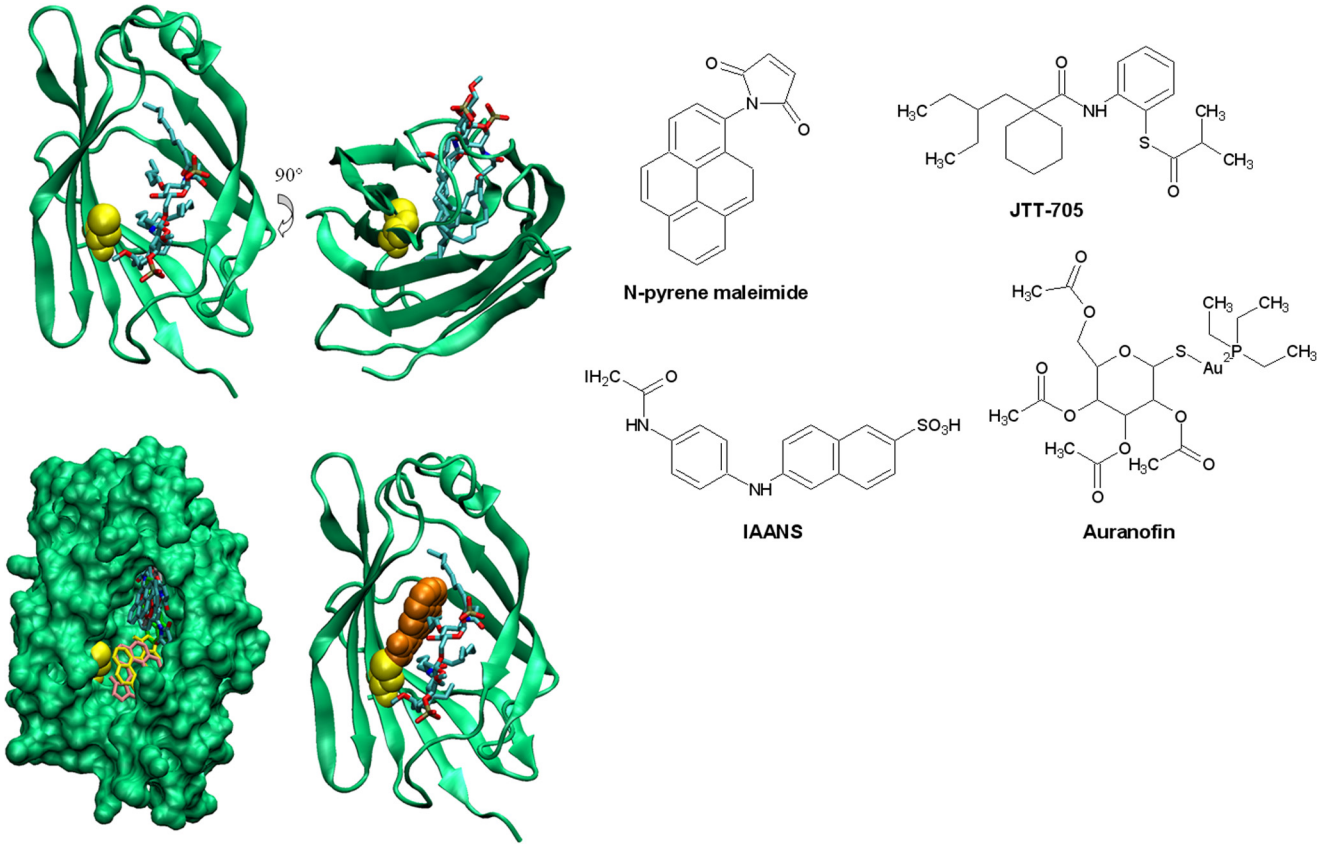

FIGURE 1. **Free cysteine residue is positioned within the binding pocket of MD-2, which can react with thiol-reactive compounds.** *Left*, MD-2 structure reveals an internal hydrophobic cavity with a cysteine residue 133 (van der Waals radii shown in *yellow*), which is located inside the cavity near the bound lipid IVa antagonist and presents a target for compounds with a thiol-reactive group. *Bottom*, surface representation of MD-2 with docked pyrene-maleimide molecules. *Right image*, MD-2 with covalently bound pyrene-maleimide, indicating steric overlap with bound lipid IVa. *Right*, tested compounds comprising a hydrophobic moiety and a thiol-reactive group. *N*-Pyrene maleimide and IAANS were used as fluorescent probes, whereas JTT-705 and auranofin are drugs already used in therapy or in clinical trials.

decrease of fluorescence (Fig. 2*C*), as expected for its binding to MD-2.

Covalent Binding of N-Pyrene Maleimide to MD-2—*N*-Pyrene maleimide contains a maleimide group that can form a thioester with free thiol-containing polypeptides. We have therefore tested our hypothesis that *N*-pyrene maleimide forms a covalent bond with MD-2 using extraction of *N*-pyrene maleimide into the organic solvent. Essentially all of the reagent transferred from the aqueous into the organic phase. The addition of WT MD-2, MD-2C133F mutant, or WT MD-2 with blocked free thiol group retained a significant amount of *N*-pyrene maleimide in the aqueous phase (Fig. 3*A*), which can be attributed to the affinity of *N*-pyrene maleimide for the binding site on MD-2. Chemical unfolding of protein should disrupt the binding site and release the noncovalently bound fluorophore, whereas the covalently bound compound, on the other hand, should remain with the protein in the aqueous phase. Fig. 3*B* shows that unfolding of WT MD-2 by the addition of 6 M guanidine HCl, subsequent to the formation of a MD-2·*N*-pyrene maleimide complex, indeed retained N-pyrene maleimide in the aqueous phase after extraction, demonstrating the formation of a covalent bond. In the case of the C133F mutant or when the thiol group of WT MD-2 was carboxymethylated, complete transfer of *N*-pyrene maleimide into the organic phase occurred from the denatured protein solution.

Binding of Thiol-reactive Compounds on MD-2 Overlaps with the LPS-binding Site and Inhibits Cell Signaling—We expected that the binding site for IAANS, *N*-pyrene maleimide, JTT-705, and auranofin on MD-2 would overlap with the binding site for LPS. To test this experimentally, we performed an ELISA type of assay where detection of MD-2·compound complex was carried out with anti-MD-2 monoclonal antibody. Since its binding to MD-2 is inhibited by LPS (24), occupation of the LPS-binding site by the compounds could also prevent recognition by antibody. We demonstrated that all tested compounds bound to MD-2 inhibited binding in a concentration-dependent manner but less efficiently than LPS (Fig. 4). Inhibition of antibody binding due to the competition with the investigated compound was maximal for IAANS (45%), followed by the *N*-pyrene maleimide (35%), auranofin (32%), and JTT-705 (30%). We may ascribe lower inhibitory capacity of compounds to their smaller size and affinity. We determined that lipid A inhibited antibody binding at 40% of the LPS value, which is probably due to the steric effects of the carbohydrates of the LPS. Based on those results, we further expected that binding of compounds to MD-2 would prevent LPS activation of macrophages. We demonstrated that IAANS, *N*-pyrene maleimide, JTT-705, and auranofin inhibit LPS stimulated NO production in mouse RAW264.7 macrophages (Fig. 5). The most efficient compounds were auranofin and *N*-pyrene maleimide with IC_{50} lower than 1 μM, whereas JTT-705 and IAANS achieved 50% inhibition

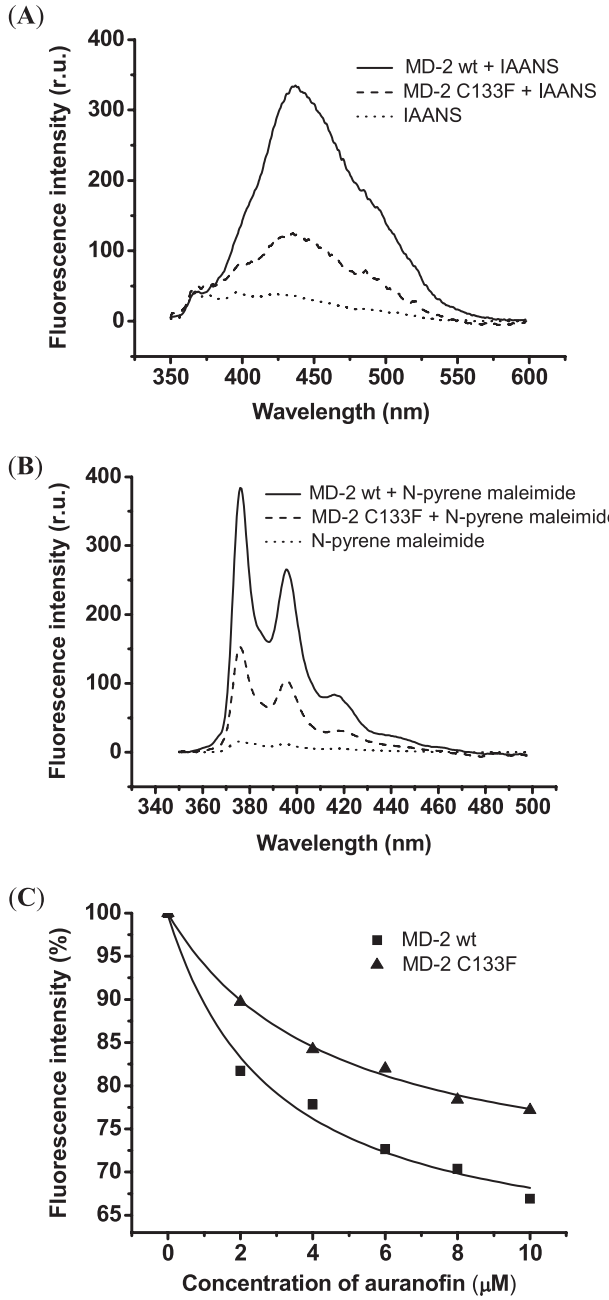

FIGURE 2. **Compounds interact with MD-2 in solution.** The addition of 1 μM MD-2 to 5 μM tested compound solution increases the fluorescence emission of hydrophobic probes IAANS (*A*) and *N*-pyrene maleimide (*B*). *C*, replacement of a fluorescent probe bis-ANS bound to MD-2 with nonfluorescent auranofin is reflected in a decrease of bis-ANS fluorescence emission. *A–C,* interaction between MD-2 and IAANS or *N*-pyrene maleimide or auranofin is impaired in mutant MD-2C133F. Fluorescence intensities are presented in relative units (*r. u.*) or a percentage (%) of maximal fluorescence intensity.

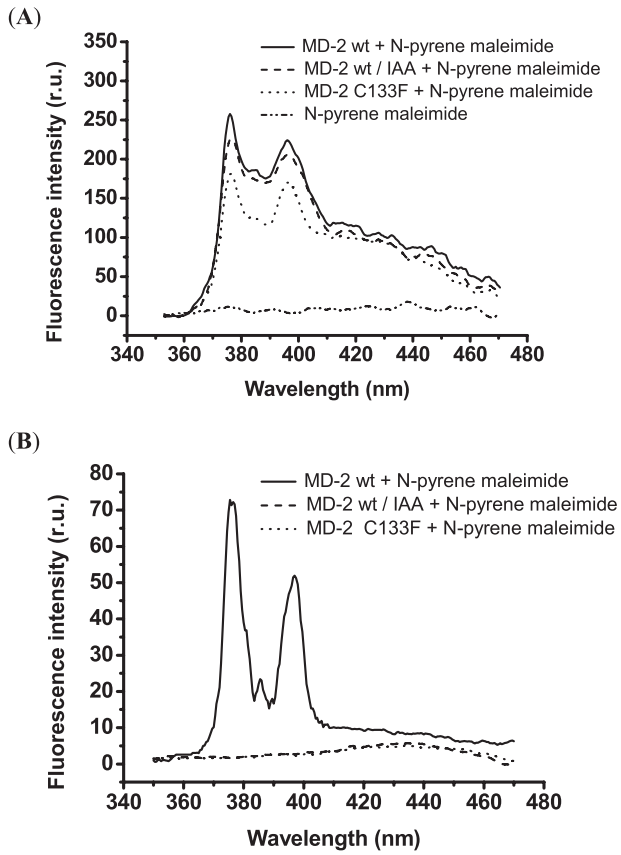

FIGURE 3. ***N*-Pyrene maleimide covalently binds to MD-2 WT.** Extraction of *N*-pyrene maleimide from the aqueous phase into the organic phase determines binding of *N*-pyrene maleimide to MD-2. *A*, *N*-pyrene maleimide was completely extracted from the buffer to chloroform. Preincubation of MD-2, either WT or WT treated with iodoacetamide or mutant C133F, with *N*-pyrene maleimide and subsequent extraction retained some *N*-pyrene maleimide in the aqueous phase as a result of tight binding. *B*, disruption of tertiary structure of MD-2 by the addition of a chemical denaturant guanidine HCl (6 M) before the extraction caused the removal of noncovalently bound *N*-pyrene maleimide from the aqueous phase. Fluorescence intensities are presented in relative units (*r. u.*).

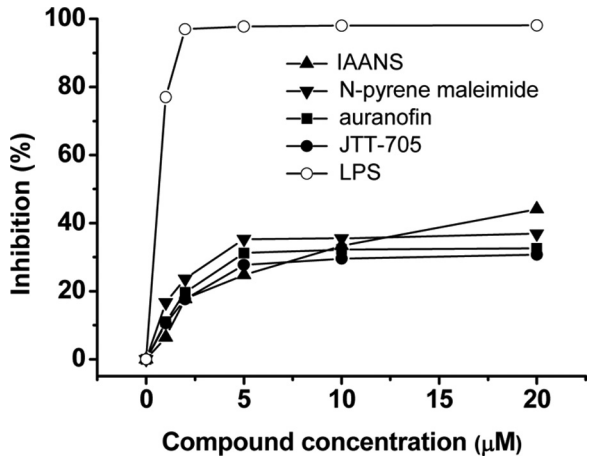

FIGURE 4. **Binding site of tested compounds on MD-2 overlaps with the LPS-binding site.** Sandwich ELISA shows competition of compound and LPS for the same binding site on MD-2. Recombinant hMD-2 WT and IAANS, *N*-pyrene maleimide, JTT-705, or auranofin was preincubated to allow the formation of complex and added to the chicken anti-MD-2 polyclonal antibody-coated plate. Detection of complex bound to polyclonal antibody was achieved by anti-MD-2 9B4 monoclonal antibody, which recognizes only LPS-free MD-2. Compound bound to MD-2 prevented 9B4 binding in a concentration-dependent manner.

above 30 μM. Since auranofin has been described as an inhibitor of TLR4 dimerization and the MyD88-dependent signaling pathway (22), inhibition of NO production could be due to the inhibition of downstream signaling. An additional experiment was performed, where MyD88 was overexpressed in HEK293T cells to investigate the potential inhibition downstream of MyD88. Auranofin and partially JTT-705 inhibited NF-κB activation induced by the overexpression of MyD88; however, 5 μM auranofin was required to significantly inhibit NF-κB activation (Fig. 6). On the other hand,

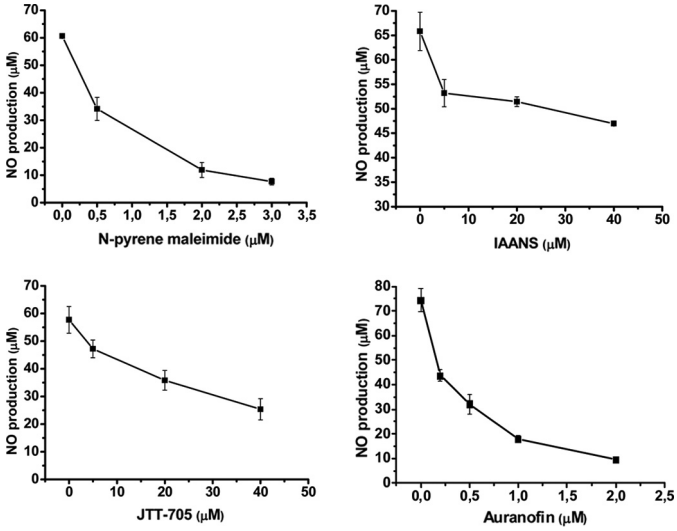

FIGURE 5. **Compounds inhibit LPS induced NO production.** RAW264.7 cells were seeded and preincubated with increasing concentrations of *N*-pyrene maleimide (*top left*), IAANS (*top right*), JTT-705 (*bottom left*), or auranofin (*bottom right*) for 1 h. After LPS stimulation for 16 h, release of NO into the medium was determined using Griess reagent, and the NO amount was calculated from the standard curve.

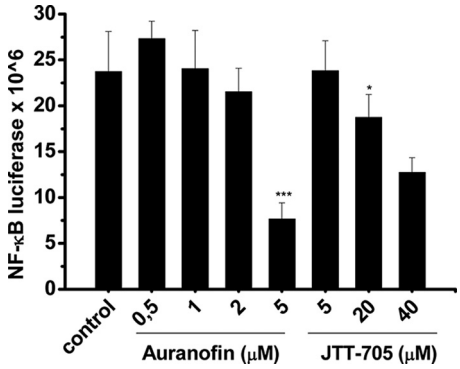

FIGURE 6. **Auranofin and JTT-705 inhibit the MyD88-dependent signaling pathway.** HEK293T cells were transfected with MyD88 and NF-κB reporter plasmids. Afterward, cells were treated with auranofin and JTT-705 for 16 h. Inhibition of MyD88 overexpression was determined by measuring relative luciferase activity in cell lysate. *, significantly different from vehicle ($p < 0.1$); ***, significantly different from vehicle ($p < 0.01$).

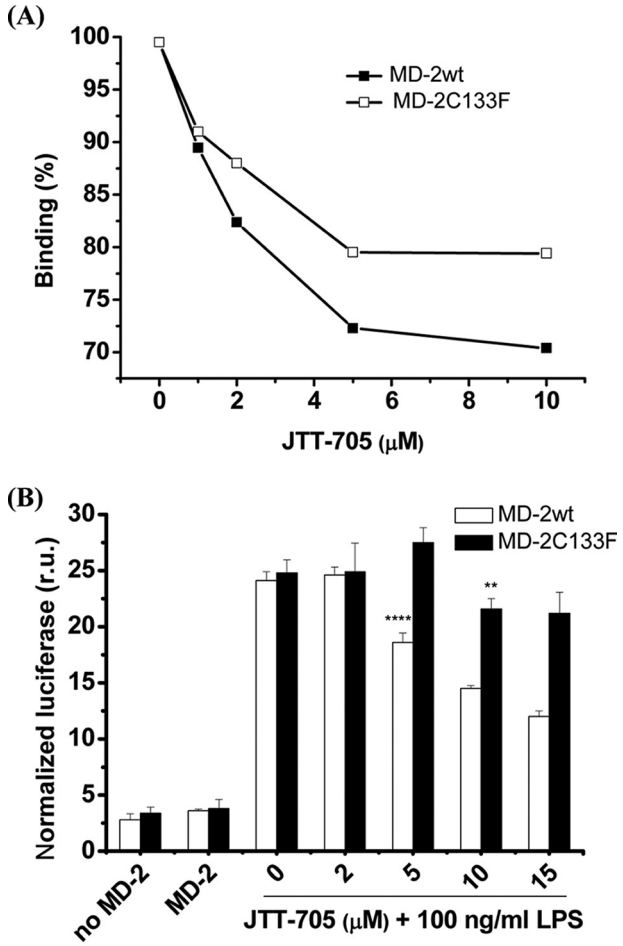

FIGURE 7. **JTT-705 inhibition of LPS signaling depends on the reactivity with free cysteine group of MD-2.** *A*, ELISA confirmed the importance of free Cys^{133}. JTT-705 was preincubated with MD-2 WT or MD-2C133F. JTT-705 bound to MD-2C133F to a lesser extent. Binding of MD-2 without a compound was defined as 100%. *B*, preincubation of recombinant human MD-2 WT with JTT-705 inhibits LPS-stimulated cell signaling, monitored by the NF-κB-responsive luciferase reporter of TLR4-transfected HEK293. The addition of preincubated MD-2C133F protein with JTT-705 to TLR4-expressing HEK293 cells had only minor influence on LPS signaling. **, significantly different from vehicle ($p < 0.05$); ****, significantly different from vehicle ($p < 0.005$).

already 2 μM auranofin completely inhibited LPS-induced NO production (Fig. 5), showing that the major inhibition target is located upstream of MyD88.

Inhibition of LPS Signaling by JTT-705 on Cell Lines and in Vivo—In order to show that JTT-705 acts through the same mechanism as *N*-pyrene maleimide via the formation of a covalent bond to Cys^{133}, we compared binding of JTT-705 to recombinant WT MD-2 and MD-2C133F mutant using the ELISA procedure as described above. JTT-705 still bound to MD-2C133F but to a significantly lower extent than to the wild type protein (Fig. 7*A*). To demonstrate that mutagenesis of Cys^{133} also affects LPS signaling in HEK293 cells, we preincubated recombinant proteins with increasing concentrations of JTT-705. MD-2·JTT-705 or MD-2C133F·JTT-705 complexes were added to HEK293 cells expressing TLR4 and stimulated with LPS. The inhibitory effect of JTT-705 on LPS signaling was only minor when MD-2C133F was applied (Fig. 7*B*). In the case of WT MD-2, cell activation was reduced by 50% at 15 μM JTT-705, which is also lower than the concentration necessary for inhibition of MyD88-induced activation. This finding confirms that the efficiency of inhibition depends on the reactivity of Cys^{133} residue with JTT-705.

For animal studies, the compound JTT-705 had to be administered to mice mixed with their feed due to its very low water solubility (see "Materials and Methods"). Animals were fed JTT-705-containing mouse pellets for 6 days, and each animal received an estimated dose of 0.6 mg of compound/day. On a body weight basis, this dose is ~2 times higher than the dose shown to be effective for inhibiting CETP activity in humans (25). After the end of the feeding period, animals were intraperitoneally inoculated with LPS from *P. aeruginosa* PAO1, and serum levels of TNFα were determined 60 min after LPS inoculation. In addition, animal mortality was monitored daily for 72 h. As the positive control of protection, a duplicate group of animals fed regular mouse pellets received an intraperitoneal injection of PMB immediately after LPS challenge. Serum levels

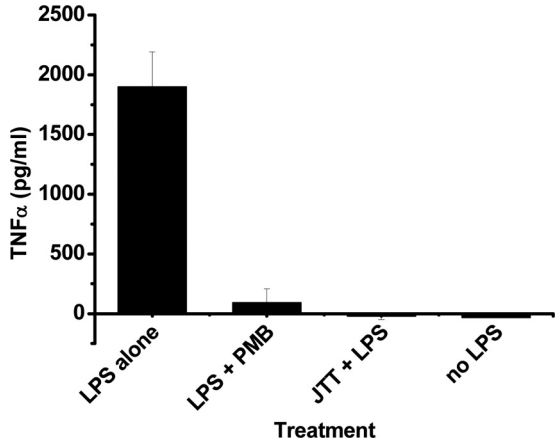

FIGURE 8. **JTT-705 inhibits TNFα production in endotoxin-treated mice.** Mice were treated intraperitoneally with 50 ng of LPS and 18 mg of galactosamine. As a positive control of protection against endotoxic shock, mice from one of the groups fed regular mouse pellets received 150 μg of PMB immediately after LPS challenge. Mice were treated with JTT705 by adding it to the food pellets 6 days before the LPS treatment. Serum levels of TNFα were determined in the animal groups ($n = 4$) at 60 min postinoculation, coinciding with the cytokine peak production as established in previous experiments.

of TNFα in animals not treated with JTT-705 were determined in an independent assay carried out under identical experimental conditions.

As shown in Fig. 8, treatment with JTT-705 completely abrogated LPS-dependent TNFα production and resulted in serum levels of TNFα indistinguishable from those measured in the PMB-treated animals. However, reduction of TNFα did not result in protection against the lethal effects of endotoxic shock, since groups treated with JTT-705 displayed a mortality at 72 h postinoculation similar (90%) to that detected in nontreated animals.

DISCUSSION

Cellular receptor for LPS TLR4 plays an important role in infection as well as in chronic diseases, such as rheumatoid arthritis (26), atherosclerosis (27, 28), and inflammation caused by tissue damage (*e.g.* by ischemia-reperfusion (29) or in hemorrhage-induced acute lung injury (30)). However, efficient inhibitors of TLR4 activation in inflammatory conditions are not yet in therapeutic use, although several of them are at the advanced stages of clinical trials (31). MD-2, as a component indispensable for LPS-induced TLR4 signaling, presents a more suitable therapeutic target for the pharmacological intervention than TLR4. MD-2 is the target of LPS antagonists, which structurally resemble lipid A, such as MPLA (32), LPS from *Rhodobacter sphaeroides* or tetraacylated lipid IVa (33), or acyclic derivatives of lipid A (AGP) (34) but also of taxoids in human MD-2 (35).

Here we demonstrated a new type of inhibitory mechanism of this important target, which is based on compounds that combine high affinity for the lipid A-binding hydrophobic pocket of MD-2 and a thiol-reactive group. We determined binding of tested compounds to MD-2, formation of covalent bond, involvement of Cys^{133} residue of MD-2, and inhibition of LPS binding and cell activation as well as proposed additional therapeutic indications of two drugs in use and in clinical trials, namely auranofin and JTT-705, respectively. Both auranofin and JTT-705 have already shown safety in clinical trials. Auranofin is used in therapy of inflammatory arthritis but less used due to the side effects. It had been known that auranofin inhibits LPS-induced interleukin-1 and TNFα production (21). Several possible targets of auranofin have been suggested previously, such as cysteine proteases of the papain family (36), IKKβ (22), and NF-κB (37), suggesting that it may target several steps in the signaling pathway, although MD-2 has not been reported to be among them. We achieved almost complete inhibition of LPS-induced NO production at 2 μM auranofin (Fig. 5). This concentration only minimally decreased the MyD88-dependent signaling pathway even after 16 h (Fig. 6), demonstrating the target upstream of MyD88 and the physiological importance of MD-2 as its target. For JTT-705 as an antihypercholesterolemic compound inhibiting CETP in humans and rabbits (14), no role in the LPS-induced cell activation has been reported before. We showed that JTT-705 significantly inhibited TNFα response to LPS in animals. It was not effective to prevent mortality under those conditions. Additional inflammatory mediators besides TNFα will have to be analyzed to clarify this discrepancy. As much as 150 μg of polymyxin B had to be administered simultaneously with LPS to the animals to prevent endotoxin-induced mortality. Polymyxin B acts further upstream and blocks interaction of endotoxin with other binding partners in the serum. Neutralization of inflammatory mediators of endotoxemia, such as TNFα or even CD14, failed in clinical trials. It has been proposed (38) that a better strategy might be to combine the inhibition of complement with neutralization of CD14 (in our case MD-2), which could attenuate the uncontrolled inflammatory reaction that leads to breakdown of homeostasis during sepsis. The inhibition of TNFα production by JTT-755 in our experiments is not likely to be due to the effect on cholesterol metabolism, since mice do not have a CETP orthologue. Additional information on the role of JTT-705 in the inhibition of TLR4-driven inflammation might become available as a "side effect" in ongoing clinical trials of JTT-705 (also known as R1658).

Further improvement of MD-2 inhibitors based on the described principles could be achieved by increasing their affinity and specificity of binding into the hydrophobic pocket (*e.g.* by the addition of negatively charged groups to interact with cationic residues at the rim of the MD-2 pocket) (10). We have demonstrated previously that curcumin inhibits LPS signaling by binding to MD-2, but in this case, it did not form a covalent bond with Cys^{133} (39). However, Youn *et al.* (40) reported that cinnamaldehyde suppresses LPS-induced homodimerization of TLR4. Although the role of MD-2 has not been investigated, it seems likely that this compound could modify the thiol group of MD-2. Recent publications indicate an additional role of TLR4 receptors in sterile inflammation caused by acute sterile injury and other disorders. TLR activation with endogenous agonists could explain the instances of systemic inflammatory response syndrome, although bacterial infection cannot be detected. Several potential endogenous ligands, especially for TLR4, have already been identified: heparan sulfate, hyaluronic acid, HMGB-1, etc. (reviewed in Refs. 41 and 42). Based on their structure, heparan sulfate and hyaluronic acid might also require MD-2 for signaling, so inhibitors that could inhibit ligand binding to MD-2 may have

broader application and increase the therapeutic options for treatment of these disorders.

Identification of the covalent mechanism of inhibition suggests that some endogenous or environmental thiol-reactive compounds could affect the LPS responsiveness by modifications of MD-2 and could thus act as an additional regulator of biological activity. Cys^{133} is no longer accessible to the solvent in the complex even with tetraacylated antagonist (Protein Data Bank code 2E59). Its thiol group is separated from the acyl groups of bound lipid IVa by less than 3 Å. All docked conformations of investigated ligands into MD-2 overlap with bound ligand, indicating that they would inhibit binding. Recently determined crystal structure of the TLR4·MD-2-RaLPS complex and a model, based on mutations of MD-2 and TLR4 demonstrated the occupation of the complete pocket of MD-2 with acyl chains of the LPS. Therefore, even a small ligand bound to its thiol group could (43, 44) interfere with lipid A binding, particularly with an agonistic chemotype, that typically contains six acyl chains. Acrolein has been reported to inhibit the NF-κB activation by endotoxin (45), and it has recently been shown that it suppresses the LPS-induced TLR4 dimerization upstream of MyD88 (46). Acrolein is an unsaturated aldehyde, produced by pyrolysis of organic material and present as an environmental toxin in fried food as well as in cigarette smoke but is also produced by myeloperoxidase in the organism at sites of inflammation (47). Acrolein is highly reactive with thiol groups and could thus also modify the MD-2 and inhibit its ability to mediate the LPS signal. Conservation of Cys^{133} in all members of the MD-2 family and variability of this residue in other ML superfamily members, including MD-1 homologues, provides further support for the function of the free cysteine residue in the binding pocket in the suppression of LPS activation.

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REFERENCES

1. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *J. Exp. Med.* **189,** 1777–1782
2. Viriyakosol, S., Tobias, P. S., Kitchens, R. L., and Kirkland, T. N. (2001) *J. Biol. Chem.* **276,** 38044–38051
3. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002) *Nat. Immunol.* **3,** 667–672
4. Saitoh, S., Akashi, S., Yamada, T., Tanimura, N., Kobayashi, M., Konno, K., Matsumoto, F., Fukase, K., Kusumoto, S., Nagai, Y., Kusumoto, Y., Kosugi, A., and Miyake, K. (2004) *Int. Immunol* **16,** 961–969
5. Visintin, A., Halmen, K. A., Latz, E., Monks, B. G., and Golenbock, D. T. (2005) *J. Immunol.* **175,** 6465–6472
6. Kawasaki, K., Nogawa, H., and Nishijima, M. (2003) *J. Immunol.* **170,** 413–420
7. Mullen, G. E., Kennedy, M. N., Visintin, A., Mazzoni, A., Leifer, C. A., Davies, D. R., and Segal, D. M. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 3919–3924
8. Gruber, A., Mancek, M., Wagner, H., Kirschning, C. J., and Jerala, R. (2004) *J. Biol. Chem.* **279,** 28475–28482
9. Gangloff, M., and Gay, N. J. (2004) *Trends Biochem. Sci.* **29,** 294–300
10. Mancek-Keber, M., and Jerala, R. (2006) *FASEB J.* **20,** 1836–1842
11. Ohto, U., Fukase, K., Miyake, K., and Satow, Y. (2007) *Science* **316,** 1632–1634
12. Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J. O. (2007) *Cell* **130,** 906–917
13. Lindvall, M. K. (2002) *Curr. Pharm. Des.* **8,** 1673–1681
14. Okamoto, H., Yonemori, F., Wakitani, K., Minowa, T., Maeda, K., and Shinkai, H. (2000) *Nature* **406,** 203–207
15. Ji, C., Kozak, K. R., and Marnett, L. J. (2001) *J. Biol. Chem.* **276,** 18223–18228
16. Fang, J., Lu, J., and Holmgren, A. (2005) *J. Biol. Chem.* **280,** 25284–25290
17. Maresso, A. W., Wu, R., Kern, J. W., Zhang, R., Janik, D., Missiakas, D. M., Duban, M. E., Joachimiak, A., and Schneewind, O. (2007) *J. Biol. Chem.* **282,** 23129–23139
18. Hirschfeld, M., Ma, Y., Weis, J. H., Vogel, S. N., and Weis, J. J. (2000) *J. Immunol.* **165,** 618–622
19. Leong, D., Diaz, R., Milner, K., Rudbach, J., and Wilson, J. B. (1970) *Infect. Immun.* **1,** 174–182
20. Galanos, C., Freudenberg, M. A., and Reutter, W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76,** 5939–5943
21. Bondeson, J., and Sundler, R. (1995) *Biochem. Pharmacol.* **50,** 1753–1759
22. Youn, H. S., Lee, J. Y., Saitoh, S. I., Miyake, K., and Hwang, D. H. (2006) *Biochem. Biophys. Res. Commun.* **350,** 866–871
23. Jeon, K. I., Byun, M. S., and Jue, D. M. (2003) *Exp. Mol. Med.* **35,** 61–66
24. Viriyakosol, S., McCray, P. B., Ashbaugh, M. E., Chu, J., Jia, H. P., Weiss, J., and Kirkland, T. N. (2006) *Hybridoma* **25,** 349–357
25. de Grooth, G. J., Kuivenhoven, J. A., Stalenhoef, A. F., de Graaf, J., Zwinderman, A. H., Posma, J. L., van Tol, A., and Kastelein, J. J. (2002) *Circulation* **105,** 2159–2165
26. Andreakos, E., Sacre, S., Foxwell, B. M., and Feldmann, M. (2005) *Front. Biosci.* **10,** 2478–2488
27. Michelsen, K. S., Wong, M. H., Shah, P. K., Zhang, W., Yano, J., Doherty, T. M., Akira, S., Rajavashisth, T. B., and Arditi, M. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101,** 10679–10684
28. Tobias, P., and Curtiss, L. K. (2005) *J. Lipid Res.* **46,** 404–411
29. Tsung, A., Hoffman, R. A., Izuishi, K., Critchlow, N. D., Nakao, A., Chan, M. H., Lotze, M. T., Geller, D. A., and Billiar, T. R. (2005) *J. Immunol.* **175,** 7661–7668
30. Barsness, K. A., Arcaroli, J., Harken, A. H., Abraham, E., Banerjee, A., Reznikov, L., and McIntyre, R. C. (2004) *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287,** R592–R599
31. Hawkins, L. D., Christ, W. J., and Rossignol, D. P. (2004) *Curr. Top. Med. Chem.* **4,** 1147–1171
32. Aybay, C., and Imir, T. (1998) *FEMS Immunol. Med. Microbiol.* **22,** 263–273
33. Feist, W., Ulmer, A. J., Wang, M. H., Musehold, J., Schlüter, C., Gerdes, J., Herzbeck, H., Brade, H., Kusumoto, S., and Diamantstein, T. (1992) *FEMS Microbiol. Immunol.* **4,** 73–89
34. Johnson, D. A. (2008) *Curr. Top. Med. Chem.* **8,** 64–79
35. Resman, N., Gradisar, H., Vasl, J., Keber, M. M., Pristovsek, P., and Jerala, R. (2008) *FEBS Lett.* **582,** 3929–3934
36. Gunatilleke, S. S., and Barrios, A. M. (2006) *J. Med. Chem.* **49,** 3933–3937
37. Yang, J. P., Merin, J. P., Nakano, T., Kato, T., Kitade, Y., and Okamoto, T. (1995) *FEBS Lett.* **361,** 89–96
38. Mollnes, T. E., Christiansen, D., Brekke, O. L., and Espevik, T. (2008) *Adv. Exp. Med. Biol.* **632,** 253–263
39. Gradisar, H., Keber, M. M., Pristovsek, P., and Jerala, R. (2007) *J. Leukocyte Biol.* **82,** 968–974
40. Youn, H. S., Lee, J. K., Choi, Y. J., Saitoh, S. I., Miyake, K., Hwang, D. H., and Lee, J. Y. (2008) *Biochem. Pharmacol.* **75,** 494–502
41. Mollen, K. P., Anand, R. J., Tsung, A., Prince, J. M., Levy, R. M., and Billiar, T. R. (2006) *Shock* **26,** 430–437
42. Rock, K. L., and Kono, H. (2008) *Annu. Rev. Pathol.* **3,** 99–126
43. Resman, N., Vasl, J., Oblak, A., Pristovsek, P., Gioannini, T. L., Weiss, J. P., and Jerala, R. (2009) *J. Biol. Chem.* **284,** 15052–15060
44. Park, B. S., Song, D. H., Kim, H. M., Choi, B. S., Lee, H., and Lee, J. O. (2009) *Nature* **458,** 1191–1195
45. Li, L., Hamilton, R. F., Jr., and Holian, A. (1999) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **277,** L550–L557
46. Lee, J. S., Lee, J. Y., Lee, M. Y., Hwang, D. H., and Youn, H. S. (2008) *Mol. Cells* **25,** 253–257
47. Anderson, M. M., Hazen, S. L., Hsu, F. F., and Heinecke, J. W. (1997) *J. Clin. Invest.* **99,** 424–432