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Activation of human TLR4/MD-2 by hypoacylated lipopolysaccharide from a clinical isolate of *Burkholderia cenocepacia*®

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*Running title: Activation of TLR4/MD-2 by *Burkholderia* LPS

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**Keywords:** Innate immunity; Lipopolysaccharide (LPS); Cystic Fibrosis; *Burkholderia*; TLR4/MD-2 complex

**Background:** The *Burkholderia cenocepacia* lipid A is hypoacylated.

**Results:** Aminoarabinose residues in lipid A contribute to *Burkholderia* lipid A binding to the TLR4/MD-2 complex.

**Conclusion:** A novel mode of *Burkholderia* lipopolysaccharide-TLR4/MD-2 interactions promotes inflammation.

**Significance:** Modifications of the lipid A structure enhance pro-inflammatory responses of hypoacylated lipopolysaccharide.
ABSTRACT

Lung infection by *Burkholderia* species, in particular *B. cenocepacia*, accelerates tissue damage and increase post-lung transplant mortality in cystic fibrosis patients. Host-microbes interplay largely depends on interactions between pathogen specific molecules and innate immune receptors such as the Toll-like receptor 4 (TLR4), which recognizes the lipid A moiety of the bacterial lipopolysaccharide (LPS). The human TLR4/MD-2 LPS receptor complex is strongly activated by hexa-acylated lipid A and poorly activated by underacylated lipid A. Here, we report that *B. cenocepacia* LPS strongly activates human TLR4/MD-2 despite its lipid A having only five acyl chains. Further, we show that aminoaabinose residues in lipid A contribute to TLR4-lipid A interactions, and experiments in a mouse model of LPS-induced endotoxic shock confirmed the pro-inflammatory potential of *B. cenocepacia* penta-acylated lipid A. Molecular modeling, combined with mutagenesis of TLR4-MD2 interactive surfaces, suggests that longer acyl chains and the aminoaabinose residues in the *B. cenocepacia* lipid A allow exposure of the fifth acyl chain on the surface of MD-2 enabling interactions with TLR4 and its dimerization. Our results provide a molecular model for activation of the human TLR4/MD-2 complex by penta-acylated lipid A, explaining the ability of hypoacylated *B. cenocepacia* LPS to promote pro-inflammatory responses associated to the severe pathogenicity of this opportunistic bacterium.

A central theme in innate immunity involves recognition of conserved microbial molecules (pathogen-associated molecular patterns) by surface receptors expressed on phagocytic cells (1,2). Pathogen recognition triggers cell signaling cascades leading to activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs), which in turn stimulate production of inflammatory cytokines (e.g. TNF-α, IL-1β, and type I interferons) (3). Lipopolysaccharide (LPS), the major component of the Gram-negative bacterial outer membrane, elicits potent innate immune responses through interactions with a receptor complex composed of Toll-like receptor 4 (TLR4) and the myeloid differentiation factor 2 (MD-2) (4). Depending on the amount and chemical nature of LPS released from the pathogen, TLR4/MD-2 recognition stimulates a protective immune response or leads to uncontrolled inflammation associated with high mortality (5,6). LPS is a complex glycolipid consisting of three distinct domains (Fig. 1) (7): lipid A, core oligosaccharide, and in many bacteria, a repeating polysaccharide moiety known as the O-antigen (8-10). Lipid A, composed of an acylated glucosamine disaccharide backbone, is the LPS moiety recognized by the TLR4/MD-2 receptor complex. Lipid A bioactivity depends on its chemical structure. The number and distribution of acyl chains, and the presence of the phosphate groups in the di-glucosamine backbone determine the agonistic and antagonistic activities of lipid A (11-19). Most enteric bacteria, such as *Escherichia coli*, produce hexa-acylated bis-phosphorylated lipid A, which has the highest cytokine-inducing capacity in mammals. In contrast, tetra-acylated lipid A (as lipid IV₅) and most of penta-acylated lipid A forms lack activity on human cells (19,20).

Lipid A interacts with a hydrophobic pocket formed by two anti-parallel β-sheets of MD-2 mediating the dimerization and activation of the TLR4/MD-2 complex (19,21). The MD-2 hydrophobic pocket can accommodate up to five acyl chains. For agonistic hexa-acylated *E. coli* lipid A, five acyl chains are buried within the pocket while the sixth chain lies on a channel of the MD-2 surface, building a hydrophobic region and the dimerization interface required for interaction with the TLR4 partner (referred to here as TLR4*). This arrangement enables hydrophobic interactions bridging the
TLR4/MD-2/LPS heterodimer and promoting the juxtaposition of the intracellular domains leading to activation of signal transduction (18,22). By contrast, antagonist lipid IVₐ binds to human MD-2 with all four acyl chains completely buried in the hydrophobic pocket precluding dimerization and subsequent activation (23). Similarly, many penta-acylated lipid As act as an antagonist of human TLR4/MD-2 (24-25). In contrast, lipid IVₐ can activate the murine TLR4/MD-2 complex (26).

Bacteria regulate the degree of lipid A acylation in response to environmental conditions. For example, *Pseudomonas aeruginosa* strains possess penta-acylated lipid A, but can produce hexa-acylated lipid A during infection in cystic fibrosis (CF) patients (27), gaining the ability to elicit stronger inflammation. Intriguingly, the non-CF pathogen *Porphyromonas gingivalis* produces a high heterogeneous LPS lipid A whose penta-acylated isoform potently activates the NF-κB pathway in human gingival fibroblasts in a similar manner to the *E. coli* LPS (28). Therefore, the molecular mechanisms underlying TLR4 dimerization and activation cannot always be predicted based on the *E. coli* lipid A-TLR4/MD2 paradigm.

*Burkholderia cenocepacia* is an opportunistic Gram-negative bacterium causing serious infections in CF patients (29). Understanding how *B. cenocepacia* infection elicits inflammation is crucial to find new ways to improve treatment of infected CF patients. The structure of the *B. cenocepacia* LPS (LPSBC) was previously described (30). The lipid A moiety of LPSBC consists of a mixture of penta-acylated and tetra-acylated, bis-phosphorylated diglucosamine backbone with two 4-amino-4-deoxy-l-arabinose (l-Ara4N) residues linked by phosphodiester linkages (Fig. 1) (30). The acyl chains are made of 3-(R)-hydroxyhexadecanoic acid C16:0 (3-OH), 3-(R)-hydroxytetradecanoic acid C14:0 (3-OH) and hydroxytetradecanoic acid C14:0 (Fig. 1) (30). The l-Ara4N decoration of lipid A is essential for *B. cenocepacia* viability (31) and represents the major determinant of resistance to cationic antimicrobial peptides (30,31). The structural features of LPSBC suggest the molecule would be a very poor TLR4/MD-2 complex agonist compared to the prototypic enterobacterial LPS. In contrast, several studies show that LPSBC, as well as biological and synthetic lipid A analogues, is highly pro-inflammatory (32-36). Therefore, the current model for binding/activation of human TLR4/MD-2 complex cannot explain why LPSBC is pro-inflammatory. In this work, we investigated the molecular basis of the mechanism of LPSBC recognition and report for the first time that longer acyl chains than those in enterobacterial lipid A together with the l-Ara4N residues allow *B. cenocepacia* lipid A (LAPC) to fit into the binding pocket of MD-2 in a manner that promotes TLR4 dimerization, leading to activation of inflammatory responses in cellular and animal models.

**EXPERIMENTAL PROCEDURES**

**LPS extraction and purification**—LPS was prepared from *B. cenocepacia* strains MH71 and MH75 (30). Both strains are isogenic derivatives of the K56-2 clinical isolate and contain a deletion of the *wbiF* gene that eliminates O-antigen production. MH75 has also a deletion removing genes involved in UDP-4-Ara4N synthesis and therefore cannot produce LPS with l-Ara4N residues (LPSBCΔAra). Both MH71 and MH75 also carry a suppressor mutation in the LPS transport gene *lptG* that allows for the transport of LPS devoid of l-Ara4N to the bacterial outer membrane (30). The *P. aeruginosa* RP73 clinical isolate was obtained from a chronically infected CF patient and kindly provided by Prof. Burkhard Tümmler (Klinische Forschergruppe, Medizinische Hochschule Hannover, Germany) (37,38). Strains were plated on trypticase soy agar (TSA) plates and cultured in trypticase soy broth (TSB) at 37 °C. For large-scale LPS purification, bacteria were treated with hot phenol/water (39). After extensive dialysis against distilled water, the extracted phases were subjected to enzymatic digestions to remove nucleic acids and protein contaminants. Water and phenol fractions were analyzed by 13.5% SDS-PAGE and silver
staining (40). The LPS fraction was exclusively found in the water phase.

Isolation of lipid A—Lipid A was obtained by hydrolysis of the LPS with 100 mM sodium acetate buffer pH 4.4, (100°C, 3 h). The solution was extracted three times with CHCl₃/MeOH/H₂O (100:100:30 v/v/v) and centrifuged (4°C, 5000 x g, 15 min). The organic phase contained lipid A and the water phase contained the core oligosaccharide. The former was further purified through several washes with distilled water and then lyophilized. The lipid A and core oligosaccharide structures of strains K56-2, MH71 and MH75 were characterized elsewhere (Fig. 1) (30,41).

Mice endotoxic studies—Animal studies were conducted according to protocols approved by San Raffaele Scientific Institute (Milan, Italy) Institutional Animal Care and Use Committee (IACUC) and adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals. Efforts were made to minimize the number of animals used and their suffering. C57BL/6 mice, 20-22 g male (Charls River) were challenged via intraperitoneal (i.p.) injection with 300 μg/mouse of LPS from E. coli and P. aeruginosa RP73, LPS_BC, and LPS_BCΔAra. Control mice were challenged with sterile saline solution. Five hours after treatment, mice were sacrificed by CO₂ administration, and blood collected from heart puncture. The blood clot was left at room temperature for 15-30 min. The clot was removed by centrifugation at 2,000 x g for 10 min at 4°C. Sera were collected and stored at -20 °C. TNF-α concentration in sera was determined by ELISA (R&D Systems), according to manufacturer instructions using antibody pairs and recombinant standards from R&D System.

HEK293 cell activation and luciferase reporter assays—Expression plasmids containing sequences of human TLR4, MD-2 and the pELAM-1 firefly luciferase plasmid were a gift from Dr. C. Kirschning (Technical University of Munich, Germany). Expression plasmid containing sequence of mouse TLR4 was purchased from InvivoGen (CA, USA). Expression plasmid for mouse MD-2 was a gift from Dr. Y. Nagai (University of Tokyo, Japan). The Renilla luciferase phRL-TK plasmid was purchased from Promega (WI, USA). Recombinant MD-2 genes were cloned into pEF-BOS with Flag and His tags on the C-terminus. Recombinant TLR4 genes were cloned into pUNO with an N-terminal HA tag. Transfection reagent JetPEI was purchased from Polyplus-Transfection (France) and was used according to the manufacturer’s instructions. The human embryonic kidney (HEK) 293 cells were provided by Dr. J. Chow (Eisai Research Institute, Andover, USA) and grown in DMEM supplemented with 10 % FBS. The MD-2 mutants were made using QuickChange site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer’s instructions. All plasmids were sequenced to confirm the appropriate mutations. For the NF-κB-luciferase reporter assay, HEK293 cells were seeded in 96-well plates at 3 x 10⁴ cells/well and incubated overnight in a humidified atmosphere (5% CO₂) at 37 °C. The next day, when cells were 60-80% confluent, they were co-transfected with the plasmids for MD-2 (10 ng), TLR4 (1 ng), NF-κB-dependent luciferase (50 ng) and constitutive Renilla luciferase (10 ng) using JetPEI transfection reagent (all amounts are in ng/well). Cells were stimulated 6 h after transfection with endotoxin preparations. Cells were lysed after 16 h of stimulation in 1x reporter assay lysis buffer (Promega, USA) and analysed for reporter gene activities using a dual-luciferase reporter assay system. Relative luciferase units (RLU) were calculated by normalizing each sample’s luciferase activity for constitutive Renilla activity measured within the same sample.

Statistical analysis—Results were expressed as mean±S.D from experiments done in triplicate. Statistical calculations and tests in vitro and in vivo were performed using Student’s t test considering P ≤ 0.05 as limit of statistical significance and a P value of 0.001 extremely significant.
coordinates were built by Maestro (42). Molecular mechanics optimization (UFF force field), semi-empirical calculations (AM1), and DFT (B3LYP/6-31G*) were subsequently applied using Gaussian03 (43). Ammonium and carboxylic acid groups were considered as ionized. Conformational analysis on DS1, DS2 and DS3 disaccharides was performed with Macromodel (44). The parameters were: MM3* force field, water solvent constant dielectric, dielectric constant of 1.0. The charges from the force field were employed, with an extended cut-off. The best conformers were selected, and from them molecular dynamics simulations (MDS) with implicit water and MM3* as force field, were performed using Schrödinger Maestro 9.3 Impact 5.8 (42,45) (MM3* force field, dielectric constant: 80.0 number of MDS steps 100, time step (ps) 0.001).

The full 3D structures of LA_{BCΔAra}, LA_{BC}, and LPS_{BC} were built with Maestro, using optimized disaccharide scaffolds obtained from the conformational analysis. FA chains, DS6 and amino-arabinoses were optimized separately using Gaussian 03 (B3LYP/6-31G*). Starting conformation for lipid A optimization was obtained from E. coli lipid A from PDB 3fxi. Full structure of LPS_{BC} was built by putting together core region disaccharides and lipid A with five aliphatic chains. Final MDS of the geometry was performed with implicit water by means of Impact (MM3* force field, dielectric constant: 80.0, number of MDS steps 100, time step (ps) 0,001). 3D Structures of lipid A molecules for validation were extracted from their corresponding PDB files (E. coli lipid A was obtained from PDB 3fxi, and Re chemotype of E. coli lipid A from PDB 3vq2), refined with the help of Maestro, and finally submitted to MDS with Impact (MM3* force field, and implicit water).

**Molecular modeling**—For docking studies with MD-2 and TLR4 proteins, 3D coordinates from MD-2 protein were obtained from PDB 3fxi (human MD-2) and 3vq2 (murine MD-2), and refined and minimized with the Protein Preparation Wizard module of Maestro, using amber force field (46). The TLR4 structure was obtained from PDB 3fxi, and treated following the same procedure after removing water molecules and any other non-standard residue.

Two different docking methodologies were used for docking studies of LA_{BCΔAra} and LA_{BC} on MD-2: AutoDock and AutoDock Vina. While AutoDock is a widely used semi-empirical docking method (47), the recently released AutoDock Vina combines empirical and knowledge-based scoring functions, with good performance and reduced computing time (48). Each ligand was docked into human and murine MD-2 protein using AutoDock 4.2 (49), and separately using AutoDock Vina 1.1.2. (48). For human MD-2 (from PDB 3fxi), the Autogrid grid point spacing was set at 0.375 Å, center coordinates of the grid box were 29.00, -7.00, 17.875(x, y, z), and number of grid points in xyz was 41, 53, 83. For murine MD-2 (from PDB 3vq2), the Autogrid grid point spacing was set at 0.375 Å, center coordinates of the grid box were -27.50, -15.50, 22.00 (x, y, z), leading to 75 x 40 x 60 (x, y, z) grid points. The best result from each docking job with LA_{BC} was used as starting geometry for subsequent docking calculations. Different combinations of allowed rotatable bonds were also considered for the ligands. Docking calculations with AutoDock were performed using Genetic Algorithm (number of individuals in population 150, maximum number of energy evaluations 2500000-5000000, maximum number of generations 27000, number of top individuals to survive to next generation 1, rate of gene mutation 0.02, rate of crossover 0.8, window size 10, Alpha parameter of Cauchy distribution 0.0, Beta parameter Cauchy distribution 1.0). When docking LPS_{BC}, flexible docking was also performed considering Asp101, Glu120 and Glu122 as flexible residues. Docking calculations with AutoDock Vina were also performed. Coordinates and dimensions of grid boxes, starting geometries and general methodology were the same as for AutoDock. When docking LPS_{BC}, flexible docking was also performed considering Asp101 as flexible residue. 3D structures of the docked complexes

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were optimized by MDS with Impact (implicit water, and AMBER* force field).

For docking studies the TLR4/MD-2 complex was built by merging docked MD-2/LPS core complex with docked LPS inner core/TLR4 using PDB 3fxi as template. The resulting structure was optimized by MDS with implicit water (AMBER* force field). Coupling of two TLR4/MD-2/LPS complexes was finally performed also using PDB 3fxi as template. The full 3D structure of the docked complex was carried out by MDS with Impact (implicit water, and AMBER* force field).

The full complex of human TLR4/MD-2 with B. cenocepacia LPS core was built by merging docked MD-2/LPS core complex with docked LPS inner core/TLR4 using PDB 3fxi as template. The resulting structure was optimized by MDS with implicit water (AMBER* force field). Coupling of two TLR4/MD-2/LPS BC complexes was finally performed also using PDB 3fxi as template. The full 3D structure of the dimer complex was submitted to MDS with implicit water and AMBER* force field (charges from: force field, cut-off: extended, method: PRCG, maximum iterations: 500, converge on: gradient, convergence threshold: 0.05). This final structure for the TLR4/MD-2/LPS BC complex was used to generate the mutant TLR4/MD-2/LPS BC complex (D294A, R322A, S415A*, and S416A*) by changing the corresponding side chains to Ala side chain. The resulting structure was submitted to MDS with implicit water and AMBER* force field. Energy analysis was performed by means of the MM-ISMSA method (50).

RESULTS

LPS and lipid A from B. cenocepacia activate the TLR4/MD-2 complex and have pro-inflammatory activity in vivo. TLR4/MD-2 activation by LPS BC and LA BC was examined using the NF-kB reporter luciferase assay in transiently transfected HEK293 and HEK293T cells co-expressing murine or human MD-2 (mMD-2, hMD-2) and murine or human TLR4 (mTLR4, hTLR4). Initial results revealed that LPS BC and LA BC activate both human and mouse TLR4/MD-2 complexes (Figs. 2, 3A and 3B). Since LA BC possesses longer acyl chains than those in E. coli lipid A and constitutively carries one or two L-Ara4N residues, to further understand the structure-function relationship of LPS BC, we utilized LPS from a mutant strain lacking the ability to produce L-Ara4N (LPS BCΔara). NF-kB reporter luciferase assays demonstrated that at 5 ng/ml, LPS BC and LPS BCΔara induced activation of TLR4/MD-2 complexes (Fig. 2). Similar results were obtained in dose-response experiments with purified LA BC and LA BCΔara (at 5, 10, and 50 ng/ml) in cells co-expressing mTLR4/mMD-2 and hTLR4/hMD-2 (Figs 3A and 3B, respectively), which also demonstrated consistent agonistic activity at the lowest concentration of 5 ng/ml. However, NF-kB activation by mouse (Fig. 3A) and human (Fig. 3B) complexes was significantly lower with LA BCΔara, suggesting that the L-Ara4N modification of the lipid A plays a role in bioactivity of the LPS molecule.

Comparisons with NF-kB activation elicited by synthetic E. coli-type hexa-acylated lipid A revealed significantly less activation by LPS BC and LA BCΔara (all at 50 ng/ml) (Figs. 3A and 3B). Lower activation by LPS BC and LPS BCΔara could depend on weaker binding to MD-2 or less efficient activation of TLR4. Therefore, we examined whether LPS BC and LPS BCΔara could interfere with TLR4 signaling elicited by the E. coli LPS by a competition assay in which HEK293 cells transfected with hTLR4/hMD-2 were pre-incubated with LPS BC or LPS BCΔara for 1 h and then re-stimulated with E. coli LPS for 4 h (Fig. 4). Pre-incubation of cells with 10 ng/ml of LPS BC followed by stimulation by 1 ng/ml of E. coli LPS, revealed weaker NF-kB activation than that with E. coli LPS alone (P < 0.05; Fig. 4). Interestingly, a two-fold reduction in NF-kB activation was evident in the case of pre-incubation with 10 ng/ml of LPS BCΔara (P < 0.01; Fig. 4). Further, a significant difference was found in activation by 10 ng/ml of E. coli LPS alone compared to that obtained after pre-incubation of cells with 100 ng/ml of LPS BC and LPS BCΔara (P < 0.01; Fig. 4). In contrast, this
inhibitory effect was reduced by increasing the concentration of the pre-incubated LPS$_{\text{BC}}$ and LPS$_{\text{BCΔAra}}$ (100 ng/ml) added to the 1 ng/ml of E. coli LPS; such effect is probably due to the contribution of a weak activation of MD-2/TLR4 by LPS$_{\text{BC}}$ and LPS$_{\text{BCΔAra}}$. Collectively, these results suggest that LPS$_{\text{BCΔAra}}$ efficiently binds to TLR4/MD-2 but is a weaker agonist compared to the E. coli LPS and LPS$_{\text{BC}}$.

The pro-inflammatory activity of LPS from B. cenocepacia was assessed using an established in vivo model of endotoxic shock. C57Bl/6 mice were challenged by intraperitoneal injection of LPS from E. coli, P. aeruginosa RP73 (a CF strain expressing penta- and tetra-acylated lipid A (37)), LPS$_{\text{BC}}$ and LPS$_{\text{BCΔAra}}$. After 5 h of treatment, TNF-α levels in sera were measured to evaluate pro-inflammatory and endotoxic potential. Compared to the saline solution control, penta-acylated LPS$_{\text{BC}}$ induced robust TNF-α production (LPS$_{\text{BC}}$ vs Ctrl $P < 0.01$; Fig. 5), while LPS$_{\text{BCΔAra}}$ induced three-fold less systemic release of TNF-α in sera (LPS$_{\text{BCΔAra}}$ vs LPS$_{\text{BC}}$ $P < 0.01$, Fig. 5), suggesting a role for L-Ara4N residues in the endotoxic LPS response, also in agreement with the in vitro results (Fig. 2).

Identification of MD-2 amino acid residues involved in the interaction with LPS$_{\text{BC}}$—To further investigate the molecular recognition of LPS$_{\text{BC}}$ and LPS$_{\text{BCΔAra}}$ by TLR4/MD-2, we constructed targeted hMD2 and mMD-2 mutants that were co-expressed with hTLR4 or mTLR4 in transfected HEK293 cells. We focused on residues at the dimerization interface of the MD-2 protein (22,51). In particular, we examined valine-82 (Val82), located in the proximity to the pocket entrance at a loop between β-strands 5 and 6. This region contains several conserved, solvent-exposed hydrophobic residues that contribute to crucial hydrophobic interactions in the activated receptor complex (22). Val82 was replaced by phenylalanine (V82F) to augment the hydrophobic interactions of the MD-2/lipid A complex with TLR4. In comparison to HEK293 cells transfected with parental MD-2, hTLR4/hMD-2 V82F led to increased activation upon stimulation with LPS$_{\text{BC}}$ and LPS$_{\text{BCΔAra}}$, while E. coli LPS activation was not significantly modified (Fig. 6). This agrees with the notion that hexa-acylated lipid A provides stronger hydrophobic interactions with TLR4, and therefore increased hydrophobicity at position 82 augments the activation by penta-acylated LPS. No significant differences were detected between stimulation with LPS$_{\text{BC}}$ and LA$_{\text{BCΔAra}}$ on hTLR4/hMD-2 V82F. Hydrophobic interactions between mTLR4/mMD-2 and LA are expected to be similar to those involving the hTLR4/hMD-2 complex since nearly all the hydrophobic residues are conserved (51). However, the electrostatic interactions that contribute to receptor selectivity differ between mMD-2 and hMD-2 proteins. Particularly, hMD-2 has a positively charged lysine at position 122 near the pocket entrance, whereas mMD-2 has a negatively charged glutamic acid (51). Moreover, hMD-2 has a positively charged lysine at position 125, while mMD-2 has a hydrophobic leucine (51). In the complex, lysine residues 122 and 125 are close to the disaccharide backbone of LA (52) and are important for the species-specific differences in the recognition of lipid IV$_{A}$ between hMD2 and mMD-2. We therefore introduced amino acid replacements at positions 122 and 125 in both MD-2 homologues to test whether the substitutions could affect LA binding and the selectivity of TLR4 activation by the LA$_{\text{BC}}$ variants. The E122K replacement in mMD-2, introducing the equivalent lysine of hMD-2, had little or no effect on the activation induced by LA$_{\text{BC}}$, LA$_{\text{BCΔAra}}$ or the hexa-acylated lipid A. In contrast, this replacement significantly decreased activation by lipid IV$_{A}$ ($P < 0.001$; Fig. 7), likely due to removal of the repulsive forces towards its bis-phosphorylated disaccharide backbone. These repulsive interactions have been proposed to be important for positioning of lipid IV$_{A}$ in an appropriate orientation for TLR4 dimerization (52). Thus, the unchanged LA$_{\text{BC}}$/LA$_{\text{BCΔAra}}$ responsiveness suggests that the single residue at position 122 of the murine MD-2 protein is not critical for LA$_{\text{BC}}$/LA$_{\text{BCΔAra}}$ signaling.
Stimulation of cells expressing the double E122K/L125K mMD-2 replacement (Fig. 7) showed substantially increased activity of the mutant MD-2 regardless of the LPS variant (Hexa-acylated lipid A vs Hexa-acylated lipid A $P < 0.05$, LA$_{BC}$ vs LA$_{BC}$ $P < 0.001$, LA$_{BCAara}$ vs LA$_{BCAara}$ $P < 0.001$ respectively on mTLR4/mMD-2 and mTLR4/mMD-2 E122K L125K; Fig. 7), except with lipid IV$_A$ whose activity was strongly impaired ($P < 0.001$; Fig. 7). On the other hand, hMD-2 in combination with mTLR4 exhibited significantly stronger activation by LPS$_{BCAara}$ ($P < 0.05$; Fig. 7), demonstrating that the L-Ara4N affects interaction with the TLR4 ectodomain. Moreover, the hMD-2 K122E mutant had an additive effect in combination with mTLR4 on the activation induced by LA$_{BCAara}$ showing an increment of the NF-$\kappa$B activity (LA$_{BCAara}$ mTLR4/hMD-2 K122E vs LA$_{BCAara}$ hTLR4/hMD-2 $P < 0.001$; LA$_{BCAara}$ mTLR4/hMD-2 K122E vs LA$_{BCAara}$ mTLR4/hMD-2 $P < 0.05$; Fig. 7). This probably reflects the presence of repulsive forces towards the phosphate groups of the lipid A, which might facilitate the orientation toward the Cys95/Cys105 loop (52,53).

Together, these experiments using hMD-2 and mMD-2 mutants support the notion that the peculiar structure of the LA$_{BC}$ plays a key role in its interaction with MD-2, as well as in the dimerization process. Our data suggest that the L-Ara4N molecules on the di-glucosamine backbone influence the binding of lipid A to the TLR4/MD-2 complex, although their absence is not sufficient to impair signaling.

Molecular modeling of LPS$_{BC}$ binding to TLR4/MD-2—Our previous results suggested that different MD-2 binding affinities drive the agonist activity of LPS. To provide a model for the interactions of LPS$_{BC}$ and LPS$_{BCAara}$, docking calculations were undertaken and the two ligands were docked into hMD-2 and mMD-2 proteins. Validation of the docking protocols was performed with AutoDock and AutoDock Vina by docking E. coli lipid A into the MD-2 protein from their corresponding crystallographic structures (PDB codes 3fxi –human and 3vq2 –murine). Values of root-mean-square deviation and predicted free energy of binding values indicated an excellent performance of both programs in predicting the crystallographic binding pose for both hMD-2 and mMD-2 proteins (Table 1).

LA$_{BC}$ and LA$_{BCAara}$ were predicted to bind both MD-2 homologues with binding poses that agreed with the crystallographic binding poses for E. coli lipid A (superimposition with E. coli lipid A in Figs. 8 and 9). Docking poses consisted of four fatty acid chains deeply immersed into the hydrophobic pocket of MD-2, establishing van der Waals and CH-$\pi$ interactions with the side chains of most of the lipophilic residues of the MD-2 pocket, mainly consisting of aliphatic Leu, Ile and Val residues, aromatic Phe (numbering 76, 104, 119, 121, 126, 147, and 151), and Tyr (numbering 102, and 131) residues. All docked poses were found to participate in hydrophobic interactions with most of these residues. Higher efficiency in establishing lipophilic interactions can be deduced given the length of these four fatty acid chains. The fifth chain is placed into the groove or channel defined by Phe126, Leu87, Val82 and Arg90. This channel has been identified in the X-ray structures of the complex of TLR4/MD-2 with E. coli lipid A (18) as the allocation site for one lipid chain, allowing to complete the hydrophobic interface required for dimerization with the second TLR4/MD-2/ligand partner (referred to here as TLR4*/MD-2*). This precludes formation of the activated TLR4/MD-2/ligand multimer. Also Phe126 has been proposed as a switch controlling the agonist/antagonist conformation of MD-2, since Phe126 mutation prevents dimerization and abolishes downstream signaling (18,53).

Therefore, the model predicts that higher length of the five fatty acid chains compared to E. coli lipid A places the fifth chain outside the MD-2 pocket, thus building the dimerization interface in the Phe126, Leu87, Val82 and Arg90 groove, analogously to the sixth FA of E. coli lipid A, while the other four chains remain inside the MD-2 pocket, providing stabilizing interactions.
Further, the disaccharide scaffolds of LA\textsubscript{BC} and LA\textsubscript{BCΔAra} together with the phosphate groups, are predicted to be in the outer region and to estabish electrostatic interactions with the polar residues which define the rim of the MD-2 pocket (Fig. 10). The higher predicted affinity for LPS\textsubscript{BC} could be explained in terms of additional anchorag points arising from the l-Ara4N residues. In particular, these l-Ara4N molecules can establish additional interactions with MD-2. The hydroxyl (OH) group from Tyr102 establishes bridged H-bonding with the OH at position 2 of l-Ara4N and the OH from the acyl chain (Fig. 10), while OH at position 3 from the same l-Ara4N establishes a hydrogen bond with the Ser118 OH group. These interactions could also be identified in the complex with the mMD-2 (data not shown). This predicted binding mode of LA\textsubscript{BC} could thus explain the unexpected significant level of activation in the biological assays. The lack of some lipophilic interactions arising from the absent sixth fatty acid chain, in comparison to E. coli lipid A, could also be counterbalanced by these extra polar interactions from the l-Ara4N residues.

*Molecular model of the hTLR4/MD-2 dimer in complex with LPS\textsubscript{BC}—To develop a structural model for the TLR4/MD-2 dimer in complex with LPS\textsubscript{BC} we first docked the inner core oligosaccharide moiety of LPS\textsubscript{BC} (Fig. 1) on hTLR4, using as a guide the region where E. coli LPS inner core binds. The best binding pose was selected (theoretical binding free energy of -2.8 kcal mol\textsuperscript{-1}, by AutoDock Vina), taking also into account a proper orientation for the building of the full complex. The docked pose, superimposed with E. coli LPS core from PDB 3fxi, is shown in Fig. 11. The hTLR4/core complex was then assembled to the MD-2/LA\textsubscript{BC} complex (best result from AutoDock Vina calculations), leading to a full hTLR4/MD-2/LPS\textsubscript{BC} complex. The receptor dimer was built using PDB 3fxi as a template. The full 3D structure of the dimer complex (Fig. 12) was subjected to MDS, which did not show meaningful differences with the docked complexes (data not shown). The 3D model of the full complex supports the key interactions for the molecular recognition of LPS\textsubscript{BC} that were described above and also confirms that the l-Ara4N residues (including the one present in the LPS core region, Fig. 1) play a fundamental role in the complex formation, as they participate in H-bonding at the dimerization interfaces (Fig. 13). First, both ammonium groups from l-Ara4N 2 and 3 (Fig. 13) (this latter is attached to a D-glycero-D-talo-2-octulosonic acid in the core moiety, Fig. 1) establish hydrogen bonds with the TLR4 Arg264 and Asp294 carboxylate groups. Second, the ammonium group of l-Ara4N 1 is close to the Asp395 carboxylate and the Ser416 OH group, which belong to the opposite TLR4*. Third, the OH-3 from l-Ara4N 1 establishes a H bond with Ser 415 CO group of TLR4* (Fig. 13). The 3D models from docking could suggest higher activation for LPS\textsubscript{BC} vs LPS\textsubscript{BCΔAra} arising from the extra anchorage points from arabinoses. This agrees with the higher endotoxic potential of LPS\textsubscript{BC} in the biological assays. Based on this TLR4/MD-2/LPS\textsubscript{BC} model, we have built a new model with the following four mutations: D294A, R322A, S415A*, and S416A*. These mutations involve the main residues establishing interactions with the ammonium groups from the three Ara4N residues, and the OH group from terminal glucose (Fig. 13): (i) D294 side chain establishes polar interactions reinforced by hydrogen bonds with the ammonium group from Ara4N-3, and is in the proximity of the ammonium group from Ara4N-2, so this residue can be considered as a main anchorage point for TLR4/MD-2/LPS\textsubscript{BC}; Asp294 has been mutated to Ala to abolish this interaction; (ii) S415* and S416* are placed in the partner TLR4* and provide an anchorage point for ammonium group from Ara4N-1 through polar interactions with the side chain of S415* and the backbone CO groups; both Ser
residues have been mutated to Ala; (iii) although not directly contacting with any of the Ara4N moieties, R322 side chain establishes a hydrogen bond with the OH group at position 3 from the terminal glucose, and we mutated it to compare its contribution to the global energy of the system; this interaction is absent in the complex of TLR4/MD2 with E. coli LPS (PDB-ID 3fxi) and could be considered as a distinctive interaction for LPS\_BC core. MDS and energy calculations of both systems (TLR4/MD-2/LPS\_BC and mutant TLR4/MD-2/LPS\_BC) have shown an important difference in the global energy: the wild type complex (TLR4/MD-2/LPS\_BC model) is around 32 kcal mol\(^{-1}\) more stable than the mutant counterpart, mainly due to the coulombic term, pointing to the presence of important polar interactions (Table 2). Analysis of the contributions of each residue to the ligand binding energy reveals that this energy difference is mainly due to the lack of the interactions involving the mutated residues: (i) interaction with D294 is the highest interaction in the TLR4/MD-2/LPS\_BC system (around 22 kcal mol\(^{-1}\)) and is absent in the mutant; (ii) also the interaction with R322 is missing, being one of the main interactions in the TLR4/MD-2/LPS\_BC system (around 9.6 kcal mol\(^{-1}\)); (iii) the lower coulombic contribution to the interaction energy in the mutant TLR4/MD-2/LPS\_BC complex is due to the absence of the interaction between the S415* side chain and the ammonium group. These results give reasonable evidences that the Ara4N ammonium groups provide additional anchorage interactions accounting for the final stability of the TLR4/MD-2/LPS\_BC complex. Abolishing these interactions leads to a less stable complex, suggesting they are crucial for the binding.

**DISCUSSION**

Lipid A is a major determinant of cytokine induction in host immune cells (11-19,54). Generally, the highest immunostimulatory activity of lipid A correlates with its hexa-acylation pattern (11-19,54), since the sixth acyl chain protrudes from the MD-2 binding pocket bridging TLR4/MD-2 complex dimerization. On the contrary, underacylated lipid A molecules are not (or poorly) sensed by human TLR4/MD-2 being potentially accommodated within the MD-2 pocket. Here, we demonstrate a role for L-Ara4N residues in lipid A that explains why the *B. cenocepacia* LPS (naturally consisting of a mixture of penta- and tetra-acylated forms) acts as a strong TLR4/MD-2 agonist. Our results agree with a recent report by Hollaus *et al.* (36), demonstrating that a synthetic *Burkholderia* lipid A substituted with L-Ara4N exclusively at the anomeric phosphate acts as a potent human TLR4/MD-2 agonist.

Our *in vitro* and *in vivo* results explain and expand the above conclusions and show that *B. cenocepacia* penta-acylated lipid A (both LA\_BC and LA\_BC\_Ara) elicits an inflammatory response activating the TLR4/MD-2 complex, suggesting that the model of the receptor complex activation should also include other structural components. Further, molecular modeling experiments indicated that the increased length of the two amide-linked acyl chains of the *B. cenocepacia* lipid A moiety (3-(R)-hydroxyhexadecanoic acid C16:0 (3-OH) vs 3-(R)-hydroxytetradecanoic acid C14:0 (3-OH) from the *E. coli* lipid A) compensates the lack of one fatty acid chain filling the hydrophobic binding pocket of MD-2 protein, allowing the placement into the channel of the fifth acyl chain responsible for TLR4 dimerization. The replacement in *E. coli* lipid A of the secondary dodecanoic acid C12:0 with a hexadecanoic acid C16:0 results in a weaker LPS agonist (55). However, our data indicate not only the increased length of the acyl chain but also its position is important for agonist activity.

Further, our mutagenesis studies using two *B. cenocepacia* lipid A forms, LA\_BC and LA\_BC\_Ara, uncovered differential responsiveness on transfected HEK293 cells indicating an important role also for the L-AraN residues into the TLR4/LA\_BC binding process. Particularly, the significantly different immunostimulatory activity of LA\_BC compared to LA\_BC\_Ara (Fig. 7), observed in presence of the human MD-2 K122E mutation, might be related to the occurrence on LA\_BC of the L-Ara4N residues, which could mask the negatively charged
phosphate groups and then reduce the repulsive forces introduced with the K122E mutation. In contrast, the absence of L-Ara4N in L_{B_{c}C_{a}A_{a}r} might explain its increased agonistic activity when tested on HEK293 cells transfected with mTLR4/hMD-2 K122E, since the repulsive forces introduced might facilitate the orientation toward the Cys95/Cys105 loop (52-53).

Therefore, it is reasonable to assume that the L-Ara4N-modification with its positively charged ammonium group could favor the electrostatic interactions allowing receptor/lipid A binding. Intriguingly, recent work by Maeshima et al. (56) revealed several key charged amino acid residues in TLR4 and MD-2 mediating host-specific responses to the glucosamine-modified penta-acylated lipid A from *B. pertussis* and its unmodified counterpart by human and mouse TLR4/MD-2 complexes. This further underscores the importance of positively charged residues decorating penta-acylated lipid A and interacting with human TLR-4/MD-2 charged amino acid residues to promote complex activation (56).

Our molecular modeling experiments further supported the notion that L-Ara4N residues provide additional polar interactions affecting the LA_{B_{c}C_{a}r} binding to the TLR4/MD-2 and contribute to anchoring the lipid A into the receptor complex. The network of hydrogen bonds and polar interactions contributing to anchoring the lipid A into the TLR4/MD-2 involves not only the sugar residues from the core oligosaccharide (for example, hydrogen bonds involving Arg322, Fig. 13), but also the three L-Ara4N residues and the partner TLR4*. As stated above, mutagenesis studies demonstrate that several Lys residues involved in LPS binding play indispensable roles through the polar interactions with the phosphate groups (56-59). In our dimer model, Lys122 is involved in binding the lipid A moiety of the LPS_{B_{c}r} through polar interactions with the core; as for V82F, it has been already mentioned its role in building the groove that accommodates the acyl chain from lipid A, completing the interaction surface required for dimerization. The replacement of Val82 by phenylalanine involves changes of van der Waals interactions into CH-π interactions, which may favor the binding of this chain, longer than the corresponding one on *E. coli* LPS.

Thus, the structural peculiarities of *B. cenocepacia* i.e., its acylation pattern and the presence of the L-Ara4N residues in the lipid A region exert a synergistic effect in the activation and dimerization of the LPS receptor opening new insights into the comprehension of the molecular recognition of LPS by TLR4/MD-2.

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**Authors contributions:** AM designed the research, FDL executed the chemistry and immunochemistry experiments, LK and SMS performed the MD experiments, all the authors have contributed with analytical tools or reagents or particular experiments, FDL, MAV, SMS and AM analyzed data and wrote the paper. All authors analyzed the results and approved the final version of the manuscript.
REFERENCES


FOOTNOTES
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11The abbreviations used are: CF, cystic fibrosis; FA, fatty acid; LA, lipid A; L-Ara4N, 4-amino-4-deoxy-L-arabinose; LA_{BC}, B. cenocepacia lipid A; LA_{BCAran}, B. cenocepacia lipid A lacking L-Ara4N; LPS, lipopolysaccharide; LPS_{BC}, B cenocepacia LPS; LPS_{BCAran}, B cenocepacia LPS lacking L-Ara4N; MD-2, myeloid differentiation factor 2; MDS, molecular dynamics simulation; PAMP, pathogen-associated molecular pattern; TLR4, Toll-Like Receptor 4.
Activation of TLR4/MD-2 by Burkholderia LPS

FIGURE LEGENDS

FIGURE 1. Structure of Burkholderia cenocepacia LPS inner core and lipid A. B. cenocepacia lipid A is heterogeneous, being composed of a mixture of penta- and tetra-acylated species (30,40). Lipid A fatty acids are two 3-(R)-hydroxhexadecanoic acids, two 3-(R)-hydroxytetradecanoic acids and one tetradecanoic acid (30,40). The dotted lines indicate the non-stoichiometric substitution.

FIGURE 2. LPSBC activates the murine and human TLR4/MD-2 complexes. NF-κB activation upon stimulation of HEK293 mTLR4/mMD-2 and hTLR4/hMD-2 after 6 h with 5 ng/ml of LPSBC and LPSBCAra. Stimulation for 6 h with E. coli LPS was used as control. The data are pooled from three independent experiments done in triplicate. Bars indicate standard deviation; significance was calculated in comparison to stimulation with E. coli LPS (*P <0.05, **P <0.01, ***P <0.001). Curly brackets indicate significance calculated comparing LPSBC and LPSBCAra (*P <0.05, **P <0.01, ***P <0.001).

FIGURE 3. Lipid A activation of murine and human TLR4/MD2-complexes. A. NF-κB activation upon stimulation of HEK293 mTLR4/mMD-2 after 6 h with LA and LAAra. Stimmulations for 6 h with E. coli LPS, hexa-acylated lipid A and lipid IV were used as controls. The data are pooled from three independent experiments done in triplicate. Bars indicate standard deviation; significance was calculated in comparison to stimulation with hexa-acylated E. coli lipid A (*P <0.05, **P <0.01, ***P <0.001); curly brackets indicate significance calculated comparing LPSBC and LPSBCAra (*P <0.05, **P <0.01, ***P <0.001). B. NF-κB activation upon stimulation of HEK293 hTLR4/hMD-2 after 6 h with LA and LAAra. Stimmulations for 6 h with E. coli LPS, hexa-acylated lipid A and lipid IV were used as controls. The data are pooled from three independent experiments done in triplicate. Bars indicate standard deviation; significance was calculated in comparison to stimulation with hexa-acylated E. coli lipid A (*P <0.05, **P <0.01, ***P <0.001); curly brackets indicate significance calculated comparing LPSBC and LPSBCAra (*P <0.05, **P <0.01, ***P <0.001).

FIGURE 4. Burkholderia cenocepacia LPSBCAra effects on the E. coli LPS agonist activity. Assay on the potential antagonist activity of LPSBC and LPSBCAra on hexa-acylated E. coli LPS. NF-κB activation upon stimulation of HEK293 hTLR4 after 1 h with LPSBC (1, 10 and 100 ng/ml) and LPSBCAra (1, 10 and 100 ng/ml) and then exposed to E. coli LPS (1 and 10 ng/ml) for 4 h. The data are pooled from three independent experiments done in triplicate. Bars indicate standard deviation; significance was calculated in comparison to stimulation with E. coli LPS (1 and 10 ng/ml) (*P <0.05, **P <0.01, ***P <0.001).

FIGURE 5. Pro-inflammatory and endotoxic potential of LPSBC in C57Bl/6 mice. From three mice to five per group were challenged via intraperitoneal injection with 300 μg/mouse of LPS from E. coli, P. aeruginosa RP73, LPSBC and LPSBCAra. TNF-α levels in sera were quantified after 5 h of treatment. Treatment with sterile saline solution was used as control (Ctrl). The data are pooled from two independent experiments. Results are reported as mean±S.D. Statistical analysis was made for pair wise comparisons (*P <0.05, **P <0.01, ***P <0.001).

FIGURE 6. Burkholderia cenocepacia LPS acylation pattern is responsible for the NF-κB activation. NF-κB activation upon stimulation of HEK293 hTLR4/hMD-2 after 6 h with LPSBC and LPSBCAra. Stimulation for 6 h with E. coli LPS was used as control. The same protocol was used to stimulate HEK293 hTLR4/hMD-2 V82F cells. The data are pooled from three independent experiments done in triplicate. Bars indicate standard deviation; significance was calculated in comparison to E. coli LPS (*P <0.05, **P <0.01, ***P <0.001).
FIGURE 7. Lys-122 and Lys-125 in the B. cenocepacia LPS signaling on murine and human TLR4/MD-2 complexes. NF-κB-luciferase reporter assay executed stimulating HEK293 hTLR4/hMD-2, HEK293 mTLR4/mMD-2 and mTLR4/hMD-2 after 6 h with LPSBC and LPSBCΔAra. Stimulations for 6 h with E. coli LPS and with lipid IV_A were used as controls. The same protocol was used to stimulate HEK293 mTLR4/mMD-2 E1222K and HEK293 mTLR4/mMD-2 E122K L125K cells. The data are pooled from two independent experiments done in triplicate. Bars indicate standard deviation; statistical analysis was calculated for pair wise comparisons (*P <0.05, **P <0.01, ***P <0.001).

FIGURE 8. Predicted binding mode of the LPSBC core to hTLR4/MD-2. Computational model from docking followed by MDS. LPSBC docked to MD-2 is shown in CPK colors. Superimposed fatty acids chains from E. coli lipid A are shown in different colors. Some representative residues from the MD-2 binding site are represented with carbon atoms in green.

FIGURE 9. Predicted binding mode of the LPSBC core to hTLR4/MD-2. Details of LPSBC (CPK colours) docked to MD-2. Superimposed fatty acids chains from E. coli lipid A are shown with carbon atoms in blue.

FIGURE 10. Predicted binding mode of the LPSBC core to hTLR4/MD-2. Detail of some H-bond interactions between the LPSBC inner core and TLR4/MD-2 involving Tyr102. LPSBC is represented in CPK colors and MD-2 protein is represented in green.

FIGURE 11. Superimposition of best dock results of LPSBC core. The docking was obtained using AutoDock Vina (depicted in red) and E. coli LPS core from PDB 3fxi (depicted in green). TLR4 is not shown for the sake of clarity.


FIGURE 13. Predicted binding mode of the LPSBC core to hTLR4/MD-2. Details of some interactions between the LPSBC inner core and TLR4/MD-2 from the computational model.
Table 1. Theoretical free energy of binding for the docking calculations with AutoDock 4.2 and AutoDock Vina (in bold). Energy values are in kcal mol$^{-1}$. Root-mean-square deviation (RMSD) values are in Å.

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Table 2. Analysis of the per residue contributions to the total ligand binding energy for both complexes TLR4/MD-2/LPS<sub>BC</sub> and the mutated TLR4/MD-2/LPS<sub>BC</sub> (D294A, R322A, S415A*, and S416A*). Energy calculations come from the MDS. Only the top 22 contributions are shown (top 20 in the case of the mutant). Residues from TLR4 are underlined. Residues from the partner TLR4 are also marked with *.

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**Activation of TLR4/MD-2 by Burkholderia LPS**
Figure 1
Activation of TLR4/MD-2 by Burkholderia LPS

Figure 2

RLA (Relative Luciferase Activity)

**

* *

**

* NS E. coli LPS 5 ng/mL

LPSBC 5 ng/mL

LPSBCΔAra 5 ng/mL

hTLR4/hMD-2

mTLR4/mMD-2

by guest on July 11, 2015 http://www.jbc.org/ Downloaded from
Figure 3A
Activation of TLR4/MD-2 by Burkholderia LPS

Figure 3B

![Graph showing Activation of TLR4/MD-2 by Burkholderia LPS](image-url)
Figure 4
Figure 5

Activation of TLR4/MD-2 by Burkholderia LPS

** TNF-α serum levels (pg/mL)
**Figure 6**

The figure shows the relative luciferase activity (RLA) for different combinations of hTLR4 and hMD-2 constructs. The constructs are hTLR4/hMD-2 and hTLR4/hMD-2 V82F. The x-axis represents time in hours (hMD-2/hTLR4, hMD-2 V82F/hTLR4). The y-axis represents RLA.

- **NS** indicates no statistically significant difference.
- **E. coli LPS** is represented by black bars.
- **LPSBC** is represented by dark gray bars.
- **LPSBCΔAra** is represented by light gray bars.

Statistical significance is indicated by:
- **NS**
- *** (p < 0.05)**
- **** (p < 0.01)
- ***** (p < 0.001)**
Figure 7

RLU (Relative Luciferase Activity)

- hMD2/TLR4
- hMD2/TLR4
- mMD2/TLR4
- mMD2/TLR4
- mMD2/TLR4
- mMD2/TLR4

Legend:
- NS
- Hexa-acylated E. coli LA
- LNA
- LAβCΔAra
- Lipid IVα

Significance:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
Figure 8
Figure 9
Figure 12
Figure 13
Glycobiology and Extracellular Matrices: Activation of human TLR4/MD-2 by hypoacylated lipopolysaccharide from a clinical isolate of Burkholderia cenocepacia

Flaviana Di Lorenzo, Łukasz Kubik, Alja Oblak, Nicola Ivan Lorè, Cristina Cigana, Rosa Lanzetta, Michelangelo Parrilli, Mohamad A. Hamad, Anthony De Soyza, Alba Silipo, Roman Jerala, Alessandra Bragonzi, Miguel A. Valvano, Sonsoles Martin-Santamaría and Antonio Molinaro

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