RESEARCH ARTICLE

Does the production of NF-κβ, IRF3, and IFN-β after LOS/LPS exposures in naive-HIV dendritic cells depend on TLR4-MD2 receptor pathway? *In vitro* and *in silico* study

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Lipopolysaccharide (LPS) and lipooligosaccharide (LOS) are inflammatory response inducers triggering downstream inflammatory signals by binding to the TLR4-MD2 complex. LPS/LOS exposure through TLR4 receptor on dendritic cells (DC) causes an increase in interferon (IFN)-β expression *via* an increase in interferon regulatory factor (IRF)3 expression and a decrease in nuclear factor (NF)-κβ, which result in HIV replication inhibition. This study aimed to compare the binding affinity between *Escherichia coli* LPS and *Neisseria gonorrhoeae* LOS toward TLR4-MD2 *in silico* and the production of NF-κβ, IRF3, and IFN-β from naive-HIV monocyte-derived dendric cells (MDDCs) after LPS and LOS exposure *in vitro*, to find a deeper understanding of host immune response, disease pathogenesis, and trends of drug development in the future. Molecular docking and dynamics were analyzed using AutoDock Vina and YASARA, respectively. The naive-HIV DC culture was exposed to LPS at 50, 100, and 200 ng/mL, or LOS at concentrations of 2.5, 5, and 10% for 24-h. Levels of NF-κβ, IRF3, and IFN- β were measured by Enzyme-linked Immunosorbent Assay (ELISA). The results showed that LOS demonstrated higher binding affinity to TLR4-MD2 complex than LPS, although the docking between LOS and TLR4-MD2 complex involved fewer amino acids. Inversely, LPS exposure significantly increased IRF3 and IFN-β production ($P < 0.01$). Production of IFN-β was significantly increased with higher doses of LOS ($P < 0.01$). Different doses of LPS induced significant differences in NF-κβ and IFN-β levels ($P < 0.01$ and $P < 0.05$, respectively). Despite LOS showing higher binding affinity to TLR4-MD2 complex, LPS exposure induced higher production of IRF3 and IFN-β from MDDCs. These findings provided information for deeper apprehension of immune responses and disease pathogenesis during HIV co-infections.

Keywords: lipooligosaccharide; lipopolysaccharide; TLR4-MD2 binding; dendritic cells; naïve-HIV.

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Introduction

Lipopolysaccharide (LPS) is one of the renowned pathogen-associated molecular patterns (PAMP) found in the outermost membrane of gram-negative bacteria. Playing role as a potent inflammatory response inducer in the case of Gram-negative bacterial infection, host cells recognize LPS through its interaction with Toll-like receptor 4 (TLR4) and myeloid differentiation protein (MD)2 in the innate immune cells. The interaction between LPS and innate immune cells consequently induces production and release of endogenous mediators to kick start inflammation and immunity responses. *Escherichia coli* (*E. coli*) LPS has been widely utilized as a model for LPS recognition by TLR4-MD2 complex due to its strong agonist properties for TLR4 signaling [1]. Lipooligosaccharide (LOS) is a component of the outer membrane of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) that triggers innate immunity *via* TLR4 activation. The local and systemic effects of LOS in gonococcal infection show that LOS is relevant in HIV coinfections. A previous study showed that HIV-1 replication could be inhibited by the activation of the TLR-4 pathway by binding its LOS, which, in turn, increased the activation of the innate immune system of the host [2].

TLR4 and MD2 form a heterodimer capable of recognizing common patterns of the various types of LPS. The TLR4-MD2 heterodimer has a complex ligand specificity and could be activated by structurally varied LPS [3]. LPS from bacterial membrane is transferred to TLR4-MD2 complex by using two accessory proteins, namely LPS-binding protein (LBP) and CD14 [2]. TLR4 exhibits a horseshoe-like structure resembling a leucine-rich repeats (LRR), while MD2 has a β-cup folded structure consisting of two anti-parallel β layers that form a large hydrophobic pocket. The hydrophobic pocket functions as a ligand binding structure. LPS that binds this structure would directly mediate dimerization between two TLR-MD2 complexes [4]. Generally, LPS and LOS directly binds MD2 through the lipid A component which then induces TLR4-MD2 to form an m-shaped dimer. Therefore, the toxicity of lipid A is influenced by its aliphatic tail composition [5]. LOS differs from LPS in their polysaccharide chains, with LOS possessing simpler unrepeating polysaccharide chains. Meanwhile, their lipid A structures differ in the acylation pattern, composition, and chain length. Thus, the binding of lipid A structure from LOS and LPS to MD2 produces different profiles of endotoxicity commonly recognized as structure-activity relationship [6, 7]. Stimulation of TLR4 by LPS/LOS induces the release of proinflammatory cytokines required to activate the natural immune response. TLR 4 activates the nuclear factor-kappa B (NF-κβ) after recognizing pathogenic infection or tissue damage [7]. The binding of LPS to TLR4 activates the TNF receptor-associated modulator (TRAM) pathway by downstream signaling to the TIR-domain-containing adapter-inducing interferon β (TRIF) to increase interferon regulatory factor (IRF) 3 expression and induce an increase in interferon (IFN)-β [8]. A study showed that *E. coli* LPS induced higher pro-inflammatory cytokine production than *N. gonorrhoeae* and *N. meningitidis* LOS exposure, while, contrastingly, another study showed that gonococcal LOS elicited higher inflammatory cytokines than *E. coli* LPS [9].

Dendritic cells are the most important antigen presenting cells (APC) for activating naïve T cells and play a role in the natural immune response to infection. Dendritic cells are the first cells to become infected after mucosal exposure to HIV. Dendritic cells have coreceptors for HIV entry *via* TLR4. Natural immune cells, namely dendritic cells and natural killer (NK) cells, are the first lines of defense that HIV encounters when it enters the body. Dendritic cells present processed antigens to T lymphocytes in the lymph nodes. Epidermal dendritic cells are the first immune cells to fight HIV on mucosal surfaces and transfer HIV from the site of infection to lymphoid tissue. Follicular dendritic cells, which are found in lymphoid tissue, are also APCs that capture and present antigens on their cell surface [10]. In this study, the binding affinity between LPS from *E. coli* and LOS from *N. gonorrhoeae*

toward TLR4-MD2 complex *in silico* were compared. We also verified whether the binding affinity was analogous to the production of NF-κβ, IRF3, and IFN-β from myeloid derived dendritic cells (MDDCs) *in vitro* as downstream products of TLR4 activation, following a 24-h exposure of LPS and LOS. The results of this study could pave the road toward a deeper understanding of host immune response, disease pathogenesis, and the development of alternative drugs to treat HIV infection.

Materials and Methods

1. *In silico* study of the interaction between TLR4-MD2 and the ligands

(1) Database collection

E. coli LPS and *N. gonorrhoeae* LOS were set as ligands with E5564 (Eritoran), a synthetic lipid A analogue as a control, to compare the binding positions of all ligands at their receptors [3]. The data of all ligands were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov) as Sybil Data Files (SDF) format and converted into a 3D structure with a Protein Data Bank (PDB) format using BIOVIA Discovery Studio 2019 (Dassault Systemes BIOVIA, Vélizy-Villacoublay, Yvelines, France). The molecular weight of *E. coli* LPS (PubChem CID 46878506), *N. gonorrhoeae* LOS (PubChem CID 45266821), and E5564 (PubChem CID 6912404) were 1,273.0, 2,486.8, and 1,313.7 g/mol, respectively. All ligands were able to bind with the 3D structure of TLR4-MD2 receptor (PDB ID 2Z65) obtained from the PDB database (https://www.rcsb.org/).

(2) Molecular docking analysis and ligand-receptor interaction

Molecular docking was used to determine the interaction between TLR4-MD2 and the ligand as indicated by the binding affinity value and the amino acid residues involved. Ligand-receptor interactions were analyzed using AutoDock Vina (Molecular Graphics Lab, The Scripps Research Institute, San Diego, California, USA), which was integrated into PyRx version 0.9.5 (https://pyrx.sourceforge.io/). The docking results, bond positions, and amino acid residues formed between ligand-receptors were analyzed using BIOVIA Discovery Studio 2019.

(3) Molecular dynamic simulation

Yet Another Scientific Artificial Reality Application (YASARA) (YASARA Biosciences GmbH, Vienna, Austria) was used for molecular dynamic (MD) simulations to compare the ligand with the lowest binding affinity value and E5564 (Eritoran) as the control. The ligands used were *E. coli* LPS, *N. gonorrhoeae* LOS, and Laminaran, which was a major polysaccharide of brown algae. The simulation parameters corresponded to the cells' physiological conditions at 37°C, pressure for the simulated system at 1 atm, pH 7.4, and salt content of 0.9%. The total duration of the molecular dynamics' simulation run at 50 ns with autosaved setting every 25 picoseconds that meant the positions and velocities of the atoms were updated every 25 picoseconds during the simulation. The simulation was run by the md_run macro program (YASARA Biosciences GmbH, Vienna, Austria), and the results were displayed by the md_analyze, md_analyzeres, and md_analyzebindenergy programs (YASARA Biosciences GmbH, Vienna, Austria) [11].

2. *In vitro study* of LOS and LPS exposures to naive-HIV dendritic cells

(1) *E. coli* LPS dose preparation

A 5 mg/mL (w/v) LPS solution was prepared by adding 2 mL of sterile phosphate buffer saline (PBS) to 10 mg of *E. coli* LPS O111:B4 powder stock (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The solution was subsequently diluted using Roswell Park Memorial Institute (RPMI) medium (2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4,500 mg/L glucose, and 1,500 mg/L sodium bicarbonate) to prepare LPS doses of 50, 100, and 200 ng/mL solutions.

(2) *N. gonorrhoea* LOS extraction

LOS was extracted from *N. gonorrhoeae* (American Type Culture Collection, ATCC, 49226) GCP broth (15 g/L proteose peptone, 1 g/L soluble starch, 4 g/L potassium phosphate dibasic, 1 g/L potassium phosphate monobasic,

15 g/L sodium chloride) culture by using modified hot-phenol water method as described by Arenas [12]. Extracted LOS was diluted in 0.5% dimethyl sulfoxide (DMSO) with a concentration of 1,000 µM. Volumes of 5 µL, 10 µL, and 20 µL LOS solution was added to 195 µL, 190 µL, and 180 µL of culture media (RPMI containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin), respectively, to obtain LOS concentrations of 2.5, 5, and 10%.

(3) Sample preparation and monocyte isolation

Blood samples were taken from six male naive HIV subjects with an age range of 20 – 30 years old who clinically had HIV stage 1 according to the World Health Organization (WHO) criteria. The HIV diagnosis was confirmed through a 3-step rapid HIV test with various sensitivities and specificities. The research protocol was approved by the Health Research Ethics Committee at Dr. Saiful Anwar General Hospital, Malang, East Java, Indonesia (Approval No. 400/024/K.3/302/2020). All participating subjects signed an informed consent prior to enrolment. The monocyte isolation was performed from 20 mL venous blood using EasySep Direct Human Monocyte Isolation Kit (STEMCELL Technologies Inc., Vancouver, Canada) with the immuno-magnetic negative selection method. Briefly, 5 mL of blood was added to 50 µL EasySep Direct Monocyte Isolation Cocktail. The sample was allowed to stand for 5 mins. Then, Ca^{2+} Mg^{2+} -free buffer was added until reaching a volume of 10 mL. After being placed on the EasySep Magnet Sorter, the negative selection was performed for 3 mins. Cell-rich suspension was separated into 5 mL tubes, added with EasySep Direct Rapid Sphere, and incubated for 5 mins. The suspension was put back onto the magnet for a second separation process for 3 mins. The cell-rich suspension was taken again, put into a 5 mL tube. The tubes were then placed in a magnet for a final separation process for 3 mins. The monocyte-rich suspension was poured into a new tube for dendritic cell culture.

(4) MDDC culture and LPS and LOS exposure

Maturation and differentiation of MDDC was performed using the ImmunoCult DC Culture Kit (STEMCELL Technologies Inc., Vancoucer, Canada) according to the manufacturer's instructions. LPS (50, 100, and 200 ng/mL) or LOS (2.5, 5, and 10%) in culture medium (RPMI containing 10% FBS, 1% penicillin/streptomycin) were added on the 6th day of MDDC culture. The differentiation medium from each well was aspirated without taking the cells (approximately 190 µL per well). The cells were then washed using sterile PBS before adding 200 µL of the mixture of culture media and LPS or LOS with different concentrations to each well. The culture was incubated at 37°C, 5% CO_2 overnight. The treatment was carried out with duplicates for each dose group. On the 7th day, the LPS/LOS-stimulated MDDCs were ready to be harvested. The suspension was moved into a new tube and subjected to flowcytometry.

(5) Determination of MDDC surface markers

MDDC maturation was confirmed by detecting cell surface markers including CD14, CD83, and CD11c [13, 14] using PerCP/Cy.5.5 anti-human CD14 and APC anti-human CD83 (BioLegend, San Diego, California, USA), and FITC anti-human CD11c (eBioscience, Thermo-Fisher Scientific, Waltham, MA, USA). The viability of the dendritic cells was confirmed using the FITC Annexin V Apoptosis Detection Kit with propidium iodide (Pi) (BioLegend, San Diego, California, USA). The staining protocol was performed according to the manufacturer's instructions and read with BD FACSMelody™ Cell Sorter (BD Biosciences, Franklin Lakes, New Jersey, USA).

(6) Crude protein preparation and measurement of IRF3, NF-κB and IFN-β levels

Extraction of crude protein from MDDCs was performed using PRO-PREPTM Extraction Solution (iNtRON Biotechnology, Seongnam, Kyonggi-do, South Korea). Cells were harvested from each well and placed into separate 50 mL centrifuge tubes, then centrifuged at 800 – 1,100 x g at 4°C for 5 mins. The supernatants were collected in separate microcentrifuge tubes, while the cell pellets were washed with sterile

PBS. Then, 100 µL of a 1:10 suspension of PRO-PREP and proteinase inhibitor (v/v) was used to resuspend the cell pellets before incubating for 10 mins at -20°C. The lysate was centrifuged at 20,700 x g at 4°C for 5 mins. The supernatant was transferred to a new microcentrifuge tube and stored at -20°C until further use. The extracted crude proteins were subjected to Enzyme-linked Immunosorbent Assays (ELISA) for NF-κβ, IRF3, and IFN-β measurements by using Human NF-κβ ELISA Kit, Human IRF3 ELISA Kit, and Human Interferon-β ELISA Kit (Bioassay Technology Laboratory, Shanghai Korain Biotech Co., Ltd., Shanghai, China) according to manufacturer's instructions. Briefly, 40 µL of sample was added to the pre-coated strips. Then, 10 µL of either anti-NF-κβ antibody (NF-κβ p65 antibody F-6: sc8008), anti-IRF-3 antibody (IRF-3 SL-12: sc-33641), or anti-IFN-β antibody (IFN-β A1: sc-53968) was added to the wells followed by 50 µL of streptavidin-HRP. After incubation of 60 mins at 37°C, the plates were washed five times using washing buffer and 50 µL of substrate was added to each well. Following an incubation time of 10 mins, 50 µL of stop solution was added, and the optical density was read using a ZN-320 Microplate Reader (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, Guangdong, China) at 450 nm within 10 mins. The measurement of the standards was performed simultaneously, and the concentration of each sample was determined based on the standard curve.

3. Statistical Analysis

Data analyses were performed using the GraphPad Prism®, version 5 (GraphPad Software, Inc., Boston, Massachusetts, USA) software. Results were shown as mean ± standard error of the mean (SEM). Significant differences among the treatment groups were analyzed using the non-parametric test, one-way ANOVA, followed by a Tukey post hoc analysis. The statistically significant differences were defined as $P < 0.05$, $P < 0.01$, and $P < 0.001$ among the treatment groups and were represented in the graph as the letters above the bars. Each graph bar annotated with the same letter indicated no statistical difference.

Results

The molecular interaction of LPS/LOS and TLR-MD2

The molecular docking results showed that LPS had the highest binding affinity for TLR-MD2 (-5.7 kcal/mol) compared to LOS, which showed an affinity of -5.5 kcal/mol. However, both ligands had lower binding affinity than the synthetic lipid A analogue, Eritoran, used as a control (Table 1). The molecular docking results were then visualized to determine the binding sites of the ligands and TLR4-MD2. Visualization with Discovery Studio showed that LPS and LOS had the same binding site as the control, which was indicated by several amino acid residues interacting through hydrogen and hydrophobic interactions. This indicated that they might have the same potency as Eritoran.

TYR102 is an amino acid residue with hydrogen interactions formed between TLR4-MD2 and Eritoran or LPS (Figure 1A and 1B), while SER120 formed hydrogen bonds between TLR4-MD2 and LOS (Figure 1C). Several other amino acid residues with hydrophobic interactions strengthened the bond between TLR4-MD2 and the ligand. The same amino acid residues shared between the ligands and control were ILE32, VAL48, ILE52, LEU61, PHE76, ILE117, PHE119, SER120, PHE121, CYS133, VAL135, PHE151. Based on the results of molecular docking and visualization, LPS and LOS might have a high potential to bind to TLR4-MD2.

The molecular dynamic simulation of LPS/LOS and TLR4-MD2 complexes

The stability of the ligand complex with TLR4-MD2 was determined based on molecular dynamic (MD) simulations, since the stability of the protein-ligand complex interaction during the simulation process is indicated by the binding energy of MD [15]. Analysis of 2D images revealed that the LOS-TLR4-MD2 complex at 25

Table 1. Molecular docking result and amino acid residues between ligands and TLR4-MD2 interaction.

Interaction		Binding affinity	Amino acid residues	
Receptor	**Ligand**	**(kcal/mol)**	**Hydrogen interaction**	**Hydrophobic interaction**
TLR4-MD2	E5564 (Eritoran)	7.0	TYR102	ILE32, VAL48, ILE52, LEU61, ILE63, TYR65, LEU71, LEU74, PHE76, ILE94, PHE104, VAL113, THR115, ILE117, PHE119, SER120, PHE121, CYS133, VAL135, LEU146, PHE147, PHE151
	LPS *E. coli*	5.7	TYR102	ILE32, VAL48, ILE52, LEU54, LEU61, PHE76, LEU78, ILE80, ILE117, SER118, PHE119, SER120, PHE121, LYS122, TYR131, CYS133, VAL135, PHE151, ILE153
	LOS *N. gonorrhoeae*	5.5	SER120, LYS122	VAL24, ILE32, ILE46, VAL48, ILE52, LEU54, LEU61, PHE76, LEU78, ILE80, GLU92, PHE119, PHE121, GLY123, ILE124, TYR131, CYS133, VAL135, PHE151, ILE153

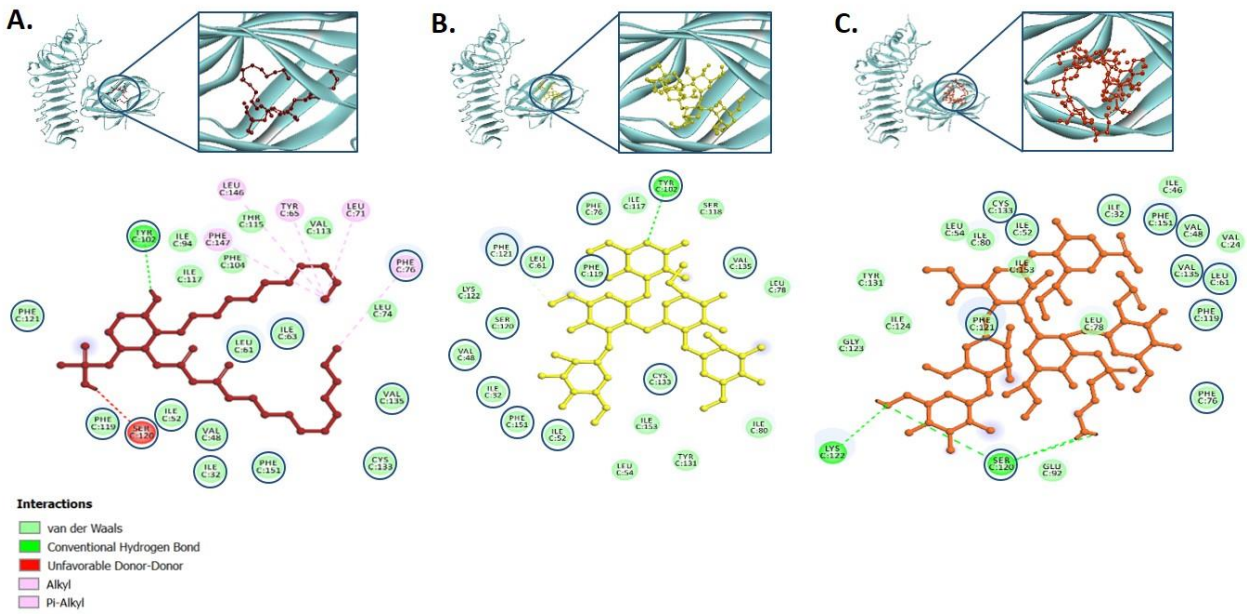

Figure 1. Interactions and amino acid residue between TLR4-MD2 and Eritoran (**A**), TLR4-MD2 and LPS (**B**), TLR4-MD2 and LOS (**C**). Several other amino acid residues with hydrophobic interactions strengthened the bond between TLR4-MD2 and the ligand.

ns until the end of the simulation was unstable, indicated by the binding energy value, which was increasingly negative and significantly different from other ligand-protein complexes (Figure 2A). MD analysis also showed that the number of hydrogen bonds in all ligand-protein complexes was not significantly different and had the same values (Figure 2B).

The Eritoran-TLR4-MD2, Laminaran-TLR4-MD2, and LPS-TLR4-MD2 binding complexes were stable until the end of the simulation (50 ns) with a root-mean-square deviation (RMSD) of 3 Å. The

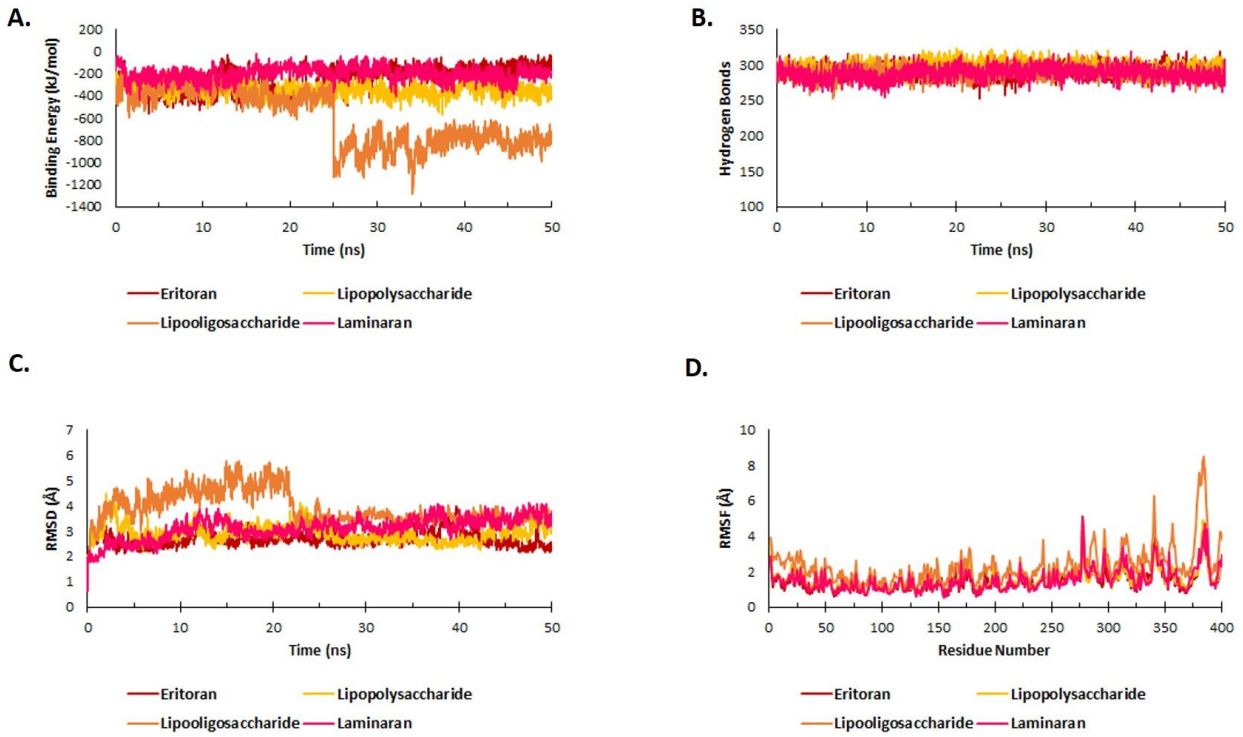

Figure 2. The stability of the ligand complex with TLR4-MD2 was determined based on molecular dynamic (MD) simulations. The stability of the protein-ligand complex interaction during the simulation process is indicated by the binding energy (**A**), the number of hydrogen bonds in all ligand-protein complexes (**B**), root-mean-square deviation (RMSD) (**C**), and root-mean-square fluctuation (RMSF) (**D**).

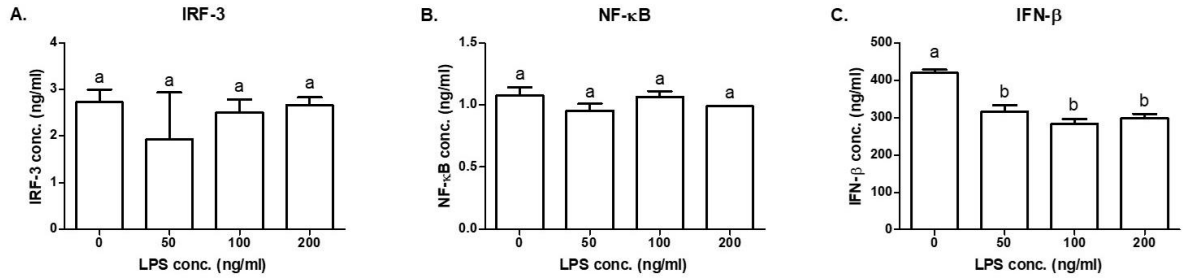

Figure 3. Mean concentration comparison of NF-κβ (**A**), IRF3 (**B**), and IFN-β (**C**) produced by MDDCs after 24-h exposure of different doses of LPS (50, 100, and 200 ng/mL). Different notation was considered significant ($P < 0.05$).

MD simulation showed that the LOS-TLR4-MD2 complex at the beginning of the simulation up to approximately 23 ns was unstable (RMSD value > 3). However, at the end of the simulation, the complex was stable. The root-mean-square fluctuation (RMSF) results showed that three amino acid residues (ASN83, LYS122, and SER127) in the LOS-TLR4-MD2 complex fluctuated. However, the movement during the simulation was still comparable to that of the control (Figure 2C and 2D).

The effect of *E. coli* LPS and *N. gonorrhoeae* LOS exposure on NF-κB, IRF3, and IFN-β levels

Following 24-h exposure of different doses of *E. coli* LPS, there were no significant differences on the IRF-3 and NF-κβ concentrations expressed by the MDDC (Figure 3A and 3B). However, incubation with different dose of LPS significantly reduced the IFN-β concentration, though there were no significant differences observed among the three doses (Figure 3C). Interestingly, 24-h of *N. gonorrhoeae* LOS exposure on MDDC

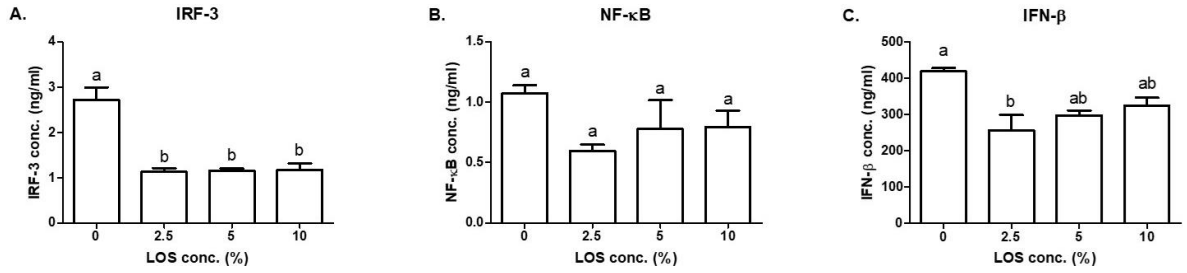

Figure 4. Mean concentration comparison of NF-κβ (**A**), IRF3 (**B**), and IFN-β (**C**) produced by MDDCs after 24-h exposure of different doses of LOS (2.5, 5, and 10%). Different notation was considered significant difference ($P < 0.05$).

significantly reduced the concentration of IRF-3. However, no significant differences were observed among the three doses (Figure 4A). Furthermore, there was no significant difference on NF-κβ expressed by MDDC following LOS exposure (Figure 4B). Notably, the concentration of IFN-β was significantly reduced by the exposure of 2.5% LOS only, but not by other doses (Figure 4C).

Discussion

Binding affinity and molecular dynamic of *E. coli* LPS and *N. gonorrhoeae* LOS

Bacteria, either pathogenic or commensal, produce PAMPs which could be recognized by different kinds of pattern recognition receptor (PRR). Detection of PAMPs by PRR would eventually lead to an inflammatory response to eliminate the microbes. TLR4, as one of the PRR, requires interaction with LBP, CD14, and MD2 to fully interact with LPS or other PAMPs [16]. The binding between TLR4-MD2 complex and its ligands (LPS/LOS) becomes a starting process for inflammation. The binding complex activates the intracellular signalling system, either through myeloid-differentiation primary response (MyD88) dependent pathway, or through TRIF-dependent pathway [1, 5, 6]. These signalling pathways eventually initiate the activation of many transcription factors, such as NF-κβ and IRF3, which subsequently also induce the formation of IFN-β [17].

LPS/LOS, through lipid A, directly binds MD2 molecule from TLR4-MD2 complex to form a dimer on the extracellular part of the cell membrane, which activates an intracellular signal [4]. The inner core of LPS has 2 – 3 units of 3-deoxy-α-D-manno-oct-2-ulopyranosonic acid (Kdo) that bind to glucosamine (GlcN) in the distal part of lipid A through β-(2→6) binding and 3 units of Hep. Meanwhile, the outer core of LPS consists of monosaccharides, such as glucose (Glc) and galactose (Gal) [18]. However, previous studies did not indicate significant influences of the numbers of the sugar cores of LPS/LOS in the endotoxicity process [5, 19, 20], since, unlike lipid A that binds MD2, the sugar core only interacts with the TLR4 molecule. Nevertheless, the sugar core contributes to increase the binding affinity and specificity of LPS/LOS to TLR4-MD2 complex [5]. Some studies suggested that complete LPS structures elicit greater activities towards MDDCs through TLR4 rather than a single lipid A structure [21]. Our study indicated that LPS from *E. coli* had a higher binding affinity to TLR4-MD2 compared to LOS from *N. gonorrhoeae*, although E55 (Eritoran) as control ligand still exerted the highest binding affinity. *N. gonorrhoeae* LOS and *E. coli* LPS bound to TLR4-MD2 at the same site as Eritoran. However, the result from MD simulation showed that the stability of TLR4-MD2 and *N. gonorrhoeae* LOS complex was comparable to *E. coli* LPS. This result suggested that the *N. gonorrhoeae* LOS might have less potential as a TLR4-MD2 antagonist compared to LPS. Meanwhile, Eritoran, as a synthetic lipid A analogue, has a selective antagonist activity toward endotoxin-mediated immune cell

activation. Eritoran antagonizes the activity of LPS by binding to the hydrophobic pocket of MD2 without any direct interaction to TLR4. Eritoran covers almost 90% of the pocket which corresponded to our results, where it showed highest binding affinity toward the TLR4-MD2 complex [3].

E. coli LPS and *N. gonorrhoeae* LOS exposure on naïve HIV MDDCs

Previous studies pointed out the importance of structure-activity relationship (SAR) of the lipid A variants from LPS/LOS [1, 5, 6]. SAR plays an important role in the immunological activity of LPS/LOS and some factors govern the SAR of LPS/LOS [2, 22]. One of the key factors of SAR is the total number of lipid chains, in which the lipid A with 6 chains has the optimal inflammatory activity, while lipid A with 5 lipid chains has 100-fold lower inflammatory activity. Lipid with 4 lipid chains does not show any agonist activity, such as Eritoran. LPS and LOS, beside having different numbers of sugar molecules, own different compositions, and lengths of lipid A chains; *E. coli* LPS mainly consisted of C14 chains while *Neisseria spp*. LOS mostly contained C12 chains. Lipid A variants also have differences in the acylation patterns, the locations of binding between lipid chain and disaccharide molecules. *E. coli* has a 4+2 pattern, while *Neisseria spp*. has 3+3 pattern, where previous studies showed more potent inflammatory and stronger endotoxic activity of the *Neisseria* lipid A compared to that of *E. coli* [5, 6]. In our study, binding affinity apparently was not associated with the capability of the ligands to trigger inflammation. Exposure of *N. gonorrhoeae* LOS significantly affected pro-inflammatory cytokines. These results contradicted the study performed by Pridmore *et al.* [20], which showed that *E. coli* LPS exposure induced higher pro-inflammatory cytokine, TNF-α, production than either *N. gonorrhoeae* or *N. meningitidis* LOS exposure. However, another study showed that gonococcal LOS elicited higher inflammatory cytokines than *E. coli* LPS [23].

Different production of pro- and anti-inflammatory cytokines by LPS and LOS exposure might be related to several factors. The molar ratios of two molecules, LOS and LPS, are different when used at the same concentration [23]. Therefore, there should be a standard dose or concentration of these two molecules. Even though sugar core molecules only interact with TLR4 without binding to MD2, several studies suggested that the number, nature, and location of the Kdo unit could modulate the molecular conformation of LPS/LOS and lipid A. The conformation is tightly linked to the endotoxic activity of LPS/LOS. Therefore, LOS with fewer Kdo sugars might have less inflammatory activity than LPS. Overall, given the contradictory results between studies, the association between the binding affinity of LPS/LOS to the TLR4-MD2 complex and the endotoxic activity measured by pro-inflammatory molecules (NF-κB, IRF3, and IFN-β) still needs to be further investigated.

Dendritic cells act as the first cell population infected by HIV upon mucosal contact and a professional APC to transfer the viral particles to CD4 T lymphocytes. Mature dendritic cells are less sensitive to HIV infection and induction of maturation using LPS and LOS have been shown to inhibit viral entry to dendritic cells. This was thought to be mediated through the TLR signalling pathways, which includes NF-κβ, activator protein-1 (AP-1), IRF-3, and IRF7, the latter two are also involved in production of type I interferons (IFN-α and IFN-β) [10]. MDDC is a subset of dendritic cells involved in the process of inflammation and infection. These cells are derived from stimulated and activated monocytes in the blood circulation [24]. A study by Cheong *et al*. mentioned that after monocytes differentiate into MDDCs, there would be an increase in the expression of TLR4 and TLR7 on their surfaces [25]. Activation of those TLRs, known as IFN receptors, subsequently induce dendritic cell maturation, thus stimulating cell mediated immunity. Maturation of dendritic cells can also be triggered by viral infection. Another study also stated that TLR4 activation induced

monocyte differentiation into MDDC, facilitating efficient antigen presentation to T cells [26].

A previous study showed that the administration of LPS to macrophages reduced the replication of HIV. LPS and LOS are components of the outer membrane of Gram-negative bacteria, such as *E. coli*. TLR4, together with MD2, will recognize LPS or LOS through oligomerization and activate downstream signals leading to the secretion of type I interferons. Intravenous administration of LPS, as natural ligand of TLR4, in fact, increased MDDCs in lymph nodes [25]. The main transcription factors activated by the TLR signalling pathway are NF-κβ, activator protein-1 (AP-1), IRF-3, and IRF7. NF-κβ and AP-1 stimulate the expression of genes encoding many molecules required for the inflammatory response, including inflammatory cytokines (e.g., TNF and IL-1), chemokines (e.g., CCL2 and CXCL8), and endothelial adhesion molecules (e.g., E-selectin). IRF3 and IRF7 activate the production of type I interferons (IFN-α and IFN-β), which are important for antiviral innate immunity response [10]. A study also found that the administration of LPS after the integration of viral genetic material decreased the activation of viral DNA. This is supported by the fact that LPS administration reduced the level of reverse transcription and integration of viral DNA [27]. In this study, neither *E. coli* LPS nor *N. gonorrhoeae* LOS affected the production of NF-κβ by MDDC. These results contradicted previous studies which showed a linear increase in NF-κβ levels along with the dose of TLR4 ligand or agonist [28-30]. Studies suggested that the transcriptional activity of NF-κβ increased with LPS administration due to increased phosphorylation of Ser536 which further increased the transcriptional activity of the p65 NF-κβ subunit [17, 29]. Administration of LPS as a TLR2 and TLR4 agonist to MDDCs increased Iκβ phosphorylation, thereby triggering nuclear translocation of NF-κβ and increased NF-κβ activation [27]. Several studies have also proven that bacterial LPS can induce HIV-1 gene expression in monocyte lineages via the NF-κβ pathway [31]. In this study, 24-h exposure of *N. gonorrhoeae* LOS decreased the level of IRF-3, which was subsequently predicted to suppress the secretion of antiviral cytokines. The IRF-3 activation pathway is associated with activation of the independent MyD88 CD14/TLR4 complex. IRF-3 activation leads to the antiviral cytokine secretion (IFN-α and IFN-β). As the MyD88 dependent pathway can be activated by LPS, the MyD88 independent pathway can also be activated by the administration of bacterial LPS, which successively activates IRF-3 [31]. The IRF-3 pathway will further suppress the production of HIV-1 virus through the production of type I interferons. In most cells, IRF-3 is stored in an inactive form. IRF-3 activation occurs *via* the PRR pathway. LPS and viral ssRNA can bind to TLR3 and TLR4 to activate the TIR-domain-containing adapter-inducing interferon-β (TRIF). TRIF together with mitochondrial antiviral-signalling protein (MAVS) and stimulator of interferon genes (STING) will trigger IRF-3 phosphorylation. IRF-3 will be rapidly activated by phosphorylation which induces subsequent dimerization. Successive IRF-3 activation will lead to interactions with additional cytosolic proteins, nuclear localization, transcriptional activity in early innate antiviral genes, and their exit from the nucleus [32, 33]. In this study, we did not find significant differences in IRF-3 levels following different concentrations of LPS exposure. Again, this finding appeared to contradict previous studies which showed that the administration of LPS increased IRF-3 levels [31, 34]. A study by Liu *et al*, which aimed to explore the effect of *E. coli* LPS exposure on IFN-β levels resulting from IRF-3 phosphorylation, found that the concentration of LPS as low as 10 ng/mL was able to increase the expression of IFN-β in dendritic cells, while LPS concentration of 100 ng/mL induced the highest level of TLR4 signalling [7]. The binding between LPS and TLR induced TLR4 through the TRIF pathway (*via* MyD88-independent adapter protein), which in turn causes phosphorylation of IRF-3 and increases the production of type I interferons. Our study also indicated that the highest levels of IFN- β was found in the negative control group and the levels of IFN-β were significantly suppressed after 24 h of LPS

administration. Production of IFN-β increased significantly after HIV infection, peaking at 24 h post-infection, which corresponded well with our study in the negative control group [35]. However, the measurement of IFN-β in the LPS-treated groups contradicted previous studies which stated that the binding of LPS to TLR4 would induce an increase in IFN-β production [8, 27, 36].

Type I interferons have been shown to inhibit HIV-1 replication in human cells, and IFN-β plays an important role in mediating LOS-induced antiviral activity via TLR4 [7, 36]. In this study, we found that 2.5% *N. gonorrhoeae* LOS exposure decreased IFN-β production of MDDC, but the higher concentrations failed to exhibit the same effect. Meanwhile, various concentration of *E. coli* LPS significantly reduced IFN-β production. Type I interferons generally have a pleiotropic effect that affects several stages of the HIV viral cycle from initial viral uptake to the release of new virions. In a study, it was mentioned that LPS played a role as strong inducer for the expression of IFN-α and IFN-β in immature dendritic cells. However, stimulation of LPS on TLR2, 5, 7, and 9 did not lead to type I interferon secretion. TLR4 induction was directly proportional to the decrease in viral production in immature dendritic cells. The administration of LPS caused an increase in the production of type I interferons drastically starting from 2 hours after exposure until reaching a peak in 6 hours. Although it is known that LPS administration also increased NF-κβ production and subsequently HIV-1 entry, this effect was neutralized by the increased antiviral activity produced by interferon/LPS stimulation [27]. Interestingly, a study found that HIV infection inhibited the maturation of MDDCs, which was indicated by the failure of surface marker detection. Although this study also mentioned similar responses of HIV-infected MDDCs to LPS stimulations, the measurement of responses was performed 1 h post-exposure. In another study, Fantuzzi *et al* suggested that HIV-exposed MDDCs produced significantly lower levels of inflammatory cytokines after LPS administration compared to healthy MDDCs. It also corresponded to the results of a study by Muthumani *et al* which showed that HIV-1 viral protein r (vpr) inhibited the maturation and activation of dendritic cells [37]. Therefore, these studies might explain our current results in which HIV-infected MDDCs failed to increase the expression NF-κβ after LPS administration.

The levels of IRF-3 and IFN-β in this study was possibly influenced by the inactivation of the transcription factor IRF-3 after the exposure of LPS or LOS, thus hindering IFN-β production. A study by Harman *et al*. indicated an inhibition of type I IFN expression in MDDCs by HIV-1 infection through IRF-3 activation inhibition by vpr [38]. Another study also confirmed that HIV vpr can decrease IRF-3 activity by stimulating IRF-3 degradation. Conversely, excessive activation of IRF-3 caused hyperphosphorylation of several serine residues in the signal response domain (SRD) which contractively leads to auto-inhibition of IRF-3 [32]. In HIV-infected cells, IRF-3 can also undergo proteosome degradation, a mechanism adopted by the virus to evade cellular antiviral responses [39]. IRF-3 degradation therefore causes failure of MDDCs to increase IRF-3 and IFN-β production in response to LPS exposure. These aforementioned factors might also explain the low levels of IFN-β in this study. The low IFN-β levels in this study was also probably influenced by the ELISA which was performed 24 h after the LPS exposure. A study by Liu *et al.* suggested that IFN-β levels would increase within 2 – 4 hours after LPS administration and reaching a peak in 8 hours [7]. IFN-β levels would then decrease tremendously within 24 hours. These results were consistent with the initiation of IFN-β secretion from dendritic cells after LPS exposure, suggesting that IFN-β production by dendritic cells rapidly induces phosphorylation of the STAT1 signalling pathway and expression of antiviral genes [34]. Therefore, the decrease of IFN-β in the current study might be correlated with the timing of measurement by which the IFN-β levels already decreased.

There are several technical limitations in this study. Further research should explore the

optimum doses of *E. coli* LPS and *N. gonorrhoeae* LOS used for MDDC stimulation, as no other studies described the effect of both stimuli on NF-κB, IRF3, and IFN-β expressed on dendritic cells. This study also did not directly measure the levels of HIV RNA in dendritic cells even though the dendritic cells were taken from naive HIV patients, thus direct relationship could not be drawn on the effect of LPS and LOS on HIV replication in dendritic cells. For future studies, the detection of CD80, CD83, and CD86 markers might be added to determine the maturation phenotype of the DCs [40].

Conclusion

LOS from *N. gonorrhoeae* had a lower binding affinity to TLR4-MD2 receptor compared to *E. coli* LPS. This binding affinity was hardly associated with the capability of the ligands to trigger inflammation. *E. coli* LPS suppressed the secretion of IFN-β from MDDCs after 24-h exposure, while the production of IRF-3 was significantly decreased by the exposure of *N. gonorrhoeae* LOS. Dose optimization is strongly recommended for future exploration to study the mechanism of inflammatory responses of the MDDC. We also suggest investigating LBP and CD14 as important receptors for *E. coli* LPS or *Neisseria gonorrhoeae* LOS in future molecular docking studies to fully analyze the capability of LPS/LOS to induce inflammation.

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