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Soluble MD2 increases TLR4 levels on the epithelial cell surface

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ABSTRACT

The accessory protein MD2 has been implicated in LPS-mediated activation of the innate immune system by functioning as a co-receptor with TLR4 for LPS binding at the cell surface. Epithelial cells that play a role in primary immune response, such as in the lung or gut, often express TLR4, but are dependent on circulating soluble MD2 (sMD2) to bind TLR4 to assemble the functional receptor. In this study, we show that sMD2 incubation with HEK293 epithelial cells transfected with TLR4 increases the cell surface levels of TLR4 in the absence of LPS. Dose response studies reveal that a threshold sMD2 concentration (~450 nM) stimulates maximal TLR4 levels on the cell surface, whereas higher concentrations of sMD2 (~1800 nM) reduce these enhanced TLR4 levels. We show evidence that MD2 multimer formation is increased at these higher concentrations of sMD2 and that addition of LPS to sMD2-stimulated cells masks the enhanced TLR4 cell surface levels, most likely due to the LPS-induced downregulation of TLR4 by endocytosis following receptor stimulation. All together, these results support a model in which sMD2 binds to TLR4 and increases TLR4 levels at the cell surface by preventing TLR4 turnover through the endocytic pathway. Thus, sMD2 may prime epithelial cells for enhanced immunoresponsive function prior to LPS exposure.

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1. Introduction

The Toll-like receptors (TLRs) are a family of innate immune receptors that recognize an array of conserved motifs in pathogens, termed pathogen-associated molecular patterns (PAMPs), and subsequently activate a potent host immune response to fight the invading pathogen [1,2]. TLRs are type I transmembrane proteins expressed at the cell surface of immunocompetent cells, such as macrophages, dendritic cells, and epithelial cells that line organs such as the lung or gut. One of the most well-characterized TLRs is TLR4, which has been implicated in mediating the host cellular response to lipopolysaccharide (LPS), an outer membrane component of Gram-negative bacteria [3,4]. Upon LPS exposure, TLR4 induces multiple signal transduction pathways that culminate in NF-κB activation, a major transcriptional regulator of the inflammatory response, and release of pro-inflammatory cytokines to further stimulate both the innate and adaptive immune systems.

LPS-mediated TLR4 signaling involves an elaborate molecular mechanism that requires the sequential transfer of LPS by three additional cellular proteins for maximal cell activation [5]. The serum lipopolysaccharide-binding protein (LBP) initially binds LPS released from the bacterial cell wall and transfers LPS monomers to an LPS binding protein, CD14, that does not itself have signaling capability [6,7]. From CD14, LPS is then transferred to a heterodimeric protein complex consisting of TLR4 and an accessory molecule MD2, which forms the complete recognition site for LPS. MD2 is a 20–25 kDa glycoporotein that is expressed as both a TLR4-bound complex on the cell surface and as an active secreted form (sMD2) in the extracellular medium consisting of a heterogeneous collection of disulfide-linked monomers and oligomers [8]. TLR4 binds to monomeric MD2 with a Kd ~12 nM in humans, which remains unchanged by the presence of LPS [9]. Given that the serum concentration of monomeric sMD2 in healthy humans has been estimated to be ~50 nM, the majority of cell surface TLR4 on immune cells in the plasma would be expected to be complexed with MD2 [8,10]. MD2 has been shown to be an absolute requirement for LPS-mediated TLR4 activation in mouse knockout studies [11–13], and directly binds LPS with a Kd ~65 nM in the absence of other LPS binding proteins [14]. Furthermore, LPS-conjugated beads are able to precipitate both sMD2 and a sTLR4/MD2 complex, but not sTLR4 by itself, suggesting that it is the MD2 in the TLR4-MD2 heterodimeric complex that interacts with LPS to activate TLR4-mediated signaling events [15].

The expression levels of TLR4, CD14, and MD2 have been shown to vary greatly among different cell types. Macrophages and monocytes express all three proteins, whereas endothelial cells express only TLR4 and MD2 and are dependent on soluble CD14 in

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the body to enhance LPS signaling [16]. Epithelial cells from the respiratory airway express only TLR4, with little or no detectable expression of MD2 [17]. Given the critical role that epithelial cells in the lung and gut play in immediate host immune responses as the primary barrier between the body and the outside environment, it has been postulated that circulating sMD2 released by macrophages or immature dendritic cells migrates into the extravascular spaces lining the epithelial cells in order to promote TLR4-mediated LPS activation [8,18]. Several studies have shown that exogenously-added sMD2 can bind TLR4 and enable LPS-dependent stimulation of epithelial cells that express TLR4 but not MD2 [10,19,20]. Indeed higher levels of sMD2 activity have been detected in plasma from patients with severe sepsis, suggesting that sMD2 is an important mediator of organ inflammation during sepsis [18,21].

In this study, we describe a novel function for sMD2, to increase steady state levels of TLR4 on the cell surface in epithelial cells that function in innate immunity. We find that incubation of sMD2 with HEK293 epithelial cells expressing TLR4 led to an increase in TLR4 levels on the cell surface in the absence of LPS. Furthermore, there exists a threshold concentration of sMD2 beyond which the increase in sMD2-induced TLR4 surface expression is inhibited, and at this threshold concentration, the population of higher order multimeric sMD2 is increased. These findings suggest a model in which sMD2 prevents TLR4 downregulation by binding to and maintaining TLR4 on the cell surface to prime immune cells for a rapid immune response to pathogen exposure.

2. Materials and methods

2.1. Cells and reagents

Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) were propagated in DMEM, 10% FBS, 2 mM glutamine at 37°, 5% CO_2 and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfection efficiencies were >90% in HEK293 cells. The pCMV-Flag1 and pMSCV-Flag TLR4 plasmid constructs were obtained from Dr. Tianyi Wang (U. Pittsburgh). The following antibodies were used: rabbit anti-MD2 (Imgenex, San Diego, CA), anti-Flag M2 (IgG1) (Stratagene, La Jolla CA), anti-HisG antibody (Stratagene), goat anti-mouse HRP (Amersham, Piscataway, NJ) and FITC-labeled goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA or BD Pharmingen, San Diego, CA). The monoclonal mouse anti-MD2 antibody 18H10 (IgG2 b) was a kind gift from Dr. Greg Elson (NovImmune SA, Geneva, Switzerland). HEK293 cells were stimulated using Ultrapure lipopolysaccharide (*E.coli* 0111:B4) (InvivoGen, San Diego, CA). FITC-labeled calibration microspheres with known numbers of FITC molecules per microsphere (Quantum FITC MESF) were acquired from Bangs Laboratories (Fishers, IN).

2.2. Insect cell expression and purification of sMD2

The coding sequences for MD2 and CD14 were cloned by PCR from pDUO hMD2/TLR4 and hCD14/TLR2 (InvivoGen), respectively, and inserted in-frame into Baculovirus insect cell expression vector pAcGP67-B, which contains an N-terminal signal sequence that drives expression of proteins through the secretory pathway (BD Biosciences, San Jose, CA). A 6× HisG tag was engineered at the C-terminus of both constructs. Recombinant baculoviruses expressing MD2 and CD14 were generated according to the manufacturers' instructions in Sf21 insect cells and amplified to a high titer of 10^8 pfu/ml. This stock virus was then used to infect High Five insect cells to generate high protein levels. Supernatants from infected High Five cells were collected, concentrated using Amicon filters with a cut-off of 10 kD, and dialyzed against Buffer A (20 mM Tris–HCl pH 7.5, 100 mM NaCl). Supernatants were then incubated with TALON metal affinity resin (Clontech, Mountain View, CA) to bind the His-tagged MD2 and CD14. The beads were washed with Buffer A plus 10 mM imidazole pH 7, and the protein was eluted with Buffer A plus 100 mM imidazole, pH 5.3, and then further concentrated and dialyzed against Phosphate buffered saline (PBS). Final protein concentrations of purified sMD2 and CD14 were determined using the BCA protein assay (Pierce, Rockford, IL). Concentrations of prepared baculoviral sMD2 stocks were ~250 μg/ml, and sMD2 was estimated to be ~60% monomeric, ~30% dimeric, and ~10% multimeric by non-reducing gels. (Fig. 1B) The concentration of sMD2 used in all experiments was adjusted to reflect the concentration of monomeric sMD2.

2.3. Protein electrophoresis and Western blotting

Protein samples from the sMD2 purification scheme were boiled in sample buffer (50 mM Tris–HCl pH 6.8, 2% SDS, 100 mM DTT, 0.1% bromophenol blue), separated on a 12% gel by SDS–PAGE, and transferred to a nitrocellulose filter. The filters were blocked in 5% milk in Tris buffered saline/0.05% Tween (TBS/Tween) for 1 h and probed with a 1:5000 dilution of anti-HisG antibody overnight at 4 °C. Filters were washed 3× in TBS/Tween for a total of 30 min, probed with a 1:10,000 dilution of anti-mouse antibody conjugated to HRP for 1 h, washed again, and developed using ECL Plus chemiluminescence (Amersham). For non-reducing gels, the DTT was removed from the sample buffer during protein sample boiling.

2.4. Luciferase assay

10^5 HEK293 cells were transfected simultaneously with 0.5 μg pMSCV-TLR4 plasmid, 100 ng pNifty NF-κB luciferase reporter (Invivogen), and 50 ng *Renilla* luciferase plasmid (Promega, Madison, WI) using Lipofectamine 2000. After 18 h, all cells were incubated with 10 nM sCD14, and 10 nM sMD2 were added to the indicated samples. After an additional 6 h, cells were then stimulated with 1 μg/ml LPS for another 6 h. Protein lysates were generated by lysing cells in Passive Lysis Buffer and luciferase activity was measured using the Dual-Glow Luciferase kit (Promega) on a luminometer plate reader (Turner Systems, Sunnyvale, CA). The measurements from the *Renilla* luciferase signal were used to control for variations in cell transfection and lysis efficiency.

2.5. Characterization of antibodies

Protein concentrations of antibodies used for flow cytometry analysis were determined using the BCA protein assay (Pierce). Fluoresceinated antibodies were also measured by absorbance at 488 nm using an extinction coefficient of $8.0 \times 10^4\ M^{-1}\ cm^{-1}$ (Mercola et al., 1972). Fluorescein stock solutions were measured by absorbance at 488 nm using an extinction coefficient of $7.25 \times 10^4\ M^{-1}\ cm^{-1}$. Cuvette fluorescence measurements were made using a QM6 spectrofluorimeter (Photon Technology International, Birmingham, NJ). Spectra were acquired and integrated and the buffer background subtracted using the Felix 32 software. The fluorophore to protein ratio (F/P) was found to be 3.25 and 5.6 for FITC-labeled goat anti-mouse IgG from Santa Cruz Biotechnology and BD Pharmingen, respectively. The quantum yields of fluorescent antibodies relative to free fluorescein were determined by comparing fluorescence measurements of the absorbance-calibrated stock solutions and were 0.344 and 0.412 for the FITC-labeled goat anti-mouse IgG from Santa Cruz Biotechnology and BD Pharmingen, respectively.