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MD-2 Mediates the Ability of Tetra-Acylated and Penta-Acylated Lipopolysaccharides to Antagonize *Escherichia coli* Lipopolysaccharide at the TLR4 Signaling Complex[1]

Stephen R. Coats,[2]* Thu-Thao T. Pham,* Brian W. Bainbridge,*† Robert A. Reife,*† and Richard P. Darveau*†

We have demonstrated previously that tetra-acylated LPS derived from the oral bacterium, *Porphyromonas gingivalis*, and penta-acylated *msbB* LPS derived from a mutant strain of *Escherichia coli* can antagonize the ability of canonical hexa-acylated *E. coli* LPS to signal through the TLR4 signaling complex in human endothelial cells. Activation of the TLR4 signaling complex requires the coordinated function of LPS binding protein (LBP), CD14, MD-2, and TLR4. To elucidate the specific molecular components that mediate antagonism, we developed a recombinant human TLR4 signaling complex that displayed efficient LPS-dependent antagonism of *E. coli* LPS in HEK293 cells. Notably, changes in the expression levels of TLR4 in HEK293 cells modulated the efficiency of antagonism by *P. gingivalis* LPS. Both soluble (s) CD14 and membrane (m) CD14 supported efficient *P. gingivalis* LPS-dependent and *msbB* LPS-dependent antagonism of *E. coli* LPS in the recombinant TLR4 system. When cells expressing TLR4, MD-2, and mCD14 were exposed to LPS in the absence of serum-derived LBP, efficient LPS-dependent antagonism of *E. coli* LPS was still observed indicating that LPS-dependent antagonism occurs downstream of LBP. Experiments using immunoprecipitates of sCD14 or sMD-2 that had been pre-exposed to agonist and antagonist indicated that LPS-dependent antagonism occurs partially at sCD14 and potently at sMD-2. This study provides novel evidence that expression levels of TLR4 can modulate the efficiency of LPS-dependent antagonism. However, MD-2 represents the principal molecular component that tetra-acylated *P. gingivalis* LPS and penta-acylated *msbB* LPS use to antagonize hexa-acylated *E. coli* LPS at the TLR4 signaling complex. *The Journal of Immunology,* 2005, 175: 4490–4498.

The TLR4 signaling complex comprises an important part of the mammalian innate immune system that is responsible for the initial ability of cells to detect and respond to LPSs from Gram-negative bacteria (1–3). The TLR4 complex consists of the serum-derived LPS binding protein (LBP)[3] and serum-derived or cell membrane-bound forms of CD14, which bind and transfer LPS to the LBP, MD-2, which is associated at the cell surface with TLR4 (4–14). Subsequently, TLR4 transmits intracellular immune response signals resulting in the activation of transcription factors such as NF-κB, which trigger host cytokine inflammatory responses (15, 16). *Escherichia coli* LPS is the best known example of a potent agonist for TLR4, and it has been used extensively to study this signaling pathway. Several LPS antagonists have been reported. For example the tetra-acylated lipid A precursor of *E. coli* LPS, lipid IVa, exhibits potent antagonist activity to *E. coli* LPS in human monocytes (17). A penta-acylated form of *E. coli* LPS that is derived from an *E. coli* strain bearing a mutation in the *msb*B gene can efficiently antagonize the ability of hexa-acylated *E. coli* LPS to activate human endothelial cells (18). Penta-acylated LPS isolated from *Rhodobacter sphaeroides* also exhibits the ability to antagonize *E. coli* LPS (17). In addition, synthetic penta-acylated lipid A-like compound, E5531, is an antagonist for LPS-dependent cell activation that has been considered as a potential therapeutic agent for bacterial-induced septic shock (19, 20).

LPS antagonists have received significant attention as potential therapeutic agents for septic shock (21). However, the authentic physiological role of natural LPS antagonists in human biology remains enigmatic but has recently received increased attention. For example, LPS isolates from the oral pathogen, *P. gingivalis*, have been shown to antagonize *E. coli* LPS-dependent induction of E-selectin expression, p38 MAPK activation, and NF-κB activation in human endothelial cells (22–24). *P. gingivalis* LPS has also been shown to inhibit *E. coli* LPS-stimulated release of TNF-α, IL-β, and IL-6 in human monocytes, as well as ICAM-1 expression in human gingival fibroblasts (25, 26). Recently, our laboratory performed structural analyses of antagonistic *P. gingivalis* LPS preparations and found that they are comprised mainly of tetra-acylated lipid A structures (27, 28). The ability of antagonistic *P. gingivalis* LPS to down-regulate critical innate immune responses has been postulated to play an important role in this oral pathogen's ability to override the normal surveillance system that is essential to maintaining periodontal health (22, 23, 26, 29). In addition, recent study has suggested that antagonist forms of LPS isolated from *Helicobacter pylori* play a role in the ability of this microbe to induce gastric inflammation (30). Given the potential biological importance of LPS-dependent antagonism, further research into the molecular mechanism of antagonism is necessary.

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[3] Abbreviations used in this paper: LBP, LPS binding protein; sCD14, soluble CD14; mCD14, membrane CD14; sMD-2, soluble MD-2; HEK, human embryonic kidney.

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We recently reported that both a tetra-acylated *P. gingivalis* LPS preparation as well as penta-acylated *msbB* LPS preparation antagonize *E. coli* LPS at the TLR4 signaling complex of human endothelial cells (24). However, in that study, we were unable to resolve the precise molecular site(s) required to mediate LPS-dependent antagonism due to limitations imposed by endogenous gene expression of the key signaling components, TLR4 and MD-2, in human endothelial cells. In the present study, we developed a recombinant TLR4 signaling system in HEK293 cells that recapitulated endothelial cell type antagonism to elucidate the molecular sites required for *P. gingivalis* LPS and *msb*B LPS to antagonize *E. coli* LPS. Through systematic examination of each of the major TLR4 signaling components, including LBP, CD14, and MD-2, we demonstrate that antagonism occurs partially at CD14 but that MD-2 is the principal molecular site that mediates LPS-dependent antagonism. Unexpectedly, we also observed that the expression levels of TLR4 determine the efficiency of antagonism in this recombinant system. The implications of these findings are discussed in the context of current models of LPS-dependent antagonism.

Materials and Methods

Bacterial strains, LPS preparations, and cytokines

E. coli O111:B4 LPS was obtained from Sigma-Aldrich. This preparation was further purified by the method of Manthey and Vogel (31) to remove trace contaminating proteins. *msbB* LPS was isolated from the *E. coli*, mutant strain, BMS67C12, by the hot-phenol method as described previously (18). MALDI-TOF mass spectrometry analyses confirmed the presence of a negative mass ion consistent with the previously published diphosphorylated penta-acylated lipid A structure (18) (data not shown). *P. gingivalis* ATCC was obtained from the American Type Culture Collection, stocked, and grown as described previously (27). *P. gingivalis* LPS was prepared by the cold $MgCl_2$-ethanol procedure (32) followed by lipid extraction (33) and conversion to sodium salts (34). The preparation used in this study was characterized by MALDI-TOF mass spectrometry and gas chromatography-mass spectroscopy and found to consist of monophosphorylated, tetra-acylated lipid A structures (27).

Cell lines

The human embryonic kidney cell lines HEK293 and HEK293T were obtained from the American Type Culture Collection. Both of these cell lines were maintained in DMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml). The human endothelial cell line, HMEC-1 (passage 11), was obtained from F. J. Candal (Centers for Disease Control and Prevention, Atlanta, GA). HMEC-1 cells were maintained in MCDB-131 medium (Invitrogen Life Technologies) supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid constructs

The expression plasmid bearing a cDNA of human MD-2 (pMD-2) encodes both a FLAG epitope and a 6× His at the C-terminal end of the protein and was obtained from K. Miyake (University of Tokyo, Tokyo, Japan). pNF-κB-TA-Luc was obtained from BD Biosciences (San Jose, CA). pβ-Actin *Renilla* Luc has been described previously (35). The expression construct encoding human membrane CD14 (pmCD14) was described previously (35). The expression vector pcDNA3.1(-) is from Invitrogen Life Technologies. The modified version of the expression vector, pDisplay, has been described previously (35). The expression plasmid construct, psCD14, encodes a FLAG epitope and a 6× His epitope at the C-terminal portion of the recombinant protein. This construct was generated by using PCR to amplify the coding region of mCD14 such that the last eight native amino acids encoded by human mCD14 are replaced with nucleotide sequences encoding the FLAG epitope followed by the 6× His epitope. The primers used to generate this construct are as follows: sense primer, 5′-TTACAGCGGATCCGAGGTTCGGAAGACTTATCGAC-3′; and antisense primer, 5′-ATCATCTAAGCTTCAGTGGTGGTGGTGG TGGTGCTTGTCATCGTCGTCCTTGTAGTCCTTCAGCACCAGGGT TCCCGAC-3′. The PCR product resulting from the amplification of pmCD14 with the indicated primers was then digested with *Hin*dIII and *Bam*HI and subsequently ligated into *Hin*dIII and *Bam*HI-digested pcDNA3.1(-) to generate psCD14. The human TLR4 expression constructs used in this study were generated as follows: a search of the National Center for Biotechnology Information database revealed four predicted mRNA splice variants for human TLR4. The accession numbers for the splice variants are as follows: TLR4 splice variant 1 (SV1) encodes aa 1–839, no. NM_138554; TLR4 SV2 NM_138556; TLR4 SV3 encodes aa 41–839, NM_003266; and TLR4 SV4 encodes aa 201–839, NM_138557. Subsequent analyses of total RNA from HUVECs or HMEC-1 cells by RT-PCR revealed the presence of mRNAs corresponding to TLR4 SV1, TLR4 SV3, and TLR4 SV4 in both cell types (data not shown). To generate pTLR4SV4, the following primer set was used to amplify pHATLR4, a plasmid encoding an N-terminal hemagglutinin epitope-tagged version of human TLR4 (aa 27–839) (36): sense primer, 5′-AGATTCGCTAGCG TATTTATTGCACAGACTTGCG-3′; antisense primer 5′-AGAT TCAAGCTTCAGATAGATGTTGCTTCCTGC-3′. The resulting PCR amplification product containing a *Nhe*I site at the 5′-end and a *Hin*dIII site at the 3′-end was ligated into pcDNA3.1(-) that had been digested with *Nhe*I and *Hin*dIII. The resulting construct, pTLR4SV4, contains an open reading frame encoding aa 201–839 of human TLR4. To generate pTLR4SV1 and pTLR4SV3 expression constructs, HUVEC total RNA was isolated as previously described (24) and was amplified by RT-PCR using the following primer sets: sense primer, 5′-TTCGCTAGCACTGCTCG GTCAGACGGTGATAGC-3′; and antisense primer, 5′-ATCGAGGTAG TAGTCTAAGTATGC-3′. The resulting PCR products corresponded to the cDNA fragments encoding the N-terminal region of either TLR4 SV1 or TLR4 SV3 and contained a *Nhe*I site at the 5′-end and an *Eco*RI site toward the 3′-end of each fragment. These fragments were isolated and cloned into the pGem-T easy vector (Promega). The resulting plasmid constructs were then digested with *Nhe*I and *Eco*RI and the respective TLR4 SV1 and TLR4 SV3 inserts isolated. pTLR4SV4 was digested with *Nhe*I and *Eco*RI to remove the SV4 N-terminal fragment leaving the C-terminal portion of TLR4 that was shared by all of the splice variants. Subsequently, the *Nhe*I/*Eco*RI fragments of TLR4 SV1 and SV3 were ligated into pTLR4SV4 (*Nhe*I/*Eco*RI) to yield pTLR4SV1 and pTLR4SV3, which encode human TLR4 aa 1–839 and 41–839, respectively. The nucleotide sequence integrity of all plasmid constructs created in this study was confirmed by Big Dye Terminator DNA sequencing at the University of Washington DNA sequencing facility.

Luciferase reporter assays

HEK293 cells were plated in 96-well plates at a density of 4×10^4 cells/well and transfected the following day by the standard calcium phosphate precipitation method. The transfected cells were allowed to grow 20–24 h until stimulations were performed. The test wells were stimulated in triplicate for 4 h at 37°C and 5% CO_2 with the indicated doses and mixtures of LPSs in DMEM containing either 10% FBS, 10% human serum, or serum-free DMEM as indicated in the text and figure legends. The total amount of DNA transfected into each well of the 96-well plate was 0.1 μg. Standard amounts of the plasmid constructs used to transfect HEK293 cells per well of the 96-well plate were as follows: pβ-actin *Renilla* Luc (0.0003 μg), pNF-κB-TA-Luc (0.002 μg), pTLR4SV1 (0.006 μg), and pMD-2 (0.0025 μg). However, when pmCD14 (0.0025 μg) was included in the mixture, the standard amount of pTLR4SV1 used was (0.002 μg). The empty expression vector, pDisplay, was used to adjust the total amount of DNA transfected per well up to 0.1 μg. After stimulation, the cells were rinsed with PBS and lysed with 50 μl of passive lysis buffer (Promega). Luciferase activity of 10 μl of each lysate was measured using the Dual Luciferase Assay Reporter System (Promega). Data are expressed as fold increase of NF-κB activity, which represents the ratio of NF-κB-dependent firefly luciferase activity to β-actin promoter-dependent *Renilla* luciferase activity. Transfections of HMEC-1 cells, stimulations, and luciferase reporter assays were performed exactly as described previously (24).

Functional immunoprecipitation analysis of antagonism

To determine the ability of sCD14 bearing a C-terminal FLAG epitope to mediate LPS-dependent antagonism at TLR4, the following procedure was used: HEK293T cells were plated in 10-cm plates, grown to 50% confluency, and transfected with 20 μg of psCD14/plate by the calcium phosphate method. The following day, growth medium was removed and replaced with serum-free medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml), and the cells were incubated for an additional 2 days before harvesting the supernatant. Expression of recombinant sCD14 was verified by Western blotting analysis (data not shown). Preimmunoprecipitation antagonism experiments were performed by combining sCD14 supernatant with DMEM containing 10% human serum. *E. coli* LPS doses alone or in combination with *P. gingivalis* LPS or *msb*B LPS were added to 1.6 ml of the sCD14 containing medium and incubated for

3–4 h at 2°C. Immunoprecipitations of the sCD14-LPS complexes were performed by adding 40 μl of washed EZview Red Anti-FLAG M2 affinity gel (Sigma-Aldrich) to 1 ml of each sample and agitating the suspension overnight at 4°C. To remove unbound LPS, the suspensions were centrifuged at 8000 $\times$ *g* for 30 s, and the supernatants were removed. The pellets were then washed twice with 1 ml of ice-cold PBS. To recover sCD14 from the agarose beads, the pellets were resuspended in 340 μl of serum-free DMEM containing FLAG peptide (200 μg/ml) (Sigma-Aldrich) and agitated at 21°C for 30 min. The sCD14-LPS complexes were then used to stimulate HEK293 cells that had been transfected with standard amounts of pTLR4SV1, and pMD-2, and luciferase reporter assays were performed as described above. To determine the role of recombinant sMD-2 bearing a C-terminal FLAG epitope in mediating LPS-dependent antagonism at TLR4, the same procedure that was used for sCD14 was followed, except that the sMD-2-LPS complexes were used to stimulate HEK293 cells that had been transfected with standard amounts of pTLR4SV1 alone.

Results

TLR4 splice variants in human endothelial cells

We previously reported that tetra-acylated *P. gingivalis* LPS antagonizes *E. coli* LPS at an unidentified extracellular point of the TLR4 signaling pathway in human endothelial cells (24). To dissect the molecular components required for LPS-dependent antagonism in the TLR4 signaling pathway, we initially attempted to develop a recombinant TLR4 signaling system that displayed efficient *P. gingivalis* LPS-dependent antagonism by using a recombinant TLR4 construct used in other studies (27, 36). However, none of the experiments using HEK293 cells transiently transfected with the recombinant TLR4 construct, which generates a receptor bearing a N-terminal hemagglutinin epitope, exhibited significant LPS-dependent antagonism (data not shown). Because TLR4 is a central component of the LPS receptor complex, we suspected that either the N-terminal epitope was interfering with the normal function of the receptor with respect to antagonism or that endothelial cells might express an alternative form of TLR4. Subsequently, we searched GenBank for sequence information pertaining to TLR4. This search revealed four possible splice variants of the mRNA called SV1, SV2, SV3, and SV4 (Fig. 1*A*). The splice variants are predicted to generate four distinct TLR4 protein isoforms called A, B, C, and D, respectively (Fig. 1*B*). To determine the profile of TLR4 components in human endothelial cells, we analyzed RNA from HUVECs and HMEC-1 cells by RT-PCR using primer sets to detect the specific splice variants. The analyses indicated that SV1, SV3, and SV4 are readily detected in both HUVECs and HMEC-1 cells (data not shown). TLR4 SV1 is predicted to generate isoform A, which corresponds to the canonical TLR4 bearing an N-terminal signal sequence important for surface expression, whereas TLR4 SV3 and SV4 code for isoforms C and D, respectively, which lack obvious N-terminal signal sequences. Accordingly, the cDNAs for each of these splice variants were synthesized from HUVEC RNA and cloned into a mammalian expression vector to generate pTLR4SV1, pTLR4SV3, and pTLR4SV4.

A recombinant TLR4 signaling system transiently expressed in HEK293 cells supports efficient P. gingivalis *LPS-dependent antagonism of* E. coli *LPS*

We have shown previously that a preparation of *P. gingivalis* LPS (1 μg/ml) consisting of tetra-acylated lipid A efficiently antagonizes *E. coli* LPS at doses of 10 and 100 ng/ml in both HUVECs and HMEC-1 cells via the TLR4 signaling complex (24, 27). To construct a recombinant "endothelial-type TLR4 signaling complex," we used HEK293 cells, which do not express TLR4, MD-2, and CD14, all of which are required components of the LPS receptor complex. The ability of human embryonic kidney (HEK) cells that had been cotransfected with pTLR4SV1, a plasmid encoding FLAG/HIS-tagged human MD-2 (pMD-2), and a NF-κB-luciferase reporter plasmid (pNF-κB-Luc) to support *P. gingivalis* LPS-dependent antagonism of EcLPS was tested by exposing cells to different doses of *E. coli* LPS alone or mixed with *P. gingivalis* LPS (1 μg/ml) in the presence of 10% serum as a source of sCD14 and LBP (Fig. 2*A*). The results of this experiment indicate that *P. gingivalis* LPS (1 μg/ml) significantly antagonized both *E. coli* LPS (10 ng/ml) and *E. coli* LPS (100 ng/ml)-dependent activation of NF-κB. In contrast, *P. gingivalis* LPS did not antagonize the ability of IL-1β (10 ng/ml) to stimulate NF-κB activity through the endogenous IL-1R demonstrating that antagonism cannot be explained by postreceptor signaling intermediates shared by the TLR4 and the IL-1R signaling pathways. We also observed that *P. gingivalis* LPS preparations, which exhibit a weak ability to activate cells through TLR2, are unable to antagonize the more potent ability of peptidoglycan to stimulate TLR2-transfected HEK293 cells, thus indicating specificity for *P. gingivalis* LPS-dependent antagonism on the TLR4 signaling pathway (data not shown).

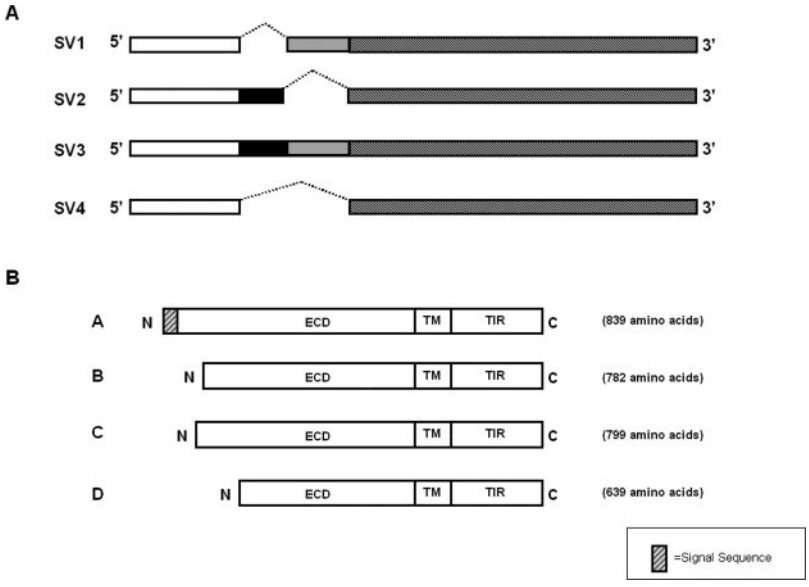

FIGURE 1. Human endothelial cells express three splice variants of TLR4 mRNA. *A*, Schematic representation of TLR4 mRNA splice variants obtained from GenBank. Shaded rectangles represent differentially spliced exons. *B*, Schematic representation of TLR4 isoforms predicted from the coding sequences of the splice variants. ECD, extracellular domain; TM, transmembrane domain; TIR, TLR/IL receptor homology domain.

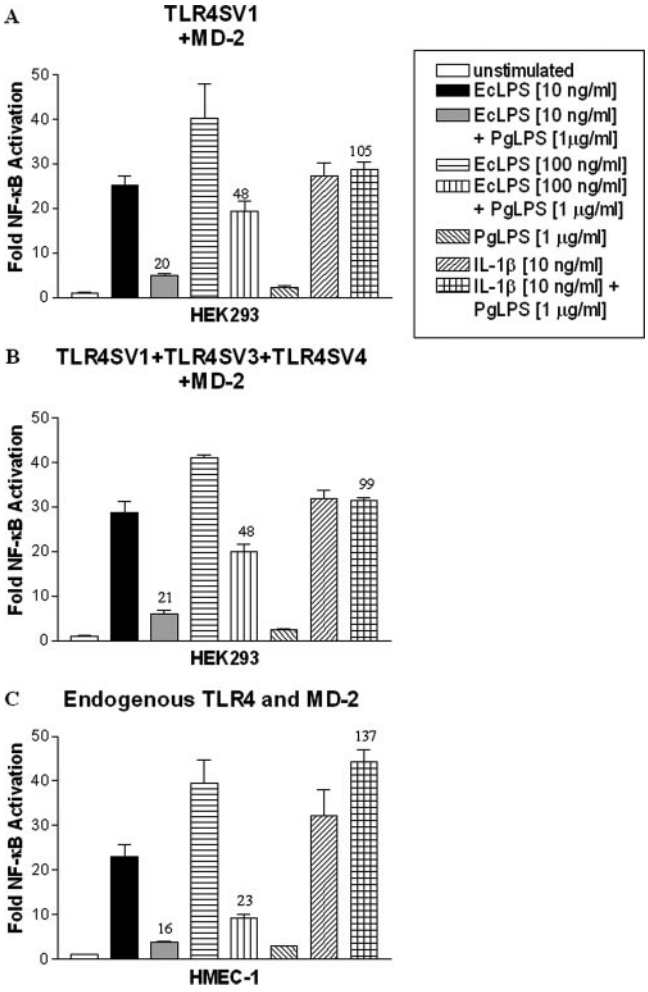

FIGURE 2. Transient expression of a recombinant TLR4/MD-2 signaling complex in HEK293 cells recapitulates endothelial cell-type LPS-dependent antagonism. *A*, HEK293 cells were cotransfected with pTLR4SV1 and pMD-2 and stimulated with the indicated doses and mixtures of *E. coli* LPS, *P. gingivalis* LPS, or IL-1β in the presence of 10% FBS. *B*, HEK293 cells were cotransfected with pTLR4SV1, pTLR4SV3, pTLR4SV4, and pMD-2 and subsequently stimulated with the indicated doses and mixtures of LPS or IL-1β in the presence of 10% FBS. *C*, HMEC-1 cells expressing endogenous TLR4 and MD-2 were transfected with pNF-κB-TA Luc that was used to detect TLR4-stimulated NF-κB activation. Cells were stimulated with the indicated doses and mixtures of LPS or IL-1β in the presence of 10% human serum. Results of the luciferase assay are shown as fold NF-κB activation. Numbers *above* the bars indicate the percent activation obtained for each respective dose of *E. coli* LPS in combination with *P. gingivalis* LPS. Results are presented as the means ± the SDs of triplicate determinations and are representative of three independent experiments.

P. gingivalis LPS (1 μg/ml) alone elicited only minor NF-κB activation in HEK293 cells transfected with pTLR4SV1 (Fig. 2*A*). Similar effects of *P. gingivalis* LPS have been observed in human endothelial cells (24). To test the effects of the other TLR4 isoforms on LPS-dependent antagonism, HEK293 cells were cotransfected with pTLR4SV1, pTLR4SV3, pTLR4SV4, phMD-2, and pNF-κB-Luc and subjected LPS treatments identical to those shown in Fig. 2*A* (Fig. 2*B*). As seen in Fig. 2*B*, coexpression of the three TLR4 splice variants yielded results that were very similar to the results depicted in Fig. 2*A*. In addition, when tested independently, neither pTLR4SV3 nor pTLR4SV4 yielded detectable NF-κB activity in response to LPS in the HEK293 cell system (data not shown). These results indicate that only pTLR4SV1 is required to support efficient antagonism in the recombinant system in HEK293 cells. Fig. 2*C* illustrates a comparable antagonism experiment performed in the endothelial cell line, HMEC-1. Clearly, similar trends of antagonism and specificity are apparent when the results in Fig. 2, *A* and *B*, are compared with the results in Fig. 2*C*. We conclude that the recombinant TLR4 signaling system in HEK293 cells displays *P. gingivalis* LPS-dependent antagonism of *E. coli* LPS that resembles the phenotype observed for the HMEC-1 endothelial cell system.

Changes in expression levels of TLR4 modulate the efficiency of P. gingivalis *LPS-dependent antagonism of* E. coli *LPS*

Although the recombinant TLR4 system clearly displays efficient PgLPS-dependent antagonism of *E. coli* LPS, we noted that the efficiency of antagonism for comparable doses of LPS was consistently greater in endothelial cells than in the HEK293 cells. For example, 1 μg/ml *P. gingivalis* LPS reduces 100 ng/ml *E. coli*-dependent NF-κB activation to 48% of maximal levels in HEK293 cells (Fig. 2, *A* and *B*), whereas similar stimulation of endothelial cells results in NF-κB activation at 23% of maximal levels (Fig. 2*C*). We hypothesized that the expression levels of TLR4 and MD-2 in the context of a transient expression system are likely to be higher relative to the levels of the respective endogenous proteins in endothelial cells, and this might explain the reduced efficiency. To address this question, we varied the amounts of either pTLR4SV1 or pMD-2 by 10-fold relative to standard amounts transfected into HEK293 cells and performed antagonism experiments by stimulating transfected cells with *E. coli* LPS alone or in combination with *P. gingivalis* LPS (Fig. 3). When pTLR4SV1 and pMD-2 were cotransfected into HEK293 cells at standard concentrations (1×), *P. gingivalis* LPS (1 μg/ml) antagonized 1, 10, and 100 ng/ml *E. coli* LPS-dependent activation of NF-κB to 25, 32, and 59% the maximal levels, respectively (Fig. 3*A*). When 10× the standard concentration of pMD-2 was cotransfected with the standard amount of pTLR4SV1, the resulting degree of *P. gingivalis* LPS-dependent antagonism observed was not significantly altered (Fig. 3*B*). However, cotransfecting 10× TLR4SV1 together with 1× pMD-2 resulted in significantly reduced efficiencies of antagonism (Fig. 3*C*). Cotransfecting 10× pMD-2 with 10× pTLR4SV1 yielded no further changes in the efficiency of antagonism (Fig. 3*D*). These experiments demonstrate that the efficiency of antagonism is modulated by the amounts of TLR4 expressed in this recombinant system.

Influence of mCD14 on LPS-dependent antagonism of E. coli *LPS*

CD14 is an integral part of the TLR4 signal transduction cascade that is required for efficient transfer of LPS to MD-2/TLR4. In myeloid cells such as human monocytes, the membrane form of CD14 (mCD14) is abundantly expressed on the cell surface and plays an important role in LPS-dependent activation (6). In non-myeloid cells such as human endothelial cells, soluble CD14 derived from serum is required for optimal LPS signaling responses (7). We have previously shown that tetra-acylated *P. gingivalis* LPS is capable of only a very weak activation of NF-κB in TLR4/MD-2-transfected HEK293 cells when soluble CD14 is used, whereas significant activation of NF-κB is observed when mCD14 is coexpressed in this system (27). These observations suggest that the presence of mCD14 may influence the ability of *P. gingivalis* LPS to antagonize *E. coli* LPS in the recombinant TLR4SV1/MD-2-transfected HEK293 cells. To test this we examined the ability of *P. gingivalis* LPS to antagonize *E. coli* LPS in cells that were expressing TLR4SV1, MD-2, and mCD14 (Fig. 4*A*). When mCD14 was present in the system, efficient antagonism was observed. As seen in other studies, increased sensitivity of the TLR4

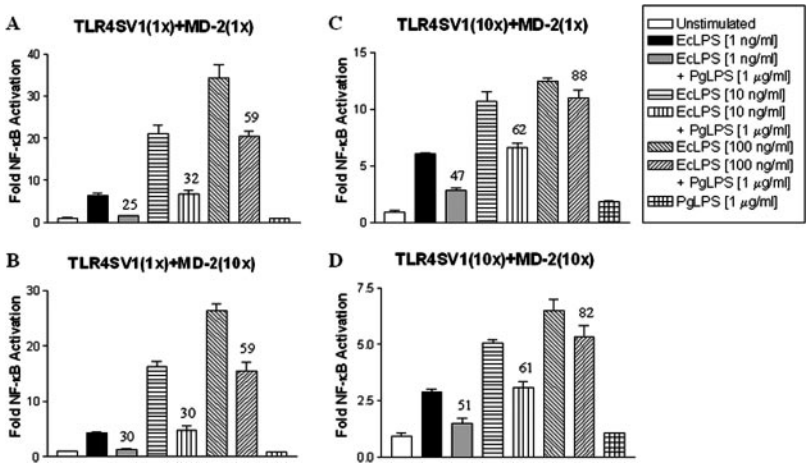

FIGURE 3. Increased expression of TLR4 modulates the efficiency of LPS-dependent antagonism. HEK293 cells were cotransfected with different amounts of pTLR4SV1 and pMD-2 indicated and subsequently stimulated with the indicated doses and combinations of *E. coli* LPS and *P. gingivalis* LPS in the presence of 10% FBS. *A*, HEK293 cells were transfected with standard (1×) amounts of both pTLR4SV1 and pMD-2. *B*, HEK293 cells were transfected with 1× amounts of pTLR4SV1 and 10× amounts of pMD-2. *C*, HEK293 cells were transfected with 10× amounts of pTLR4SV1 and 1× amounts of pMD-2. *D*, HEK293 cells were transfected with 10× amounts of both pTLR4SV1 and pMD-2. Results of the luciferase assay are shown as fold NF-κB activation. Numbers above the bars indicate the percent NF-κB activation obtained for each respective dose of *E. coli* LPS in combination with *P. gingivalis* LPS. Results are presented as the means ± the SDs of triplicate determinations and are representative of three independent experiments.

signaling complex to low concentrations of LPS (1 ng/ml) was observed when both mCD14 and sCD14 were present during cell stimulation. In addition, the ability of tetra-acylated *P. gingivalis* LPS to stimulate TLR4-dependent activation of NF-κB was enhanced in the presence of mCD14 consistent with other results obtained from experiments in our laboratory (27). Similar to experiments that used only sCD14, the expression levels of TLR4 also influenced the efficiency of antagonism when mCD14 was present in the recombinant system (data not shown). We conclude that both sCD14 and mCD14 support efficient *P. gingivalis* LPS-dependent antagonism in the recombinant TLR4 signaling system.

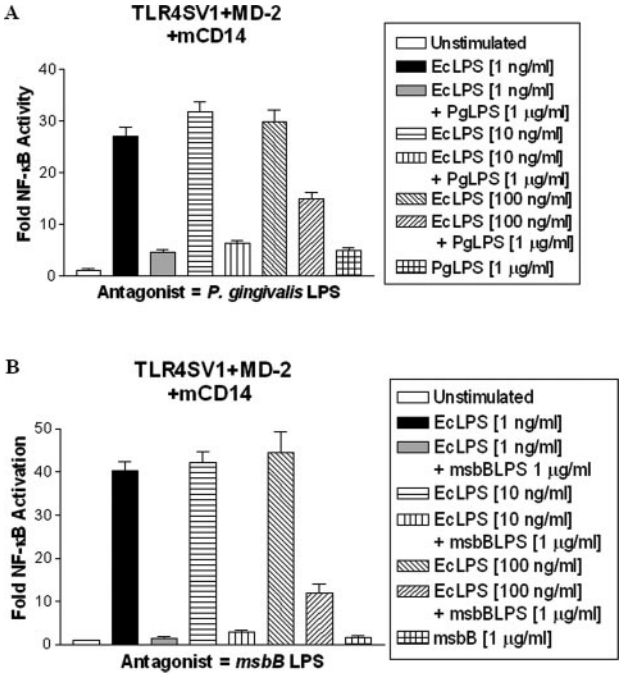

FIGURE 4. Membrane CD14 supports LPS-dependent antagonism of *E. coli* LPS at the TLR4 signaling complex. *A*, HEK293 cells were cotransfected with pTLR4SV1, pMD-2, and pmCD14 and stimulated with the indicated doses and mixtures of *E. coli* LPS and *P. gingivalis* LPS in the presence of 10% FBS. *B*, HEK293 cells were cotransfected with pTLR4SV1, pMD-2, and pmCD14 and stimulated with the indicated doses and mixtures of *E. coli* LPS and *msbB* LPS in the presence of 10% FBS. Results of the luciferase assay are shown as fold NF-κB activation. Results are presented as the means ± the SDs of triplicate determinations and are representative of three independent experiments.

One of the main goals of this study was to determine the molecular component(s) of the TLR4 signaling pathway that tetra-acylated *P. gingivalis* LPS uses to antagonize hexa-acylated *E. coli* LPS. Because there are multiple potential molecular sites for antagonism to occur, it is of interest to determine whether another LPS antagonist exhibits different or similar patterns of action to more clearly delineate the general requirements for LPS-dependent antagonism of *E. coli* LPS. To address this question, we chose to analyze the ability the *E. coli* LPS variant, *msbB* LPS, to function as an antagonist in the recombinant TLR4 system because this LPS was shown previously to antagonize the ability of *E. coli* LPS to stimulate human endothelial cells (18, 24). *msbB* LPS is generated from a mutant strain of *E. coli* bearing a mutation in the *msbB* locus of the LPS synthesis pathway (18). As a result of this mutation, the lipid A of this LPS is penta-acylated, lacking a secondary myristic acid at the primary hydroxy-myristic acid at the 3′ position of the glucosamine disaccharide residue. Antagonism experiments using 1 μg/ml *msbB* LPS combined with doses of *E. coli* LPS ranging from 1 to 100 ng/ml were performed in HEK293 cells that were expressing TLR4SV1, MD-2, and mCD14 (Fig. 4*B*). Under these conditions, *msbB* LPS functions as a strong antagonist against *E. coli* LPS. Thus, both tetra-acylated *P. gingivalis* LPS and penta-acylated *msbB* LPS function as effective antagonists to *E. coli* LPS in this recombinant TLR4 signaling system.

LBP is not required for tetra-acylated or penta-acylated LPSs to antagonize E. coli *LPS*

Having established the recombinant TLR4 signaling system as a vehicle for exploring the mechanism of antagonism, we set out to dissect the individual steps involved in LPS signaling to identify the required molecular components for antagonism. One of the earliest events in the LPS signaling pathway is the capture of LPS

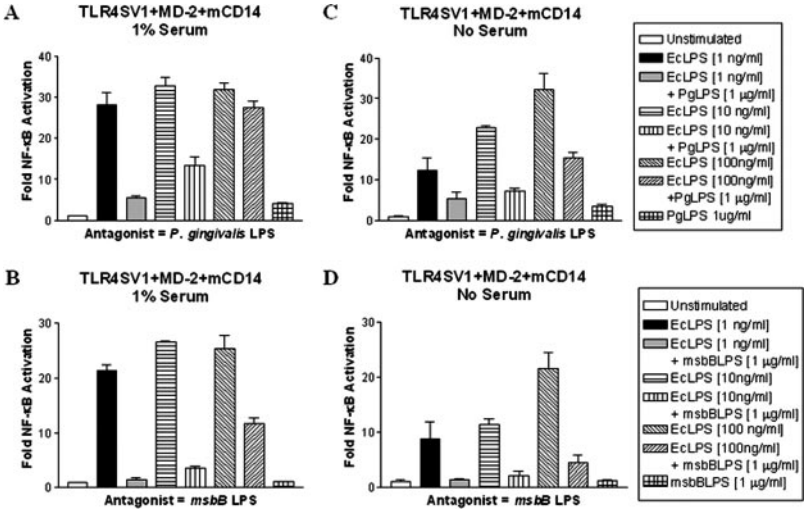

FIGURE 5. *P. gingivalis* LPS-dependent and *msbB* LPS-dependent antagonism of *E. coli* LPS at the TLR4 complex does not require LBP. *A*, HEK293 cells were cotransfected with pTLR4SV1, pMD-2, and pmCD14 and stimulated with the indicated doses and mixtures of *E. coli* LPS and *P. gingivalis* LPS in the presence of 1% FBS. *B*, HEK293 cells were cotransfected with pTLR4SV1, pMD-2, and pmCD14 and stimulated with the indicated doses and mixtures of *E. coli* LPS and *msbB* LPS in the presence of 1% FBS. *C*, HEK293 cells were cotransfected with pTLR4SV1, pMD-2, and pmCD14 and stimulated with the indicated doses and mixtures of *E. coli* LPS and *P. gingivalis* LPS in the absence of serum. *D*, HEK293 cells were cotransfected with pTLR4SV1, pMD-2, and pmCD14 and stimulated with the indicated doses and mixtures of *E. coli* LPS and *msbB* LPS in the absence of serum. Results of the luciferase assay are shown as fold NF-κB activation. Results are presented as the means ± the SDs of triplicate determinations and are representative of three independent experiments.

by serum-derived LBP (5). To determine the role of LBP in mediating LPS-dependent antagonism, HEK293 cells were transfected with pTLR4SV1, pMD-2, and pmCD14. Subsequently, various doses of *E. coli* LPS alone or in combination with either *P. gingivalis* LPS or *msbB* LPS were mixed in medium containing 1% serum or serum-free medium before stimulation of the transfected HEK293 cells (Fig. 5). Cell stimulations conducted in the presence of 1% serum demonstrated typical efficient LPS-dependent antagonism for both *P. gingivalis* LPS (Fig. 5*A*) and *msbB* LPS (Fig. 5*B*). When cell stimulations were performed in the absence of serum, both *P. gingivalis* LPS (Fig. 5*C*) and *msbB* LPS (Fig. 5*D*) still efficiently antagonized *E. coli* LPS, although the ability of *E. coli* LPS to activate NF-κB was clearly diminished relative to serum containing preparations. These data indicate that serum-derived LBP is not required for the antagonistic activities of *P. gingivalis* LPS and *msbB* LPS in the recombinant TLR4 signaling system and suggest that the major molecular sites of antagonism are downstream from the LBP component of the TLR4 signaling cascade.

Roles of CD14 and MD-2 in mediating LPS-dependent antagonism are revealed by immunoprecipitation of CD14-LPS and MD-2-LPS complexes

The proximal events downstream from LBP engagement of LPS involve a transfer of LPS to CD14 and subsequent presentation of LPS to the TLR4-MD-2 complex (5–7, 11, 13, 14). To separate the requirement for each of these components in mediating LPS-dependent antagonism, we used the abilities for both CD14 and MD-2 to function as soluble molecules in LPS signaling to examine their respective roles (Fig. 6). We examined the abilities of *P. gingivalis* LPS and *msbB* LPS to antagonize *E. coli* LPS at sCD14 by expressing a C-terminal FLAG epitope-tagged version of human sCD14 in HEK293T cells. Supernatants expressing the tagged sCD14 were harvested and mixed with serum as a source of LBP. LBP was included in these experiments to ensure that *P. gingivalis* LPS had sufficient opportunity to transfer to sCD14 because earlier studies have demonstrated that *P. gingivalis* LPS transfers to sCD14 at a significantly slower rate than does *E. coli* LPS (37). The supernatants were then mixed with either varying doses of *E. coli* LPS alone or in combination with *msbB* LPS (1 μg/ml) or *P. gingivalis* LPS (1 μg/ml). Subsequently, these LPS-sCD14 complexes were subjected to immunoprecipitation with anti-FLAG Ab agarose beads and washed to remove unbound LPS. The CD14-LPS complexes were recovered and used to stimulate HEK293 cells that had been transfected previously with pTLR4SV1 and pMD-2. As shown in Fig. 6*A*, the immunoprecipitates of sCD14 that remained associated with different doses of *E. coli* LPS alone effectively stimulated NF-κB activity. The immunoprecipitates that contained either sCD14, *E. coli* LPS, and *P. gingivalis* LPS or sCD14, *E. coli* LPS, and *msbB* LPS exhibited small but significant reductions in their abilities to activate NF-κB. These data suggest that *msbB* LPS and *P. gingivalis* LPS do partially compete *E. coli* LPS at the level of sCD14 in the TLR4 signaling pathway. However, this antagonism was partial and does not account for all of the observed potency of antagonism typically observed in this recombinant system. We used a similar strategy to examine the role of MD-2 in LPS-dependent antagonism (Fig. 6*B*). In this case, HEK293T cells were transfected with pMD-2, which encodes a C-terminal FLAG epitope tag in the protein. Supernatants expressing the sMD-2 were harvested and combined with serum as a source of LBP and CD14. Subsequently, the MD-2 supernatants were treated with doses of LPSs in the presence of serum-derived LBP and sCD14, immunoprecipitated, and washed. The eluted sMD-2-LPS complexes were then exposed to HEK293 cells that had been transfected previously with pTLR4SV1 alone. HEK293 cells expressing TLR4 alone do not exhibit significant NF-κB activation in response to *E. coli* LPS and serum (data not shown). Immunoprecipitates that were generated with MD-2 and *E. coli* LPS elicited significant NF-κB activation at all of the doses tested. This result is in agreement with the reported ability of sMD-2-LPS complexes to stimulate cells transfected with TLR4 alone (38, 39). However, immunoprecipitates that were generated with MD-2 and *E. coli* LPS combined with either *msbB* LPS or *P. gingivalis* LPS

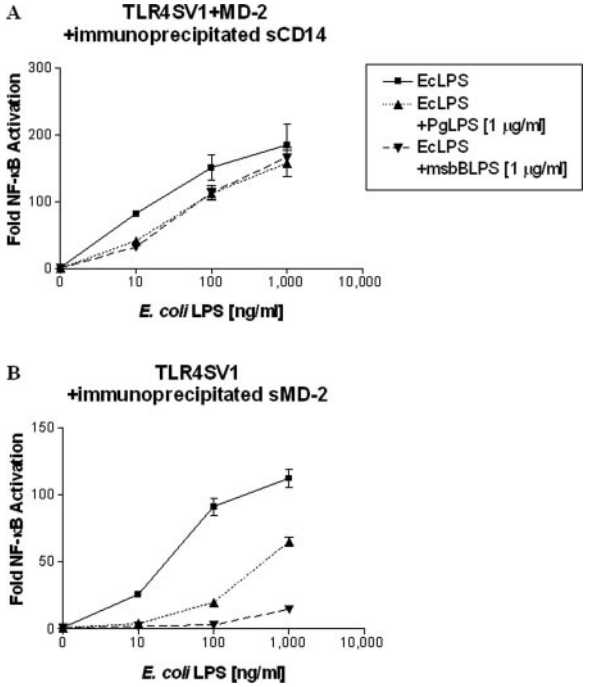

FIGURE 6. Postantagonism immunoprecipitates of sCD14 and sMD-2 reveal the molecular site of antagonism. *A*, HEK293 cells were cotransfected with pTLR4SV1 and pMD-2 and then stimulated with immunoprecipitates of sCD14 that contained the indicated doses and mixtures of *E. coli* LPS and *P. gingivalis* LPS or *E. coli* LPS and *msbB* LPS. *B*, HEK293 cells were transfected with pTLR4SV1 and then stimulated with immunoprecipitates of sMD-2 that contained the indicated doses and mixtures of *E. coli* LPS and *P. gingivalis* LPS or *E. coli* LPS and *msbB* LPS. Results of the luciferase assay are shown as fold NF-κB activation. Results are presented as the means ± the SDs of triplicate determinations and are representative of three independent experiments.

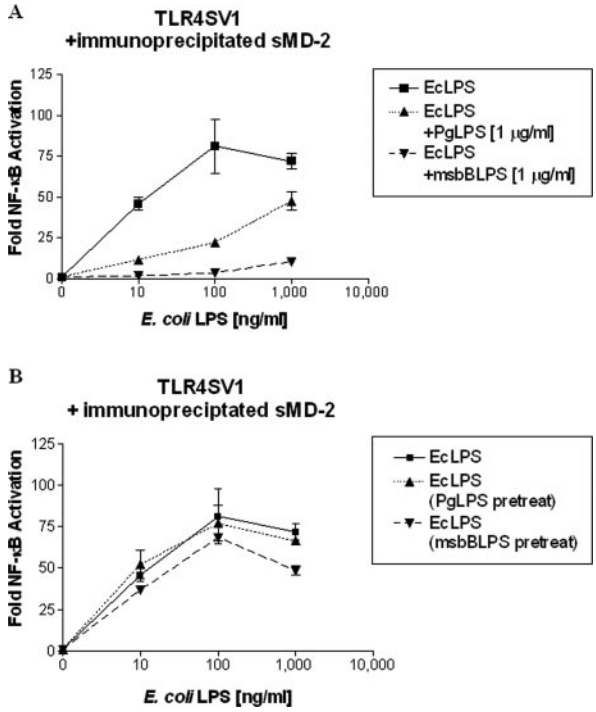

FIGURE 7. Pretreatment of HEK293 cells expressing TLR4 with *E. coli* LPS antagonists does not support efficient antagonism of *E. coli* LPS-MD-2 complexes. *A*, HEK293 cells were transfected with pTLR4SV1 and then stimulated with immunoprecipitates of sMD-2 that contained the indicated doses and mixtures of *E. coli* LPS and *P. gingivalis* LPS or *E. coli* LPS and *msbB* LPS. *B*, TLR4SV1-transfected HEK293 cells were pretreated with either *P. gingivalis* LPS (1 μg/ml) or *msbB* LPS (1 μg/ml) for 1.5 h in the presence of 10% FBS. Subsequently, the medium was removed, and the cells were immediately stimulated with *E. coli* LPS-sMD-2 complexes. Results of the luciferase assay are shown as fold NF-κB activation. Results are presented as the means ± the SDs of triplicate determinations.

were substantially inhibited in their ability to promote NF-κB activation. Given that the LPS-MD-2 associations were established in solution, in the absence of TLR4, these data indicate that TLR4 is not required for the ability of *P. gingivalis* LPS and *msbB* LPS to compete for *E. coli* LPS interaction with MD-2. Relevant to this idea, when sMD-2-*E. coli* LPS complexes were applied to pTLR4SV1-transfected HEK293 cells that had been pretreated with either (1 μg/ml) *P. gingivalis* LPS or (1 μg/ml) *msbB* LPS, significant reduction in NF-κB activation was not observed as compared to non-pretreated cells (Fig. 7*B*). These results clearly contrast with the results obtained by using sMD-2-LPS complexes that were pre-exposed to antagonists prior to immunoprecipitation (Fig. 7*A*). These data provide novel evidence that the primary molecular site of LPS-dependent antagonism of *E. coli* LPS is the MD-2 component of the TLR4 signaling pathway.

Discussion

The main goal of this study was to identify the key molecular component(s) of the TLR4 signaling pathway that mediate the ability of tetra-acylated LPS and penta-acylated LPS antagonists to block the ability of hexa-acylated *E. coli* LPS, a potent agonist, to activate human endothelial cells (24). For this purpose, we developed a recombinant TLR4 signaling system in HEK293 cells, which have been used by other laboratories as well as ours for studying LPS interactions with TLR4 and TLR2 complexes. The composition of this recombinant system was specifically designed to recapitulate the functional aspects the TLR4 signaling complex that exist in human endothelial cells. By using this system, we were able to demonstrate that the antagonistic activity of tetra-acylated LPS derived from *P. gingivalis* required only expression of the SV1 splice variant of TLR4 in combination with MD-2 to support efficient LPS-dependent antagonism. Although the other splice variants, TLR4SV3 and TLR4SV4, are readily detectable in the endothelial cell total RNA population, they do not produce LPS responsive receptor complexes nor do they influence the function of the TLR4SV1 in our recombinant HEK293 cell assay system. Based on the predicted structural properties of TLR4SV3 and TLR4SV4, neither protein is expected to be exported to the cell surface due to the absence of a signal sequence at the N terminus of each protein (Fig. 1*B*). In addition, both of these predicted proteins lack critical N-terminal amino acid residues that have been shown recently to be required for functional interaction with MD-2 and expression at the cell surface (40, 41). It would be interesting to determine whether or not these isoforms function in cell types that purportedly respond to LPS via intracellular pathways (42, 43). Clearly, additional experiments are required to determine the functional significance of the TLR4SV3 and TLR4SV4 splice variants.

During the course of developing the recombinant TLR4SV1 signaling system in the HEK293 cells, we observed that endothelial cells exhibited slightly more efficient antagonism as compared with the transiently transfected HEK293 cells (Fig. 2). Additional investigation indicated that the expression level of TLR4 is a key

determinant mediating the efficiency of *P. gingivalis* LPS-dependent antagonism in the recombinant TLR4 signaling system because increased amounts of transfected pTLR4SV1 led to more potent NF-κB activation by *E. coli* LPS and decreased efficiency of antagonism by *P. gingivalis* LPS (Fig. 3). It is currently not known how increased expression levels of TLR4 lead to reduced efficiency of antagonism. An interesting possibility is that significant increases in TLR4 density on the cell surface may lead to a higher frequency and/or prolonged duration of homomeric receptor interactions and receptor clustering induced by the fraction of *E. coli* LPS-MD-2 complexes that form productive associations with TLR4. This may result in decreased antagonism efficiency. This idea is based on the recent findings that TLR4 signaling may involve both clustering and homotypic interaction of TLR4s upon engagement of MD-2-LPS (44, 45). Regardless of the explanation, the observed concentration effect of TLR4 on antagonism efficiency may have important biological relevance to different subsets of cells that express TLR4. Specifically, myeloid cells such as human monocytes express high levels of mCD14 and TLR4 as compared with nonmyeloid cells such as human endothelial cells, which lack mCD14, depend on sCD14, and express relatively low levels of TLR4 (6, 7, 16, 46, 47). *P. gingivalis* LPS acts a very weak agonist for E-selectin expression and NF-κB activation but as a potent antagonist against *E. coli* LPS-dependent stimulation of E-selectin expression and NF-κB activation in human endothelial cells (22, 24). Similarly, in HEK293 expressing relatively low levels of TLR4 and using serum-derived sCD14, *P. gingivalis* LPS acts as a very weak agonist and strong antagonist. In contrast, *P. gingivalis* LPS acts as a more potent agonist for IL-8 expression in monocytes and a weaker antagonist for *E. coli* LPS (B. W. Bainbridge and R. P. Darveau, unpublished data). This pattern resembles the ability of *P. gingivalis* LPS to stimulate TLR4-dependent NF-κB activation more effectively in transiently transfected cells that are expressing mCD14 and high levels of TLR4 (27) (Fig. 4*A* and data not shown). Future experiments aimed at either reducing the levels of TLR4 in monocytes or increasing the levels of TLR4 expressed in endothelial cells may help to determine the relationship between endogenous levels of TLR4 expression and LPS-dependent antagonism in myeloid and nonmyeloid cell types.

The development of a recombinant human TLR4 signaling system in HEK293 cells facilitated our goal of elucidating the requirements for LPS-dependent antagonism. The experiments in this study indicated that LBP was not required for either *P. gingivalis* LPS-dependent antagonism or *msbB* LPS-dependent antagonism in the presence of mCD14 (Fig. 5). However, it cannot be ruled out that LBP plays a significant role in mediating the antagonistic activities of *P. gingivalis* LPS and *msbB* LPS in the presence of sCD14 (Fig. 6*A*) because it may participate in the delivery of poorly transferred antagonistic LPSs to sCD14 (37). It is conceivable that atypical LPS-LBP-sCD14 complexes may explain the observed antagonism at sCD14. An earlier report suggested that both LBP and CD14 played major roles in mediating the ability *R. sphaeroides* di-phosphoryl lipid A to antagonize *E. coli* LPS (48). Given the complexity of TLR4 signaling, therefore, it needs to be considered that different LPS antagonists may use multiple components of the human TLR4 signaling system.

The present finding that sCD14 plays a partial role in mediating *P. gingivalis* LPS-dependent antagonism (Fig. 6*A*) is consistent with earlier observations from our laboratory that demonstrated the ability for *P. gingivalis* LPS to antagonize *E. coli* LPS-dependent stimulation of endothelial cell E-selectin expression in the presence of mutant sCD14 mutants that interact strongly with *E. coli* LPS but poorly with *P. gingivalis* LPS (49). Also consistent with the current results, observations from other research groups have indicated that the main sites of LPS-dependent antagonism for both lipid IVa and *P. gingivalis* LPS occur in the TLR4 signaling pathway partially at the level of CD14, but other significant determinants of antagonism are downstream from CD14 (25, 44, 50, 51).

The main finding of the present study was that MD-2 represents the principal site used by both tetra-acylated *P. gingivalis* LPS and penta-acylated *msbB* LPS for antagonism of hexa-acylated *E. coli* LPS (Fig. 6*B*). MD-2 has been shown previously to play a critical role in LPS-dependent TLR4 signaling, and its role is consistent with this finding. Namely, MD-2 association with TLR4 is essential for the ability of *E. coli* LPS to elicit TLR4-dependent cell activation (8, 52). In addition, LPS physically binds to MD-2 subsequent to its transfer from LBP and CD14 (10, 14, 38). Furthermore, LPS association with TLR4 requires MD-2 (11, 14). We exploited the ability of isolated sMD-2-LPS complexes to activate TLR4 in HEK293 cells (38, 39) to determine that *P. gingivalis* LPS and *msbB* LPS antagonists can functionally block the productive association of *E. coli* LPS and sMD-2 before interacting with TLR4 (Fig. 6*B*). It has been suggested that sMD-2 might play an important role in modulating the innate responses of epithelial cells that express TLR4 but not MD-2 (39). Relevant to this finding, a recent report has suggested that plasma-derived sMD-2 might play a major role in promoting organ inflammation during the progression of Gram-negative bacterial-induced septic shock (53). Information derived from additional investigation of *msbB* LPS and *P. gingivalis* LPS interactions with LBP, CD14, and MD-2 will be critical in further elucidating the molecular mechanism of LPS-dependent antagonism. These studies should assist in the future design of therapeutic approaches to combat LPS-induced sepsis, as well as provide further insight into the role that natural LPS antagonists play in host-pathogen interactions.

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Disclosures

The authors have no financial conflict of interest.

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