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Cooperation of Toll-Like Receptor 2 and 6 for Cellular Activation by Soluble Tuberculosis Factor and *Borrelia burgdorferi* Outer Surface Protein A Lipoprotein: Role of Toll-Interacting Protein and IL-1 Receptor Signaling Molecules in Toll-Like Receptor 2 Signaling[1]

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Toll-like receptor 2 (TLR2) and TLR4 play important roles in innate immune responses to various microbial agents. We have previously shown that human dermal endothelial cells (HMEC) express TLR4, but very little TLR2, and respond to LPS, but not to *Mycobacterium tuberculosis* 19-kDa lipoprotein, unless transfected with TLR2. Here we report that HMEC are unresponsive to several additional biologically relevant TLR2 ligands, including, phenol-soluble modulin (PSM), a complex of three small secreted polypeptides from the skin commensal *Staphylococcus epidermidis*, soluble tuberculosis factor (STF), and *Borrelia burgdorferi* outer surface protein A lipoprotein (OspA-L). Expression of TLR2 renders HMEC responsive to all these ligands. We further characterized the signaling pathway in response to STF, OspA-L, and PSM in TLR2-transfected HMEC. The TLR2 signaling pathway for NF-κB *trans*-activation shares the IL-1R signaling molecules. Dominant negative constructs of TLR2 or TLR6 inhibit the responses of STF and OspA-L as well as PSM in TLR2-transfected HMEC, supporting the concept of functional cooperation between TLR2 and TLR6 for all these TLR2 ligands. Moreover, we show that Toll-interacting protein (Tollip) coimmunoprecipitates with TLR2 and TLR4 using HEK 293 cells, and overexpression of Tollip inhibits NF-κB activation in response to TLR2 and TLR4 signaling. Collectively, these findings suggest that there is functional interaction between TLR2 and TLR6 in the cellular response to STF and OspA-L in addition to *S. epidermidis* (PSM) Ags, and that engagement of TLR2 triggers a signaling cascade, which shares the IL-1R signaling molecules, similar to the TLR4-LPS signaling cascade. Our data also suggest that Tollip may be an important constituent of both the TLR2 and TLR4 signaling pathways. ***The Journal of Immunology,*** **2001, 167: 987–994.**

Invertebrates and vertebrates protect against microbial infections by sensing the presence of conserved pathogen-associated molecular patterns, such as bacterial LPS or lipoproteins (1–3). Host organisms have developed a set of receptors, known as pattern recognition receptors, that specifically recognize pathogen-associated molecular patterns and trigger a defense cascade (4–8). The discovery of the key role played by Toll in the *Drosophila* host defense as well led in 1997 to the description of the first mammalian Toll homologue, now referred to as Toll-like receptor 4 (TLR4)[4] (9). The human TLRs are pattern recognition receptors of the innate immune system and play an important role in early innate immune recognition and inflammatory responses by the host to microbial challenge (4–10). Mammalian TLRs share with Toll extracellular leucine-rich repeats flanked by characteristic cysteine-rich regions (7, 11). Although at least 10 human homologues of *Drosophila* Toll have been described, identified, or submitted to GenBank (9, 11–13), only two TLRs, TLR2 and TLR4, have known functions. TLR4 is the primary signaling receptor for LPS, whereas TLR2 has a broader role as a pattern recognition receptor and is implicated in inflammatory responses to lipoteichoic acid and bacterial, mycobacterial, and spirochetal lipoproteins. Recently, a role for TLR9 in the response to bacterial DNA has been reported (14).

In *Drosophila*, Toll initiates a signaling pathway homologous to mammalian NF-κB activation using a signaling cascade strikingly similar to the IL-1R signaling complex (3, 4, 7, 9, 15). We have previously shown that TLR4-induced NF-κB activation in human endothelial cells is mediated by IL-1R signaling molecules, including the adapter protein myeloid differentiation protein (MyD88), IL-1R-associated kinase (IRAK), and TNF receptor-associated factor 6 (TRAF6), which links TLR4 to the protein kinase NF-κB-inducing kinase (NIK) (16). A recent study described a new component of the IL-1R signaling pathway, Toll-interacting protein (Tollip), which is present in a complex with IRAK and links IRAK to the IL-1R, and limits IRAK phosphorylation and NF-κB activation (17).

Genetic and experimental studies have now established that TLR4 is the primary signaling receptor for LPS (10, 18–23). Recent reports have documented that mice lacking TLR2 respond normally to LPS (24), and that neither human nor murine TLR2

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[4] Abbreviations used in this paper: TLR, Toll-like receptor; DN, dominant negative; HMEC, human dermal microvessel endothelial cells; iNOS, inducible NO synthase; IRAK, IL-1R-associated kinase; MyD88, myeloid differentiation protein; Mtb, *Mycobacterium tuberculosis*; NIK, NF-κB-inducing kinase; Tollip, Toll-interacting protein; TRAF, TNF receptor-associated factor; PSM, phenol-soluble modulin; STF, soluble tuberculosis factor; wt, wild type; VSV, vesicular stomatitis virus; OspA-L, outer surface protein A lipoprotein.

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can transduce LPS signals in the absence of contaminating endotoxin lipoproteins (21). TLR2 has been implicated as the signaling receptor for Gram-positive cell wall components; bacterial, mycobacterial, and spirochetal lipoproteins; and fungi (24–30). Thus, a concept of division of labor between these two receptors in sensing diverse microbial pathogens and alerting the immune system has emerged. However, it is not clear how TLRs have developed specificity for a given pathogen-associated molecular pattern and to what extent cooperation between different TLRs accounts for the recognition of a specific ligand. An emerging concept in TLR signaling is that TLRs can establish a combinatorial repertoire to discriminate among the large number of pathogen-associated molecular patterns found in nature (31). Recently, the cytoplasmic domain of TLR2 was shown to interact functionally with TLR6 in the recognition of certain TLR2 ligands, such as peptidoglycan, but not to others, i.e., lipoproteins, in mouse macrophage cell lines (31).

Recent studies have shown that a protein-free and heat-stable *Mycobacterium tuberculosis* (Mtb)-conditioned culture supernatant soluble tuberculosis factor (STF) (28), and *Borrelia burgdorferi* outer surface protein A lipoprotein (OspA-L) signal through TLR2 (26, 27). Phenol-soluble modulin (PSM), a complex of three small secreted polypeptides from *Staphylococcus epidermidis* has been shown to activate NF-κB and induce cytokine release in macrophages, suggesting that they may contribute to the systemic manifestations of Gram-positive sepsis (32). PSM was recently shown to signal through TLR2 and to require TLR6 as well (33). Based on the recent data that TLR2 cooperates with TLR6 in response to certain TLR2 ligands but not others, it is unclear whether TLR2 ligands such as STF and OspA-L also require the cooperative effect of TLR6 to signal.

Endothelial cells participate in the early stages of the immune response against various microbial agents and constitute an integral component of the innate immune system. Previously we demonstrated that human dermal microvessel endothelial cells (HMEC) respond to LPS via TLR4, but express very little TLR2 and are unresponsive to Mtb 19-kDa lipoprotein, a TLR2 ligand, unless transfected with TLR2 (22). In the current study we investigate the response of endothelial cells to other biologically relevant TLR2 ligands, including PSM, STF, and OspA-L. We further address whether TLR6 is required for these TLR2-dependent responses in HMEC. We determined that TLR6 functionally cooperates with TLR2 in transducing the STF-, OspA-, and PSM-dependent signals in TLR2-transfected endothelial cells. Downstream of TLR2, our studies explore the signaling pathway leading to activation of NF-κB in response to TLR2 ligands. Our data indicate that the TLR2 signaling pathway shares the IL-1R signal transducer molecules, i.e., MyD88, IRAK, and TRAF6, to activate NF-κB, and that Tollip associates with TLR2 and TLR4 and may be an important signaling component of the TLR2 and TLR4 pathways as well as the IL-1R pathway.

Materials and Methods

Cells and reagents

The immortalized HMEC (gift from F. J. Candal, Center for Disease Control and Prevention, Atlanta, GA) (34) were cultured in MCDB-131 medium supplemented with 10% inactivated FBS, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin in 24-well plates and were used between passages 10 and 14 as previously described (22). Recombinant human IL-1β was obtained from Endogen (Cambridge, MA). PSM, which was purified by phenol extraction of supernatants of stationary *S. epidermidis,* as previously described (32), was obtained from Seymour Klebanoff (University of Washington, Seattle, WA). OspA-L was obtained from Tim Sellati and Justin Radolph (University of Connecticut, Hartford, CT). STF was obtained from Terry K. Means and Matthew J. Fenton (Boston University, Boston, MA). All reagents were verified to be LPS free by the *Limulus* amebocyte lysate assay (Pyrotell, Association of Cape Cod, MA; $<$0.03 EU/ml). Highly purified, phenol-water extracted, and protein-free ($<$ 0.0008% protein) *Escherichia coli* LPS, which was prepared according to the method described by McIntire et al. (35), was obtained from S. N. Vogel (Uniformed Services University, Bethesda, MD). The purity of this LPS preparation has been previously demonstrated (36, 37), and this preparation of LPS is active on TLR4-transfected HEK 293 cells and not on TLR2 transfectants (S. N. Vogel, unpublished observation).

Constructs

Plasmids used in transient transfections were prepared with an endotoxin-free Plasmid Maxi kit (Qiagen, Valencia, CA). Flag-tagged wild-type (wt) human TLR2 and TLR4 constructs were obtained from R. Medzhitov (Yale University, New Haven, CT). ELAM-NF-κB-luciferase construct and the dominant negative (DN) expression vectors of MyD88, IRAK, TRAF6, TRAF2, and NIK have been characterized and described previously (16, 22). Human IL-8 promoter and inducible NO synthase (iNOS) promoter luciferase constructs were provided by N. Mukaida (Kagoshima University, Kagoshima City, Japan) (38), and W. J. Murphy (University of Kansas, Kansas City, KS), respectively. DN TLR2 constructs were obtained from C. J. Kirschning (Technical University of Munich, Munich, Germany). The C3H/HeJ TLR4 cDNA was a gift from B. Beutler (The Scripps Institute, La Jolla, CA) and were described previously (16, 22). C3H/HeJ mice express DN TLR4 encoding a single missense mutation, which converts a cytoplasmic proline residue to histidine (P712H) (18). The analogous mutation was engineered in TLR6 (P691H), which works as a DN. Wild-type and DN mouse TLR6 cDNA expression vectors were provided by A. M. Hajjar and D. Underhill (University of Washington). Vesicular stomatitis virus (VSV)-Tollip cDNA expression vector was provided by J. Tschopp (University of Lausanne, Lausanne, Switzerland). All constructs were verified by sequencing.

Transfection and luciferase assays

HMEC were plated at 2×10^5 cells/24-well plate before transfection. Cells were transfected with FuGene 6 transfection reagent (Roche, Indianapolis, IN) following the manufacturer's instructions as reported previously (16, 22). Reporter genes pCMV-β-galactosidase (0.1 μg), ELAM-NF-κB-luciferase (0.5 μg), and pcDNA3 empty vector or DN mutants of MyD88, IRAK, TRAF6, TRAF2, and NIK (0.1 μg each) were used as described previously (16, 22). In addition, wt human TLR2 (0.5 μg), wt mouse TLR6 (0.5 μg), and different amounts of DN human TLR2 or mouse TLR6 cDNA constructs or wt Tollip cDNA were cotransfected. After 24-h transfection, cells were stimulated for 5 h with various TLR2 ligands, including PSM (50–200 ng/ml), STF (10–30 μl/ml), and OspA-L (10 μg/ml), or with protein-free *E. coli* LPS (20 or 50 ng/ml) in medium containing 10% FCS. Following a 5-h incubation, cells were washed once in PBS and lysed, and luciferase activity was measured with a kit (Promega, Madison, WI) and a luminometer as described previously (16, 22). β-Galactosidase activity was determined by a calorimetric method to normalize transfection efficiency as described previously (16, 22). The data shown are the mean of three independent experiments.

RT-PCR

Total RNA was isolated from HMEC or THP1 cells using a Qiagen kit following the manufacturer's instruction and treated with RNase-free DNase I as previously described (22). For reverse transcription reaction, the Moloney murine leukemia virus preamplification system (Life Technologies, Gaithersburg, MD) was applied. PCR amplification was performed with *Taq* gold polymerase (Perkin-Elmer, Foster City, CA) for 32 cycles at 95°C for 45 s, 54°C for 45 s, and 72°C for 1 min (for TLR2 and TLR4); 35 cycles at 95°C for 30 s and 65 and 72°C for 1 min (for TLR6); and 38 cycles at 95°C for 1 min, 61°C for 1 min, and 72°C for 1 min (for Tollip). The oligonucleotide primers used for RT-PCR were: TLR2, 5′-GCCAAAGTCTTGATTGATTGG and 5′-TTGAAGTTCTCCAGC TCCTG; TLR4, 5′-TGGATACGTTTCCTTATAAG and 5′-GAAATG GAGGCACCCCTTC; TLR6, 5′-AGAACTCACCAGAGGTCCAACC and 5′-GAAGGCATATCCTTCGTCATGAG; and Tollip, 5′-CAAGA ATCCCCGCTGGAATAAG and 5′-ATGGCTTTCAGGTCCTCCTCGC. GAPDH primers were obtained from Clontech (Palo Alto, CA).

Immunoprecipitation

For coimmunoprecipitation of transfected proteins, 3×10^6 HEK293 cells were plated on 10-cm dishes and transfected with 5 μg Flag-TLR2 and 5 μg VSV-Tollip or with 3.5 μg Flag-TLR4, 3.5 μg MD2, and 3.5 μg VSV-Tollip. After 24 h, cells were collected, washed with PBS, and lysed for 20 min on ice in 500 μl lysis buffer (50 mM HEPES (pH 7.9), 250 mM NaCl, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM DTT,

protease inhibitor cocktail (Calbiochem, San Diego, CA), and 1% Nonidet P-40). Lysates were clarified by centrifugation at 4°C for 15 min at 10,000 × *g*. Half the cell lysates were incubated with 3 μg anti-Flag mAb (anti M2, Sigma, St. Louis, MO) and 20 μl 50% (v/v) Protein G Plus/Protein A-agarose (Oncogene, Cambridge, MA) overnight at 4°C with gentle rocking. After extensive washing with lysis buffer, precipitated complexes were solubilized by boiling in SDS sample buffer, fractionated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes, and blotted with the indicated Abs (anti-Flag or anti-VSV (Sigma)). The reactive bands were visualized with HRP conjugated to the appropriate secondary Abs with an ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL).

Results

Transfection of TLR2 confers endothelial cell responsiveness to PSM, STF, and OspA-L

We have previously shown that HMEC express low levels of TLR2 mRNA and protein and are unresponsive to Mtb 19-kDa lipoprotein, and that transfection of TLR2 restores their responsiveness to Mtb 19 kDa (22). However, it is unknown whether this low level of TLR2 expression is sufficient for the recognition and response to other classic TLR2 ligands or whether additional TLRs can substitute functionally for TLR2 in endothelial cells. To investigate whether HMEC have a broader defect in recognition of other TLR2 ligands, we measured the responses of HMEC to three additional biologically relevant TLR2 ligands, including PSM, STF, and OspA-L. Our data demonstrate that HMEC are unresponsive to PSM, STF, and OspA-L, as measured by activation of an NF-κB-dependent reporter gene, ELAM-luciferase (Fig. 1*A*). These findings suggest that HMEC have a broad inability to respond to diverse TLR2 ligands, although they are LPS responsive (16, 22). The failure of these ligands to activate NF-κB in HMEC confirms that these ligands are not contaminated with LPS.

Recognition of pathogen-associated molecular patterns requires both the expression of the appropriate TLR as well as the presence of downstream signaling molecules, which lead to cellular responses such as activation of NF-κB, proinflammatory cytokines, or iNOS production. To test whether the low level of TLR2 expression was the reason for defective endothelial cell responses to classic TLR2 ligands, we transiently expressed TLR2 in HMEC. Transfection of wtTLR2 restored the responsiveness of HMEC to PSM, STF, and OspA-L, as measured by NF-κB-dependent ELAM-promoter reporter (Fig. 1*A*), iNOS (Fig. 1*B*), and IL-8 promoter-reporter gene activation (Fig. 1*C*). We used purified protein-free LPS (50 ng/ml) as an internal control for the TLR4 signaling pathway in HMEC. Expression of TLR2 did not affect LPS-induced response in HMEC (data not shown). These results demonstrate that the low level of TLR2 expression is the cause of the defective responses of HMEC to these ligands. These data further suggest that the intracellular pathway leading to proinflammatory responses is intact in HMEC.

TLR2 uses MyD88, IRAK, and TRAF6 to activate NF-κB in response to TLR2 ligands

We (16) and others (15, 39) have previously shown that the molecular signaling pathway induced by LPS-TLR4 interaction to

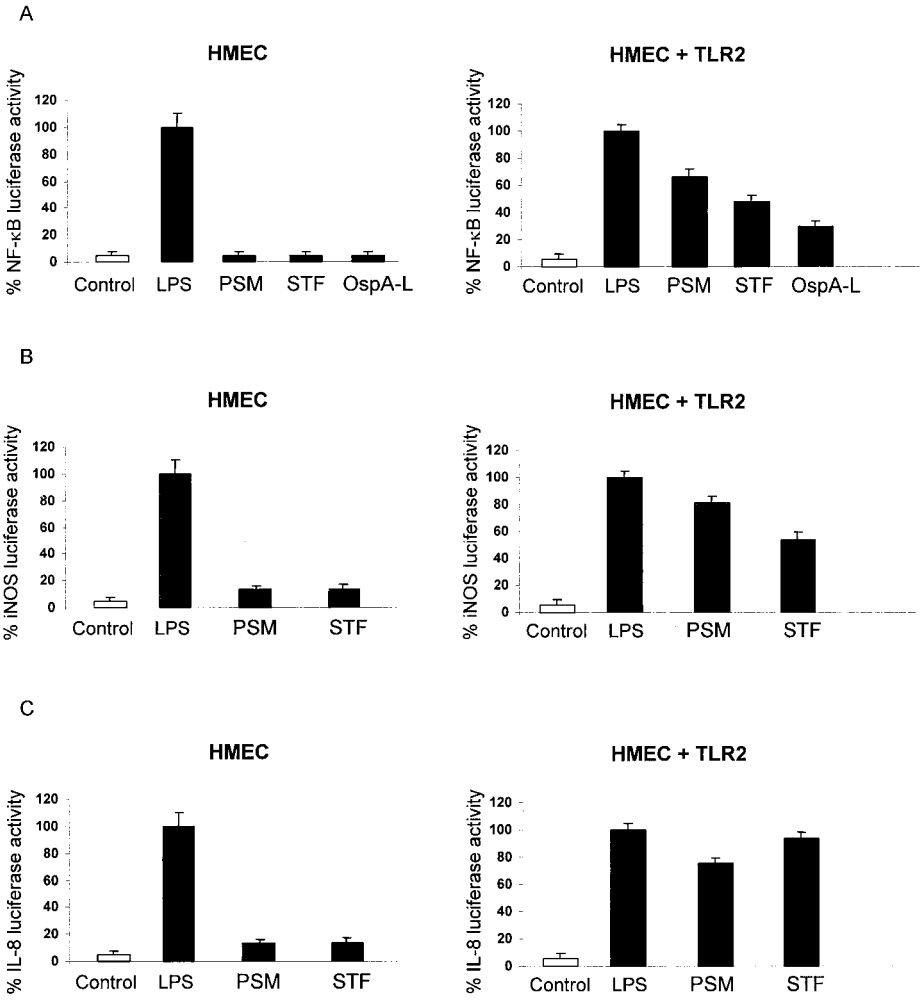

FIGURE 1. Expression of TLR2 in HMEC cells confers responsiveness to PSM, STF, and OspA-L. HMEC cells were transiently cotransfected with ELAM-NF-κB (*A*), iNOS promoter (*B*), or IL-8 promoter luciferase constructs (*C*) with flag-tagged wtTLR2, and β-galactosidase reporter vectors for 24 h. The total amount of cDNA transfected was kept constant with empty vector. Native HMEC cells and TLR2-transfected HMEC were stimulated for 5 h with PSM (100 ng/ml), STF (20 μl/ml), OspA-L (30 μg/ml), or LPS (50 ng/ml). Luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Data shown are the mean ± SD of three independent experiments and are expressed as the percentage of luciferase activity induced by LPS.

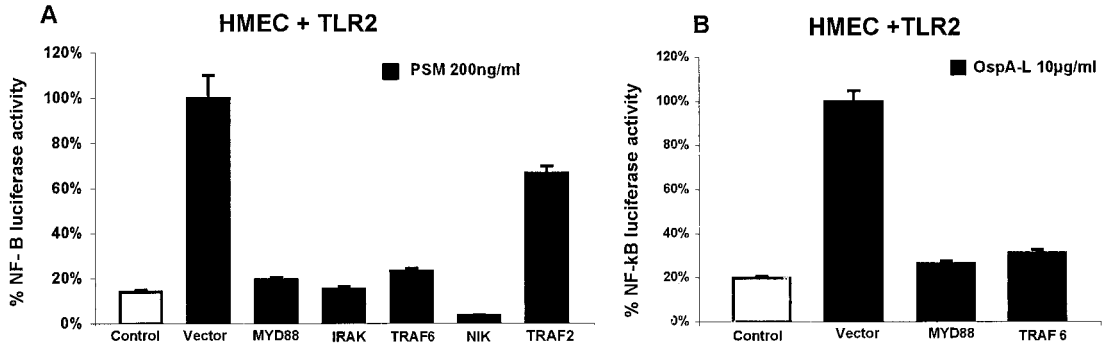

FIGURE 2. DN constructs of MyD88, IRAK, TRAF6, and NIK, but not TRAF2, inhibit PSM- and OspA-L-induced NF-κB activity in TLR2-transfected HMEC. HMEC grown on 24-well plates were cotransfected with flag-tagged wtTLR2 and DN mutants of MyD88, IRAK, TRAF6, TRAF2, and NIK as well as reporter genes for 24 h. The total amount of cDNA transfected was kept constant with empty vector. The cells were treated with 200 ng/ml PSM (*A*) or 10 μg/ml OspA-L (*B*) for 5 h. NF-κB luciferase activity was measured with a luciferase assay and was normalized with β-galactosidase activity. Data shown are the mean ± SD of three independent experiments and are expressed as the percentage of luciferase activity induced by the ligands in cells transfected with the empty vector.

activate NFκ-B shares the IL-1R signaling molecules, MyD88, IRAK, TRAF6, and NIK. It has been shown that TLR2 signaling shares the signaling molecule MyD88 in macrophages (33), but the remainder of the signaling pathway leading from TLR2 to NFκ-B has not been clearly demonstrated. Yang et al. (40) described the signaling pathway emanating from TLR2; however, they used LPS as a ligand, which was subsequently determined to signal via TLR4 and not TLR2. To determine whether TLR2 ligands use the same IL-1R signaling molecules as TLR4, we assessed the ability of DN forms of MyD88, IRAK, TRAF6, and NIK to inhibit the responses of various TLR2 ligands, i.e., PSM and OspA-L, in TLR2-transfected HMEC. This approach has been used successfully in HMEC to characterize the LPS-TLR4 signaling pathway (16). DN constructs of MyD88, IRAK, TRAF6, and NIK significantly blocked the PSM (Fig. 2*A*), OspA-L (Fig. 2*B*), and STF responses (data not shown) in TLR2-HMEC. The expression of a DN TRAF2 construct, a signaling molecule involved in TNF-α signaling, did not block the response to PSM (Fig. 2*A*), whereas it completely blocked TNF-α-induced NF-κB activation (data not shown). Because the pathways for IL-1 and TNF-α signaling converge at the level of NIK for NF-κB activation, DN NIK blocked both PSM-induced (Fig. 2*A*) and TNF-α-induced (data not shown) NF-κB activation. In control experiments DN constructs of MyD88, IRAK, TRAF6, and NIK significantly blocked the IL-1β response in HMEC (data not shown). These results indicate that the TLR2 signaling pathway shares IL-1R signaling molecules, including MyD88, IRAK, and TRAF6, to activate the transcription factor NF-κB.

TLR6 functionally cooperates with TLR2 to activate NF-κB in STF-, OspA-L-, or PSM-stimulated cells

A recent study has shown that TLR2 and TLR6 cooperate to activate macrophages in response to Gram-positive bacteria and Gram-positive bacterial peptidoglycan, whereas TLR2 is able to recognize bacterial lipopeptide without TLR6 cooperation (31). Although PSM was reported to be a TLR2 ligand, which requires TLR6, in studies using 293-HEK cells (33), it is not clear whether it requires the cooperation of TLRs in human endothelial cells. Furthermore, there are no data to suggest that other TLR2 ligands, such as STF and OspA-L, require TLR6 as well to signal. We investigated whether STF-, OspA-L-, and PSM-dependent TLR2 activations in HMEC require TLR6 cooperation. We first determined whether TLR6 is expressed in HMEC. To investigate the expression of TLR2 and TLR6 mRNA in HMEC, we used RT-PCR. We have previously shown that HMEC express TLR1, TLR3, TLR4, and TLR5 and very low levels of TLR2 mRNA (16, 22). Here we show that HMEC express high levels of TLR6 mRNA compared with TLR4, to which the cells respond (Fig. 3). To determine whether TLR6 functionally contributes to the TLR2-mediated STF, OspA-L, and PSM responses in endothelial cells, we cotransfected HMEC with TLR6 or TLR2 cDNA. Fig. 4 shows the NF-κB responses of TLR2- or TLR6-transfected HMEC. All transfectants expressed equivalent amounts of flag-tagged TLR as judged by anti-flag Western blots (data not shown). HMEC were unresponsive to STF, OspA-L, and PSM unless transfected with TLR2 as shown in Fig. 1*A*. We then asked whether overexpression of TLR6 could overcome the TLR2 signaling defect in HMEC. Transfection of TLR6 alone was unable to restore PSM responsiveness in HMEC (Fig. 4); cotransfection of both TLR2 and TLR6 did not result in an enhanced response to PSM compared with TLR2-transfected HMEC (Fig. 4). LPS-mediated induction of the NF-κB reporter construct was not different in untransfected or TLR2- and/or TLR6-transfected HMEC, and transfection of TLR2

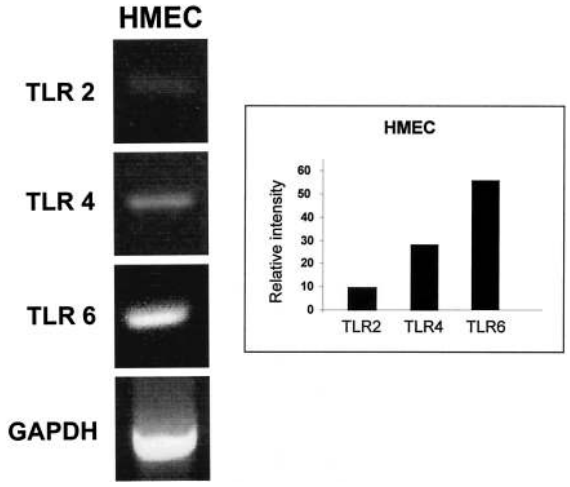

FIGURE 3. TLR2, TLR4, and TLR6 mRNA expression in HMEC. Expression of TLR2 (347 bp), TLR4 (548 bp), and TLR6 (500 bp) mRNA in HMEC was analyzed by PCR following reverse transcription. RT-PCR analysis of GAPDH expression was used as a control (*lower panel*; 983 bp). The graph shows the relative intensity of each band, which was measured by densitometry using Kodak ID Image Analysis Software (EDAS 290; Eastman Kodak, Rochester, NY).

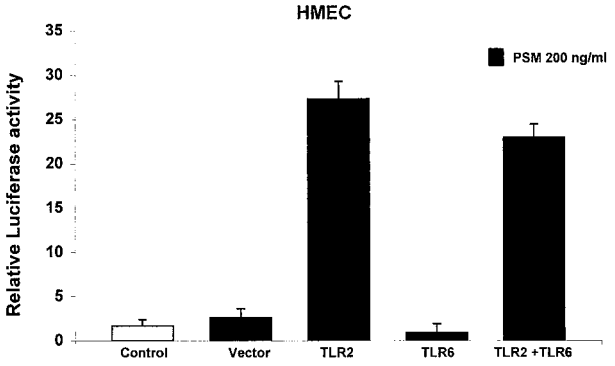

FIGURE 4. TLR2, but not TLR6, transfection confers responsiveness of HMEC to PSM. HMEC were transiently cotransfected with ELAM-NF-κB and β-galactosidase reporter vectors and with flag-tagged wtTLR2 (0.5 μg) or wtTLR6 (0.5 μg) cDNA constructs. The total amount of cDNA was kept constant with the empty vector. Cells were stimulated with PSM (200 ng/ml) for 5 h. NF-κB luciferase activity was measured and normalized with β-galactosidase activity. Data shown are the mean ± SD of three independent experiments and are presented as relative luciferase activity.

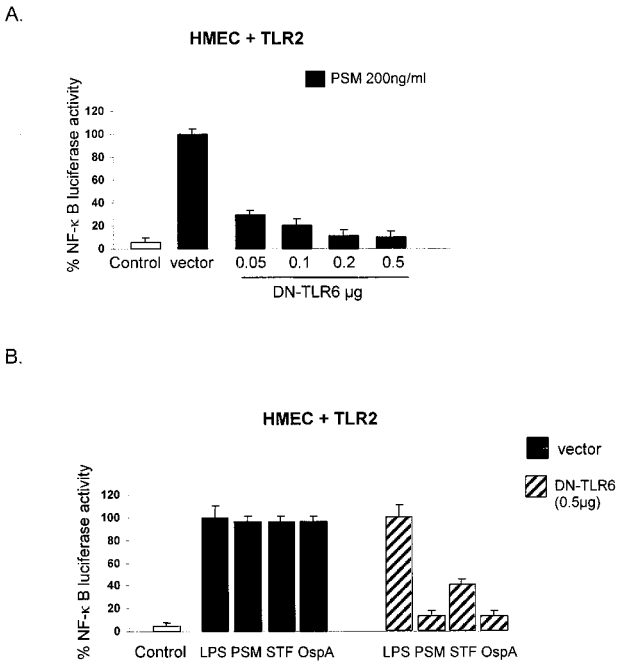

FIGURE 5. DN constructs of TLR6 inhibit the PSM, STF, and OspA-L responses. HMEC were transiently cotransfected with ELAM-NF-κB, β-galactosidase reporter vectors, flag-tagged wtTLR2 (0.5 μg), and increasing concentrations of DN TLR6. Following overnight transfection, cells were stimulated with PSM (200 ng/ml) for 5 h (*A*). *B*, A fixed concentration of DN TLR6 (0.5 μg) was cotransfected with NF-κB and β-galactosidase in TLR2-transfected HMEC cells. Cells were stimulated with LPS (20 ng/ml), PSM (200 ng/ml), STF (20 μl/ml), and OspA-L (30 μg/ml). Luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. The total amount of cDNA was kept constant with empty vector. Results are shown as the mean + SD of three independent experiments and are reported as a percentage of LPS-, PSM-, STF-, or OspA-L-stimulated NF-κB luciferase activity in cells cotransfected with a vector control.

alone or with TLR6 did not enhance the baseline activity of the reporter gene in these cells (data not shown). These findings suggest that the unresponsiveness of HMEC to various TLR2 ligands is primarily the result of their very low level expression of TLR2, and transfection of exogenous TLR6 is unable to overcome the unresponsiveness to PSM. Our data further suggest that the endogenous level of TLR6 in HMEC does not limit TLR2-dependent activation.

To characterize the functional role of endogenously expressed TLR6 in the response of TLR2-transfected HMEC to various TLR2 ligands, we assessed the ability of DN forms of TLR6 and TLR2 to inhibit the responses to STF, OspA-L, and PSM. DN TLR6 (Fig. 5) and DN TLR2 (data not shown) significantly blocked the STF, OspA-L, and PSM responses in TLR2-HMEC. Fig. 5*A* shows the dose-dependent inhibition of DN TLR6 for PSM responses. These DN mutants were unable to affect LPS-induced NF-κB activation in native (data not shown) and TLR2 transfected HMEC (Fig. 5*A*), supporting the specificity of these mutants. In additional control experiments, the DN construct of TLR4 (C3H/HeJ TLR4) (22) was unable to inhibit PSM responses in TLR2-transfected HMEC, whereas the LPS response was completely inhibited by C3H/HeJ TLR4 cDNA (data not shown). Thus, the TLR signaling complex, which recognizes STF, OspA-L, and PSM in TLR2-HMEC, can be inhibited by DN TLR2 and DN TLR6, suggesting a functional cooperation between TLR2 and TLR6 for all three TLR2 ligands tested.

Tollip associates with TLR2 and TLR4, and overexpression of Tollip inhibits TLR2- and TLR4-induced NF-κB activation

Tollip, a recently discovered signaling molecule involved in IL-1R signaling, is present in a complex with IRAK and links IRAK to the IL-1R (17). Recently, Tollip was shown to impair NF-κB activation in response to IL-1β, but not TNF-α, in 293T cells (17). The role of Tollip in TLR-dependent signaling is unknown. We hypothesized that because TLR2 and TLR4 share the IL-1R signaling molecules, Tollip may also be associated with TLR2 and TLR4 and modify TLR-mediated NF-κB activation. To test these hypothesis, we cotransfected HEK293 cells with epitope Flag-TLR2 or Flag-TLR4 and MD2 with VSV-Tollip cDNA. Cell lysates were immunoprecipitated with anti-Flag Ab and immunoblotted with anti-VSV Ab. In resting cells and in cells stimulated with PSM or LPS, we found that Tollip was coimmunoprecipitated with either TLR2 or TLR4 (Fig. 6). Furthermore, we found that endogenous Tollip is present in HMEC and THP1 cells by RT-PCR (data not shown). To investigate the effects of Tollip overexpression in endothelial cells, we cotransfected HMEC and TLR2-HMEC with ELAM-NF-κB reporter and increasing amounts of Tollip cDNA and measured luciferase activity following stimulation with LPS or TLR2 ligands (STF, OspA-L, and PSM), respectively. Expression of Tollip cDNA inhibited the LPS response via TLR4 signaling in HMEC (Fig. 7) and STF, OspA-L, and PSM responses in TLR2-transfected HMEC (Fig. 7) in a dose-dependent manner. In control experiments expression of Tollip in HMEC inhibited IL-1β-induced NF-κB activation, whereas it did not affect TNF-α-induced NF-κB activation (data not shown). These results indicate that in addition to IL-1R signaling, Tollip is an important constituent of the TLR4 and TLR2 signaling pathways.

Discussion

Endothelial cells participate in the early stages of the immune response against various microbial agents and constitute an integral component of the innate immune system. HMEC are one of the first lines of defense against invading micro-organisms, including Gram-positive bacteria and spirochetes, and actively participate in the innate immune and inflammatory responses of the host (41–45). We have previously shown that HMEC express low levels of TLR2 and do not respond to Mtb 19-kDa lipoprotein (22). Our

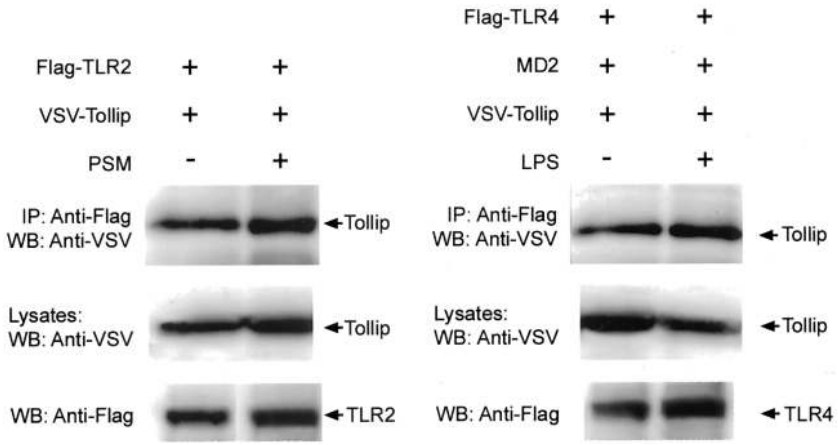

FIGURE 6. Tollip coimmunoprecipitates with TLR2 and TLR4. *Upper panel*, Tollip was coimmunoprecipitated, using an anti-flag Ab, from lysates of 293T cells cotransfected with the indicated combinations of expression plasmids, stimulated with either PSM (200 ng/ml) or LPS (50 ng/ml) for 2 min, and immunoblotted with anti-VSV Ab. IP, immunoprecipitate; WB, Western blot. *Middle* and *lower panels*, Western blotting of whole cell extracts, using anti-VSV (*middle panel*) or anti-flag (*lower panel*) Abs, to monitor expression of the various proteins.

current study demonstrates a broader defect in endothelial cell responses to known TLR2 ligands. The experiments described above indicate that HMEC do not respond to a variety of TLR2 ligands, including the polypeptide PSM, OspA-L, and STF, and that this defect can be overcome by expression of TLR2. Although endothelial cells are a front line defense against bacteremia and septicemia, our studies demonstrate that these cells may have an important defect to a wide array of TLR2 ligands, including PSM, a polypeptide from *S. epidermidis*, the most frequently isolated bacteria in intravascular catheter-associated infections (46). It is intriguing that dermal endothelial cells have down-regulated the ability to recognize Ags derived from this common skin commensal. The implications of these findings for intravascular catheter infections caused by *S. epidermidis* are unknown. We speculate that the absence of an immediate innate immune response by endothelial cells to *S. epidermidis*-derived ligands may permit the development of an established intravascular infection before an inflammatory response. An understanding of the factors that regulate TLR2 expression can aid in control of common catheter-related infections. We have recently shown that TLR2 expression in endothelial cells can be regulated at sites of infection and inflammation, either directly by LPS or indirectly by inflammatory cytokines such as TNF-α and IFN-γ (47), suggesting that the responsiveness of endothelial cells to diverse TLR2 ligands may be modulated by external stimuli.

We have previously shown in endothelial cells and monocytic cell lines that LPS-induced NF-κB activation is mediated by IL-1R signaling molecules that are engaged by TLR4 (16). The molecules transducing the signal from TLR2 to NF-κB have not been clearly reported. Here we determined that TLR2 signaling also shares the IL-1R signaling molecules, including MyD88, IRAK, and TRAF6, to activate NF-κB. MyD88 appears to represent the most upstream mediator of the IL-1-, TLR4-, and TLR2-induced signaling cascade, which ultimately activates NF-κB, thus driving transcriptional activation of several inflammatory cytokines. Therefore, MyD88 may represent a potentially useful therapeutic target to control the molecular switch from innate to the adaptive immune responses.

Tollip is a recently discovered signaling molecule involved in IL-1R signaling (17). Before IL-1β stimulation, endogenous Tollip is present in a complex with IRAK, and recruitment of Tollip-IRAK complexes to the activated IL-1R complex occurs through association of Tollip with IL-1R accessory protein (17). Corecruited MyD88 then triggers IRAK autophosphorylation, which in turn leads to rapid dissociation of IRAK from Tollip and IL-1 receptors. Burns et al. have shown that overexpression of Tollip inhibits IL-1β-induced, and not TNF-α-induced, activation of NF-κB and c-Jun N-terminal kinase in a dose-dependent manner in 293T cells (17). Therefore, Tollip not only links IRAK to IL-1R, but may also inhibit phosphorylation of IRAK, either by blocking

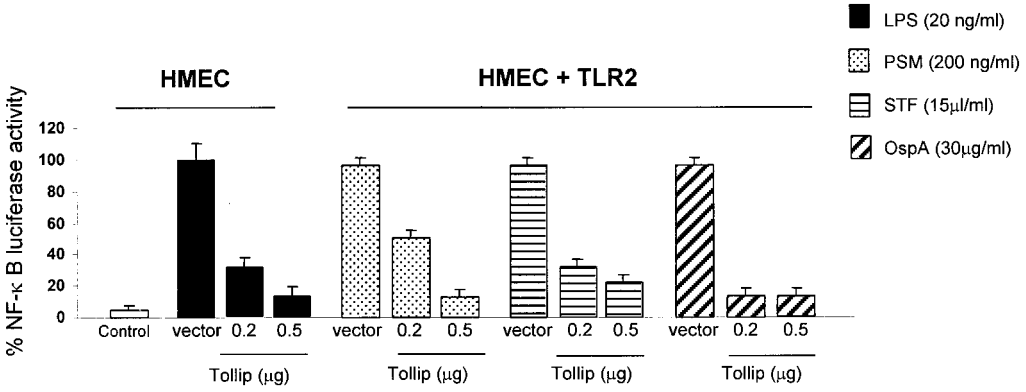

FIGURE 7. Overexpression of Tollip inhibits TLR4- and TLR2-induced NF-κB activation. Native and TLR2-transfected HMEC were cotransfected with ELAM-NF-κB and β-galactosidase reporter vectors and with increasing concentrations of Tollip cDNA for 24 h. The total amount of cDNA was kept constant with empty vector. Native cells were stimulated for 5 h with LPS (20 ng/ml), and TLR2-HMEC cells were stimulated with PSM (200 ng/ml), STF (15 μl/ml), and OspA-L (30 μg/ml). Luciferase and β-galactosidase assays were performed as described in *Materials and Methods*. Results are shown as the mean + SD of three independent experiments and are reported as a percentage of LPS- or TLR2 ligand-stimulated NF-κB promoter activity in cells cotransfected with a vector control.

IRAK autophosphorylation or by interfering with binding of a crucial signaling molecule, such as a second kinase (48). This may serve to keep IRAK in an off state before formation of an IL-1-induced complex with its activator MyD88. It is also possible that the regulated release of IRAK from Tollip is required for signal termination, to release phosphorylated IRAK for degradation by proteasomes (49). We now show that Tollip coimmunoprecipitates with TLR2 and TLR4 and has a functional role in TLR2- and TLR4-mediated signaling in HMEC. Furthermore, overexpression of Tollip inhibits TLR4- and TLR2-mediated NF-κB activation in a dose-dependent manner, suggesting that this signaling molecule is also shared among the IL-1R, TLR4, and TLR2 signaling pathways. Further studies are needed to determine the kinetics of the IRAK-Tollip complexes recruited to TLR4 and TLR2. Thus, Tollip is an endogenously expressed molecule that limits proinflammatory signals from both the innate and adaptive immune systems and may be exploited as an anti-inflammatory strategy.

PSM, a complex of three small secreted polypeptides from *S. epidermidis*, has been shown to activate NF-κB and proinflammatory cytokine release (32). Although PSM was recently shown to activate TLR2 with the cooperation of TLR6 (33), it is unknown whether additional TLR2 ligands, such as STF and OspA-L, also require the cooperative effect of TLR6 to signal. Ozinsky et al. (31) observed that TLR2 and TLR6 coordinate macrophage activation by intact Gram-positive bacteria and yeast, whereas TLR2 recognizes bacterial lipopeptide without TLR6. Here we show that in addition to the polypeptide PSM, STF and OspA-L require the functional cooperation of TLR6 and TLR2 to signal. We observed that cotransfection of TLR2 and TLR6 in HMEC did not enhance the TLR2-mediated PSM response. This observation differs from the data reported by Hajjar et al., showing that the TLR2-mediated response to PSM is enhanced by TLR6 in HEK 293 cells (33). This difference is most likely explained by cell specificity, given the relative abundance of TLR6 within HMEC, and suggests that TLR2, and not TLR6, is the limiting factor in HMEC responses to TLR2 ligands such as PSM. These observations suggest that the ratio of different TLRs within a cell type may modify the response to a given ligand. Our data support the importance of combinatorial repertoires of TLRs for innate immune recognition in diverse cell types, including endothelial cells, to discriminate among the large number of pathogen-associated molecular patterns in nature. These combinations of TLRs facilitate mammalian responsiveness to a wide array of pathogen-associated molecular patterns and diversify the repertoire of Toll-mediated responses.

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