Am J Physiol Heart Circ Physiol 301: H2246–H2253, 2011.
First published September 23, 2011; doi:10.1152/ajpheart.01194.2010.

Heterotrimeric G_i/G_o proteins modulate endothelial TLR signaling independent of the MyD88-dependent pathway

Shauna M. Dauphinee,[1,3] Verena Voelcker,[2] Zinaida Tebaykina,[2] Fred Wong,[4] and Aly Karsan[1,2,3,4]

[1]*Genome Sciences Centre and* [2]*Department of Pathology and Laboratory Medicine, British Columbia Cancer Agency, Vancouver, British Columbia, Canada; and* [3]*Experimental Medicine Program and* [4]*Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada*

Submitted 30 November 2010; accepted in final form 2 September 2011

Dauphinee SM, Voelcker V, Tebaykina Z, Wong F, Karsan A. Heterotrimeric G_i/G_o proteins modulate endothelial TLR signaling independent of the MyD88-dependent pathway. *Am J Physiol Heart Circ Physiol* 301: H2246–H2253, 2011. First published September 23, 2011; doi:10.1152/ajpheart.01194.2010.—The innate immune recognition of bacterial lipopolysaccharide (LPS) is mediated by Toll-like receptor 4 (TLR4) and results in activation of proinflammatory signaling including NF-κB and MAPK pathways. Heterotrimeric G proteins have been previously implicated in LPS signaling in macrophages and monocytes. In the present study, we show that pertussis toxin sensitive heterotrimeric G proteins ($G\alpha_{i/o}$) are involved in the activation of MAPK and Akt downstream of TLR2, TLR3, and TLR4 in endothelial cells. $G\alpha_{i/o}$ are also required for full activation of interferon signaling downstream of TLR3 and TLR4 but are not required for the activation of NF-κB. We find that $G\alpha_{i/o}$-mediated activation of the MAPK is independent of the canonical MyD88, interleukin-1 receptor-associated kinase, and tumor necrosis factor receptor-associated factor 6 signaling cascade in LPS-stimulated cells. Taken together, the data presented here suggest that heterotrimeric G proteins are widely involved in TLR pathways along a signaling cascade that is distinct from MyD88-TRAF6.

lipopolysaccharide; innate immunity; toll-like receptors; heterotrimeric G proteins; myeloid differentiation factor

THE INNATE IMMUNE RECOGNITION of bacterial and viral products is mediated by a family of transmembrane receptors known as Toll-like receptors (TLRs). Lipopolysaccharide (LPS), a key component of the outer wall of gram-negative bacteria, initiates endothelial activation through a receptor complex consisting of TLR4, CD14, and MD2 (9). Recruitment of the adaptor proteins TIR-containing adaptor molecule (TIRAP) and myeloid differentiation factor (MyD88) initiates a MyD88-dependent pathway that culminates in the activation of NF-κB and MAPK. Activation of these downstream targets requires recruitment of interleukin-1 receptor-associated kinase (IRAK4) and IRAK1 through interaction between the death domains of MyD88 and the IRAKs. The autophosphorylation and activation of IRAK1 results in the ability to bind tumor necrosis factor receptor-associated factor 6 (TRAF6) (7), which leads to oligomerization and polyubiquitination of the TRAF6 molecule (11). This facilitates activation of transforming growth factor-β (TGF-β)-activated kinase 1, which leads to activation of the IKK-NF-κB pathway and the three MAPK: p38, ERK, and JNK (39). In addition to the MyD88-dependent pathway, LPS stimulation also results in the activation of a MyD88-independent pathway, through recruitment of the adaptor molecules TIR-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule. This leads to the late-phase activation of NF-κB and interferon regulatory factor 3 (IRF3), as well as activation of the MAPKs and phosphatidylinositol 3-kinase (PI3K) (10).

The endothelium plays a major role in the pathogenesis of sepsis. Under normal conditions, the endothelium functions to maintain organ homeostasis through vasoregulation, selective vascular permeability, and providing an anticoagulant surface. During bacterial infection, the normal physiological functions of the endothelium are perturbed, contributing to the organ failure characteristic of sepsis (10). Several recent studies suggest that the LPS-mediated functions of the endothelium are a direct result of LPS binding to TLR4 on the endothelial surface rather than secondary to the production of proinflammatory mediators from LPS-primed immune cells. It has been shown that endothelial-specific expression of TLR4 leads to increased leukocyte recruitment in lung and cerebral microvessels and is sufficient to clear a bacterial infection (2, 3, 27, 45). These studies highlight the need for further examination of the signaling pathways initiated in response to LPS in the endothelium, which will be critical to our understanding of the innate immune response to gram-negative bacterial infection.

In addition to TLR4, endothelial cells express TLR2 and TLR3, which have been shown to be important mediators of inflammation (21, 38). Endothelial cells from TLR2-null mice are deficient in IL-6 production and do not increase expression of selectin molecules critical for neutrophil adhesion in response to TLR2 agonists (38). Moreover, human endothelial cells upregulate coagulation pathways in response to TLR2 ligands (38). TLR2 has also been shown to be important for aortic endothelial cell Weibel-Palade body exocytosis, thereby facilitating the cell-surface expression of P-selectin and subsequent rolling and adhesion of platelets and leukocytes (21). TLR3 mediates the expression of inflammatory molecules, such as IFN-β and soluble E-selectin, in human retinal vascular endothelial cells of patients with retinal vasculitis (25). Taken together, these studies suggest that endothelial expression and function of TLRs is critical for the regulation of inflammatory events.

Previous studies have implicated heterotrimeric guanine nucleotide binding regulatory (G) proteins in LPS signaling in macrophages and monocytes (17). Genetic deletion of the $G\alpha_i$ isoforms ($G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$) suggests that $G_{\alpha i}$ proteins differentially regulate TLR-stimulated cytokine production in a cell-type specific manner (16). However, the role of $G\alpha_{i/o}$ proteins in TLR4 signaling in endothelial cells remains less well understood and the role of $G\alpha_{i/o}$ in TLR signaling cascades other than TLR4 has not been shown. Endothelial cells

Address for reprint requests and other correspondence: A. Karsan, British Columbia Cancer Agency, 675 West 10th Ave., Vancouver, BC V5Z 1L3 Canada (e-mail: akarsan@bccrc.ca).

have been shown to express isoforms of the $G\alpha_{i/o}$ family (12) and G protein-coupled receptors that signal through $G\alpha_{i/o}$ are important mediators of vascular functions during inflammation, such as vascular permeability, adhesion molecule expression, and cytokine production (4, 6, 8, 41). Thus we wanted to determine the activation and contribution of $G\alpha_{i/o}$ to the inflammatory signaling events initiated by TLRs in endothelial cells. In this study, we find that $G\alpha_{i/o}$ are important for the activation of MAPK and Akt downstream of TLR2, TLR3, and TLR4, as well as the activation of interferons downstream of TRIF-dependent signaling from TLR3 and TLR4. Moreover, we find that the activation of downstream signaling by $G\alpha_{i/o}$ is independent of TRAF6.

MATERIALS AND METHODS

Reagents. LPS and pertussis toxin (PTx) were purchased from Sigma. Poly(I:C) and the synthetic tripalmitoylated lipopeptide, Pam3CSK4, were purchased from InvivoGen (San Diego, CA). The phospho-specific antibodies against JNK, p38, ERK1/2, and Akt and anti-Akt were purchased from Cell Signaling Technology (Beverly, MA). Anti-p38 and anti-ERK1/2 were purchased from Stressgen (Plymouth Meeting, PA). Anti-JNK1, anti-ubiquitin, and anti-TRAF6 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). TrueBlot anti-rabbit immunoglobulin immunoprecipitation beads were obtained from eBioscience (San Diego, CA).

Cell culture. The skin-derived human microvascular endothelial cell line (HMEC) was cultured in MCDB131 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 10 ng/ml EGF and 100 units each of penicillin and streptomycin. Human umbilical vein endothelial cells (HUVEC) were isolated as described previously (24) and cultured in MCDB131 supplemented with 10% heat-inactivated FCS, 10% heat-inactivated FBS, 2 mM glutamine, 20 ng/ml endothelial cell growth supplement (BD Bioscience, Mississauga, ON), 16 units/ml Heparin, and 100 units each of penicillin and streptomycin. The retroviral producer cell line AmphoPhoenix (gift from G. Nolan, Stanford University, Stanford, CA) was cultured in Dulbecco's modified Eagle medium (Sigma, St. Louis, MO) supplemented with 10% FBS, 2 mM glutamine, and 100 units each of penicillin and streptomycin.

Recombinant plasmids and transfection. TRAF6ΔN (aa289-522) was provided by Tularik (San Francisco, CA) and previously subcloned into the retroviral vector, LNCX (gift of A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) (20). The expression plasmid encoding $G\alpha_{i2}$-GFP was generated by PCR and cloned into pEGFPN2 by restriction digest. Transient transfections of the AmphoPhoenix packaging cell line were carried out using TransIT-siQUESTtransfection reagent according to the manufacturer's instructions (Mirus Bio, Madison, WI). Viral supernatants were used to transduce HMEC, and cell lines were selected using G418 (300 μg/ml). Expression of proteins was verified by immunoblotting. A small interfering (si)RNA targeting human $G\alpha_{i2}$ mRNA (NM_002070) sh$G\alpha_{i2}$ 266-GCAACCTGCAGATCGACTTTG-286 and shRandom GTTGCTTGCCACGTCCTAGAT were cloned into pLentilox3.7. Lentiviral particles were produced from HEK293T cells by cotransfection of pLentilox short hairpin (sh)RNA vector, pVSVG, pMDL g/p RRE, and RSV-REV. Viral supernatants were used to transduce target cells, and green fluorescent protein-positive cells were selected by flow sorting (FACS 440; Becton Dickson). Expression of mRNA was verified by RT-PCR.

cAMP assay. HMEC were pretreated with 1 mM IBMX for 15 min and then treated for 30 min with 10 μM forskolin (FSK) and 100 ng/ml LPS. In some instances, cells were pretreated with PTx for 1 h (16 h) before the treatment with IBMX, FSK, and LPS. Cells were lysed in a solution of 0.1 N HCl, and intracellular cAMP levels were assayed using the cAMP Direct Immunoassay Kit (Calbiochem, Gibbstown, NJ), according to manufacturer's instructions.

Immunoprecipitation. To examine covalent polyubiquitination of TRAF6, HMEC treated with or without LPS, in the presence or absence of PTx pretreatment, were lysed for 30 min in modified radioimmunoprecipitation buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 20 mM *N*-ethylmaleimide and freshly added protease inhibitor cocktail (Sigma; St. Louis, MO). Cell lysates (3 mg) were immunoprecipitated with antibody against TRAF6 (1 μg) for 3 h followed by 12 h incubation in the presence of TrueBlot anti-rabbit immunoglobulin immunoprecipitation beads. Immunoprecipitates were washed three times with lysis buffer followed by the addition of SDS sample buffer. Proteins were separated on SDS-polyacrylamide gels and subjected to immunoblotting with anti-ubiquitin and TRAF6 antibodies.

Luciferase assay. HMEC were transiently transfected with the NF-κB luciferase responsive reporter (pNF-κB-Luc), which contains five tandem NF-κB-binding sites upstream of the luciferase gene (a gift from Dr. Frank Jirik, University of Calgary, Calgary, AB, Canada) or an interferon stimulated response element (ISRE) coupled to luciferase (Stratagene, Santa Clara, CA). Transfections were performed using SuperFect Transfection Reagent (Qiagen, Frederick, MD) according to manufacturer's instructions. For normalization, cells were cotransfected with the *Renilla* luciferase plasmids, pRL-CMV (for NF-κB; Promega, Madison, WI) or pRL-TK (for ISRE; Promega, Madison, WI). HMEC were treated with or without LPS, in the presence or absence of PTx pretreatment 48 h after transfection, and dual luciferase reporter assays (Promega, Madison, WI) were performed.

Statistical analysis. Results are expressed as means ± SD. Data were analyzed using a two-tail Student's *t*-test using the GraphPad Prism statistical program. *P* values of less than 0.05 were considered significant. Error bars depict SD.

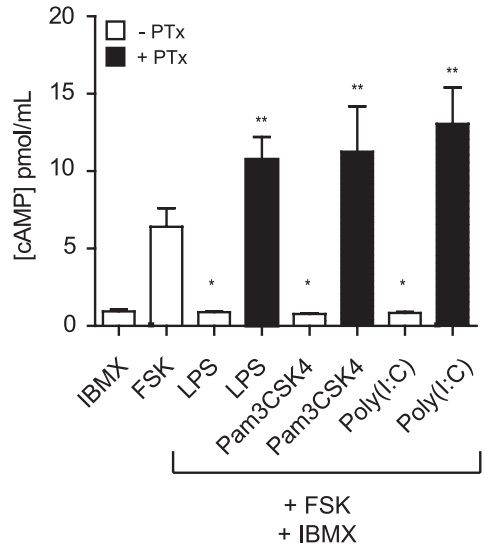

Fig. 1. Decrease in cAMP production in response to ligands for Toll-like receptors (TLR)2, TLR3, and TLR4 in endothelial cells. Human microvascular endothelial cell line (HMEC) were pretreated with 1 mM IBMX for 15 min and then treated with forskolin (FSK; 10 μM, 30 min) and either lipopolysaccharide (LPS; 100 ng/ml, 1 h), Pam3CSK4 (100 ng/ml, 1 h), or Poly(I:C) (2 μg/ml, 1 h) as indicated. In some instances, cells were pretreated with pertussis toxin (PTx) for 16 h. Cell lysates were used to determine intracellular cAMP levels. *$P < 0.001$ relative to FSK-treated cells and **$P \leq 0.001$ relative to ligand-treated cells, as determined by Student's *t*-test. Error bars indicate SD; $n = 3$ independent experiments.

RESULTS

TLR ligands activate heterotrimeric Gα$_{i/o}$ proteins in endothelial cells. Activated heterotrimeric Gα$_{i/o}$ proteins inhibit the function of adenylyl cyclase, thereby reducing intracellular cAMP levels (40). Because resting endothelial cells have been shown to express TLR2, TLR3, and TLR4 (18), we sought to determine whether Gα$_{i/o}$ proteins are activated downstream of these TLRs in HMEC by measuring intracellular cAMP in response to their respective ligands. HMEC treated with LPS (TLR4 ligand), Pam3CSK4 (TLR2/6 ligand), or Poly(I:C) (TLR3 ligand) showed decreased FSK-induced cAMP production (Fig. 1). To confirm that the decrease in cellular cAMP levels were the result of increased activation of Gα$_{i/o}$ proteins, HMEC was treated with PTx, a Gα$_{i/o}$ protein inhibitor, before the stimulation with TLR ligands. PTx treatment reversed the decrease in cAMP levels (Fig. 1), suggesting that PTx-sensitive Gα$_{i/o}$ proteins are activated downstream of TLR2, TLR3, and TLR4 in endothelial cells.

Gα$_{i/o}$ proteins contribute to TLR4-induced activation of MAPK and Akt in endothelial cells. We next determined whether Gα$_{i/o}$ proteins play a role in LPS-induced activation of MAPK and Akt in endothelial cells. HMEC and HUVEC pretreated with PTx and stimulated with LPS showed attenuated ERK1/2 activation and significantly reduced JNK activation (Fig. 2 and Supplemental Figs. S1 and S2, *A* and *B*). p38 activation was delayed in HMEC pretreated with PTx (Fig. 2 and Supplemental Fig. S2*C*), although inhibition of Gα$_{i/o}$ by PTx did not decrease activation of p38 in HUVEC (Supplemental Fig. S1). Pretreatment of HMEC with cholera toxin, an inhibitor of the stimulatory class of G proteins (Gαs), did not alter MAPK activation in response to LPS (data not shown). These results suggest that Gα$_{i/o}$ proteins regulate ERK1/2 and

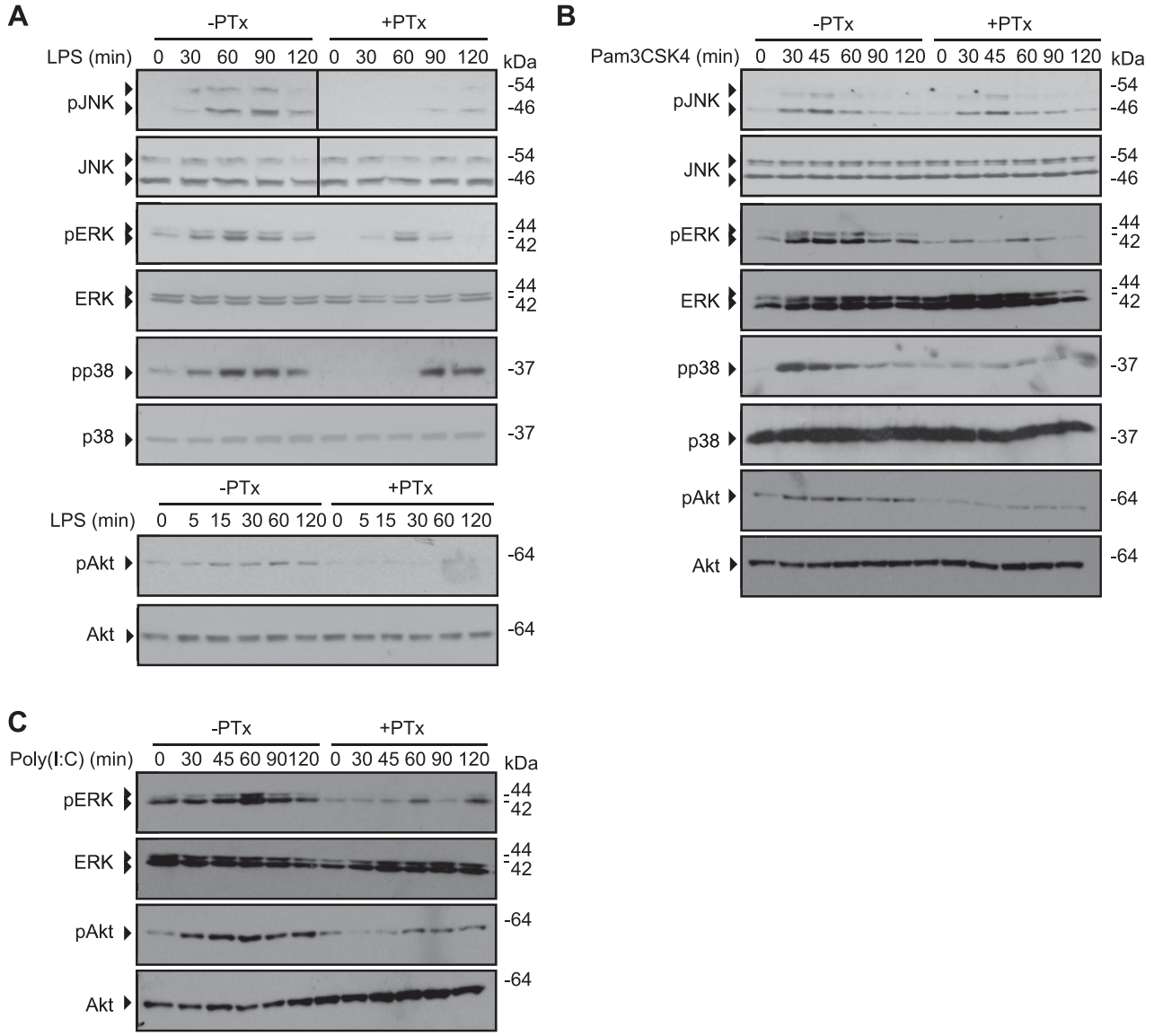

Fig. 2. PTx inhibits TLR-mediated activation of MAPK and Akt in endothelial cells. *A–C*: HMEC were pretreated with PTx (100 ng/ml, 1 h) and stimulated with LPS (*A*; 100 ng/ml), Pam3CSK4 (*B*; 100 ng/ml), or Poly(I:C) (*C*; 2 μg/ml) for the indicated times. Cell lysates were subjected to immunoblot analysis with antibodies against the phosphorylated form of ERK1/2, JNK, p38, and Akt. Total MAPK or Akt protein was used as a control for protein loading. Data are representative of $n \geq 2$ independent experiments.

JNK, but not p38, activation downstream of TLR4 in endothelial cells. This is in contrast with the ubiquitous involvement of $G\alpha_{i/o}$ proteins in MAPK activation in macrophages and monocytes (13, 17).

We, and others, have shown that PI3K and Akt are activated downstream of TLR4 in endothelial cells (20); however, the mechanism of activation of PI3K/Akt in response to LPS is not well characterized. Because the PI3K isoform, PI3K-β, is activated downstream of G protein-coupled receptors (28, 30), we wanted to determine whether $G\alpha_{i/o}$ play a role in the activation of PI3K/Akt downstream of TLR4. HMEC or HUVEC pretreated with PTx showed decreased LPS-induced activation of Akt compared with vehicle-treated cells, suggesting that the activation of $G\alpha_{i/o}$ is required for TLR4-mediated activation of Akt. (Fig. 2*A* and Supplemental Figs. S1 and S2*D*). Because similar results were obtained using both HMEC and the primary cell line, HUVEC, the remaining experiments were conducted in HMEC.

$G\alpha_{i/o}$ proteins contribute to activation of MAPK and Akt downstream of TLR2 and TLR3. Since $G\alpha_{i/o}$ proteins are activated downstream of TLR2 and TLR3 (Fig. 1), we wanted to determine whether $G\alpha_{i/o}$ proteins modulate MAPK and/or Akt activation by these TLRs. Pretreatment of HMEC with PTx decreased Pam3CSK4-induced ERK1/2, p38, and Akt, but not JNK, activation (Fig. 2*B* and Supplemental Fig. S3*A*-*C*). In contrast with TLR2 and TLR4, TLR3 signaling is initiated solely through receptor binding of the adaptor molecule TRIF, leading to activation of NF-κB, interferon regulatory factor 3 (IRF3), and the MAPKs (10). To examine whether MAPK or Akt activation downstream of TLR3 requires $G\alpha_{i/o}$ proteins, HMEC were pretreated with PTx and stimulated with Poly(I:C). This resulted in a PTx-mediated reduction of ERK1/2 and Akt activation (Fig. 2C and Supplemental Fig. S4, *A* and *B*). Activation of JNK and p38 by Poly(I:C) was weak in HMEC and unable to be assessed (data not shown). We were also unable to detect activation of MAPK downstream of TLR5 and TLR9 (data not shown), and resting HMEC do not express TLR7 and TLR8 (18). Similar to what was observed downstream of TLR4, pretreatment of HMEC with cholera toxin did not inhibit activation downstream of TLR2 or TLR3 (data not shown). These results suggest that activation of $G\alpha_{i/o}$ downstream of both MyD88-dependent and MyD88-independent TLRs leads to increased MAPK and Akt activation.

$G\alpha_{i/o}$ proteins do not contribute to LPS-induced activation of NF-κB in endothelial cells. In endothelial cells, TLR4 signals along the MyD88/IRAK axis and bifurcates downstream of TRAF6 to activate NF-κB and JNK (20). To assess the role of $G\alpha_{i/o}$ in endothelial NF-κB activation, we used an NF-κB luciferase reporter assay. HMEC pretreated with PTx did not show a decrease in LPS-induced NF-κB transcriptional activity (Fig. 3*A*). Conversely, pretreatment of HMEC with mastoparan abolished NF-κB luciferase activity, which agrees with the earlier finding that mastoparan acts indirectly on LPS signaling by neutralizing the ligand and decreasing TLR4 mRNA expression (42). Moreover, PTx also did not have an effect on NF-κB activation downstream of TLR3 (Fig. 3*B*). LPS-induced NF-κB activation requires degradation of IκBα in endothelial cells (43); thus, to confirm that $G\alpha_{i/o}$ proteins do not contribute to activation of the canonical NF-κB pathway, LPS-induced IκBα degradation was measured in PTx-treated HMEC. As expected, IκBα protein levels were decreased in

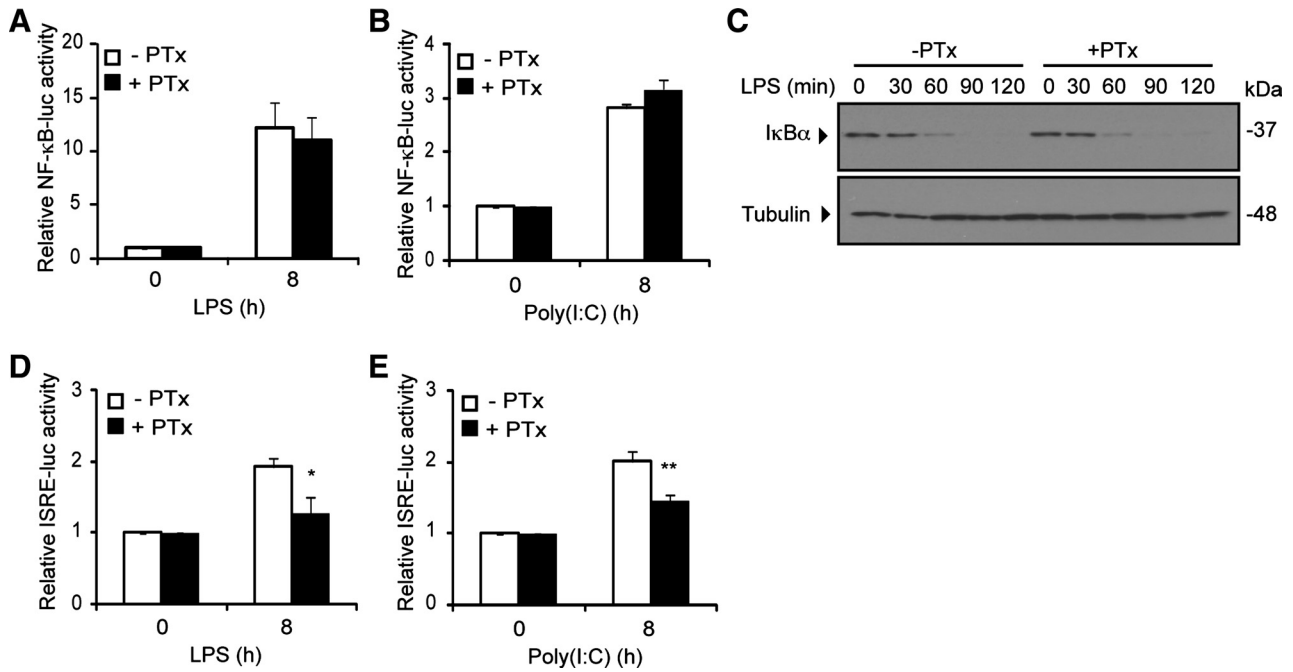

Fig. 3. $G\alpha_{i/o}$ proteins are not required for NF-κB activation in endothelial cells. *A*–*C*: HMEC were cotransfected with an NF-κB-Luc construct and a constitutively active *Renilla* luciferase plasmid (pRL-CMV). Cells were pretreated with PTx (100 ng/ml, 1 h) and stimulated with LPS (*A*; 100 ng/ml) or Poly(I:C) (*B*; 2 μg/ml) for 8 h. *C*: HMEC were pretreated with PTx (100 ng/ml, 1 h) and stimulated with LPS (100 ng/ml) for the times indicated. Cell lysates were subjected to SDS-PAGE and immunoblotted with an antibody directed against IκBα. Anti-tubulin was used as a control for protein loading. *D* and *E*: HMEC were cotransfected with an ISRE-Luc construct and a constitutively active Renilla luciferase plasmid (pRL-TK). Cells were pretreated with PTx (100 ng/ml, 1 h) and stimulated with LPS (*D*; 100 ng/ml) or Poly(I:C) (E; 2 μg/ml) for 8 h. $^*P < 0.002$ and $^{**}P < 0.005$. Error bars indicate SD; $n \geq 3$ independent experiments.

LPS-stimulated cells, and pretreatment with PTx had no effect on IκBα degradation (Fig. 3*C*).

$G\alpha_{i/o}$ proteins contribute to activation of IFNs by TLR3 and TLR4 in endothelial cells. TLR3 and TLR4 also engage TRIF for activation of IFN-responsive genes (10). Thus, to determine whether $G\alpha_{i/o}$ proteins are important for activation of interferons, we used an ISRE luciferase reporter. Inhibition of $G\alpha_{i/o}$ resulted in a decrease in LPS and Poly(I:C)-induced ISRE transcriptional activity (Fig. 3, *D* and *E*), suggesting that $G\alpha_{i/o}$ are needed for full activation of the IFN pathway downstream of TLR3 and TLR4.

$G\alpha_{i/o}$ proteins do not function along a TRAF6-dependent pathway in endothelial cells. Because PTx does not decrease NF-κB activation in HMEC, and is important for IFN activation, we hypothesized that $G\alpha_{i/o}$ proteins act independently of the MyD88-dependent signaling pathway that signals through TRAF6. Upon TLR4 activation, TRAF6 forms a complex with the ubiquitin conjugating enzyme Ubc13 and the Ubc-like protein Uev1A to catalyze the formation of a polyubiquitin chain linked through lysine 63 (K63) of ubiquitin (11). Therefore, to determine whether $G\alpha_{i/o}$ proteins are important for TRAF6 activation, we assessed TRAF6 ubiquitination in PTx-treated cells. LPS stimulation increased TRAF6 ubiquitination in vehicle control HMEC, and the degree of polyubiquitinated TRAF6 was not affected by pretreatment with PTx (Fig. 5*A*). This suggests that $G\alpha_{i/o}$ proteins do not act upstream of TRAF6. Therefore, we hypothesized that $G\alpha_{i/o}$ proteins must either converge on the signaling pathway down-

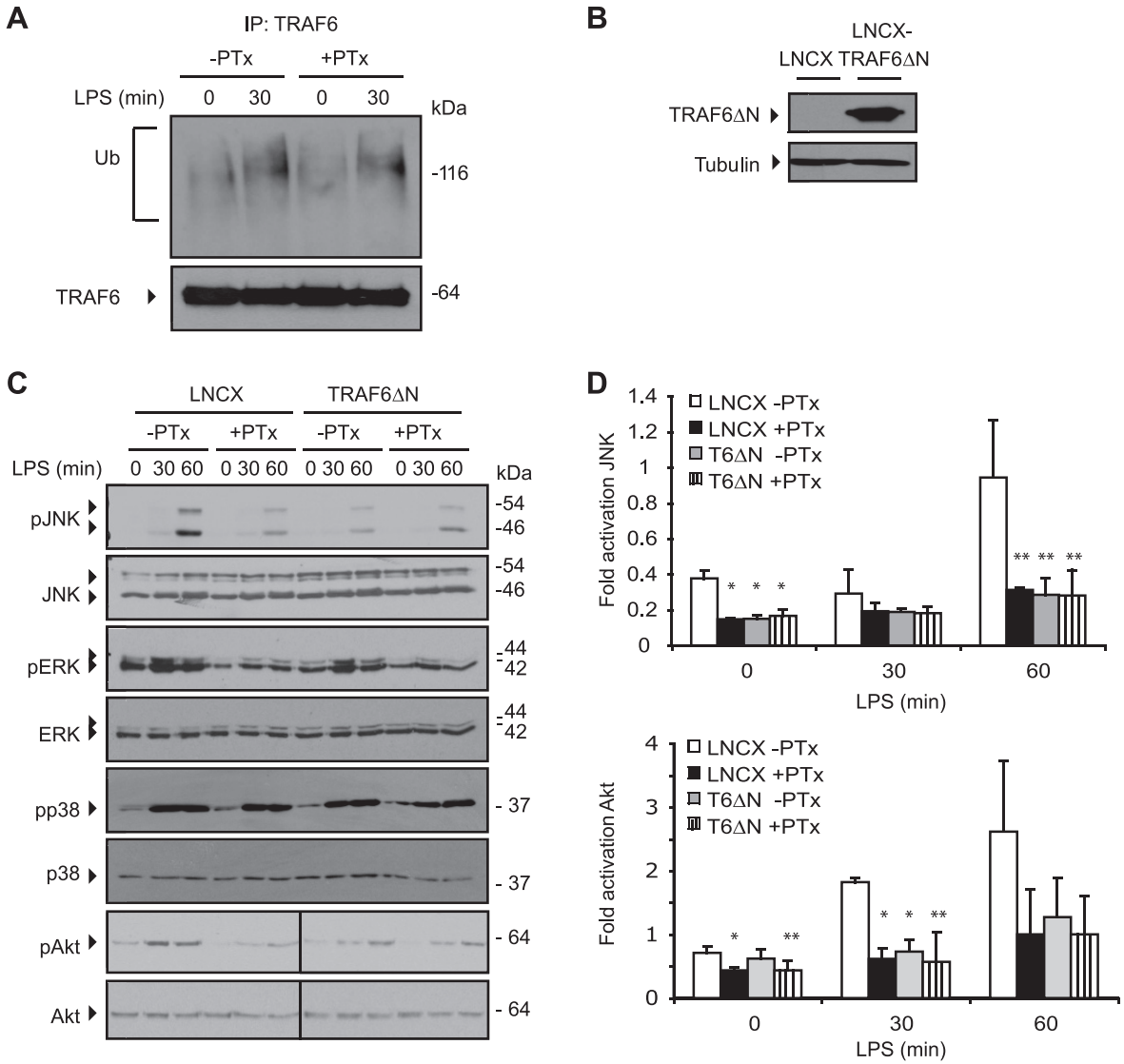

Fig. 4. $G\alpha_{i/o}$ proteins do not function along the TRAF6 signaling cascade. *A*: HMEC were pretreated with PTx (100 ng/ml, 1 h) and stimulated with LPS (100 ng/ml) for 30 min. Whole-cell lysates were prepared and immunoprecipitated with anti-TRAF6 antibody under denaturing conditions followed by immunoblotting with anti-ubiquitin and anti-TRAF6 antibodies. HMEC were transduced with the dominant negative LNCX-TRAF6ΔN or vector alone. *B*: expression of LNCX-TRAF6ΔN was confirmed by immunoblotting of cell lysates. Tubulin was used as a control for protein loading. *C*: cells were pretreated with PTx (100 ng/ml, 1 h) and stimulated with LPS (100 ng/ml) for the times indicated. Activation of MAPK and Akt was measured using phospho-specific antibodies to JNK, ERK1/2, p38, and Akt. Total MAPK and Akt was used as a positive control for protein loading. The P-Akt/Akt blot was loaded with LNCX-TRAF6ΔN lanes preceeding the LNCX lanes; thus the image was rearranged to align with the MAPK blots. *D*: densitometric analysis of the activation of JNK and Akt relative to total protein. $*P \leq 0.005$, $**P < 0.05$. Error bars indicate SD; $n = 3$ independent experiments. IP, immunoprecipitation.

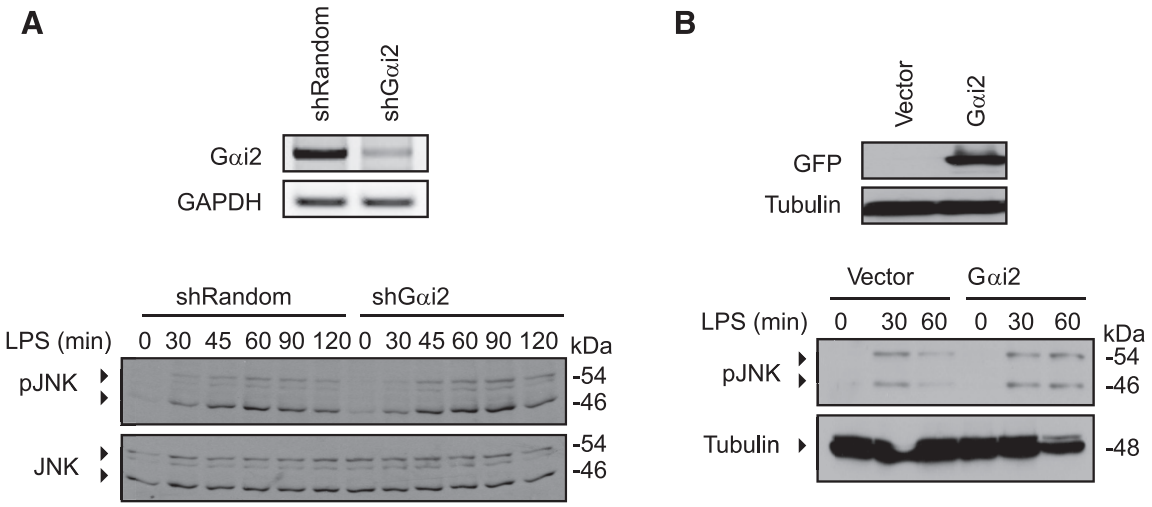

Fig. 5. $G\alpha_{i2}$ is sufficient for activation of JNK in endothelial cells. *A*: HEK293-TLR4-MD2-CD14 cells were transfected with $G\alpha_{i2}$-GFP or vector control. Expression of $G\alpha_{i2}$-GFP was confirmed by immunoblotting and JNK activation following LPS stimulation (100 ng/ml) was measured for the times indicated. *B*: HMEC were transduced with lentiviral vectors encoding shRNA as indicated and $G\alpha_{i2}$ was measured by RT-PCR. Activation of JNK following LPS stimulation (100 ng/ml) was measured for the times indicated. Data is representative of 2 independent experiments.

stream of TRAF6 or function in a parallel signaling cascade. To further explore where $G\alpha_{i/o}$ converge on the signaling pathway, we used concurrent inhibition of $G\alpha_{i/o}$ proteins with PTx, and expression of dominant negative TRAF6. The COOH-terminal fragment of TRAF6 (TRAF6ΔN) has been shown to act as a dominant-negative molecule to inhibit signaling downstream of TRAF6 in IL-1R and TLR signaling (7, 44). Expression of TRAF6ΔN in HMEC decreased activation of JNK and Akt, but this decrease was not augmented by inhibition of $G\alpha_{i/o}$ (Fig. 4, *B* and *C*). In contrast, ERK1/2 activation was unaffected by the expression of TRAF6ΔN and significantly decreased by PTx (Fig. 4*C*). Together, these results support a model where $G\alpha_{i/o}$ signaling converges downstream of TRAF6 to activate JNK and Akt, whereas $G\alpha_{i/o}$-mediated ERK1/2 activation remains independent of the TRAF6 pathway in endothelial cells.

$G\alpha_{i2}$ is sufficient, but not necessary, to activate JNK in endothelial cells. There is emerging evidence that single transmembrane spanning receptors couple to heterotrimeric G proteins (37). Thus we used genetic manipulation to determine whether TLR4 signals through $G\alpha_{i/o}$. Indeed, overexpression of $G\alpha_{i2}$ in HEK293-TLR4-MD2-CD14 expressing cells resulted in an increase in LPS-induced activation of JNK (Fig. 5*A*). However, a decrease in LPS-induced JNK activation using lentiviral-mediated RNA interference using shRNA targeting $G\alpha_{i2}$ alone was not detected in HMEC (Fig. 5*B*). Together, these findings suggest that $G\alpha_{i2}$ is sufficient, but not necessary, for signaling through TLR4, and that other $G\alpha_{i/o}$ proteins may also be involved.

DISCUSSION

There is increasing evidence demonstrating cross-talk between TLRs and signaling pathways, such as TGF-β (29, 31), β_2 integrins (23), and nuclear receptors (34). Furthermore, collaboration between TLR4 and heterotrimeric $G\alpha_{i/o}$ protein signaling has been described in cells of the immune system (13, 16, 17). Here, we show for the first time that $G\alpha_{i/o}$ proteins are activated by multiple TLRs in endothelial cells to mediate activation of MAPK, Akt, and interferons (Fig. 6). The finding that $G\alpha_{i/o}$ proteins contribute to TLR4-mediated ERK1/2 and JNK activation, but not p38, is in contrast with the ubiquitous involvement of $G\alpha_{i/o}$ proteins in MAPK activation in immune cells (13) (17). The activation of PI3K/Akt downstream of TLRs has not been extensively studied. We find that inhibition of $G\alpha_{i/o}$ is sufficient to reduce the level of Akt activation back to basal levels in response to ligands for TLR2, TLR3, and TLR4. These results show that $G\alpha_{i/o}$ proteins are important mediators of multiple signaling events downstream of TLRs and highlights the importance of cell type specific modulation of TLR signaling.

Heterotrimeric $G\alpha_{i/o}$ proteins have been suggested to be involved in TLR4, but not TLR2, signaling in endothelial cells (26). However, this study was performed using the wasp venom-derived peptide, mastoparan, to disrupt heterotrimeric G protein signaling. However, it has been shown that masto-

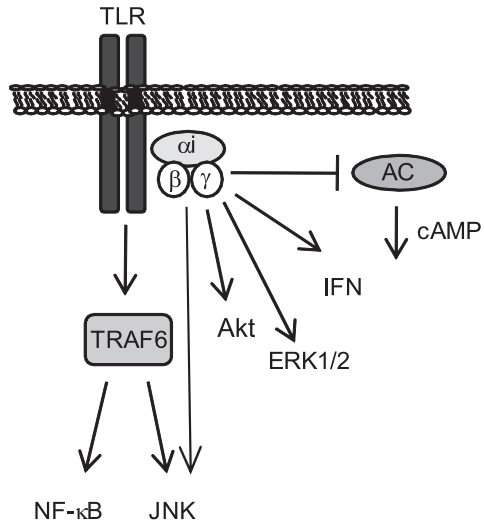

Fig. 6. A model for $G\alpha_{i/o}$ in TLR signaling. Proposed model of $G\alpha_{i/o}$-mediated signaling downstream of TLRs is shown.

paran binds the toxic lipid A portion of the LPS molecule with high affinity, effectively reducing the activity of LPS in a manner similar to that of the LPS neutralizing agent, polymyxin B (42), which is consistent with our data. Furthermore, mastoparan decreases TLR4 mRNA expression in a dose-dependent manner (42). These findings suggest that the results obtained from studies utilizing mastoparan as an inhibitor of TLR4-mediated G protein signaling should be interpreted with caution since the effects of this treatment may be to specifically bind and inhibit LPS rather than to inhibit G protein activation or signaling. Moreover, in contrast with the previous study (42), we find that TLR2 does indeed utilize $G\alpha_{i/o}$ proteins for MAPK and Akt activation.

The family of TLRs signal through various adaptor proteins (33). TLR2 engages MyD88/TIRAP and TLR3 engages TRIF alone, whereas TLR4 is coupled to all of MyD88/TIRAP and TRIF/TRIF-related adaptor molecule (33). These results suggest that $G\alpha_{i/o}$ proteins may act through a common mechanism to initiate signaling events downstream of diverse TLR signaling pathways. Fan et al. (16) have found that Gαi proteins are important for signaling events initiated by both gram-positive and gram-negative bacteria. These data support our finding that $G\alpha_{i/o}$ proteins are important for signaling from multiple TLRs. The TLR homology domain, TIR, is common to all TLRs and the IL-1R family members, IL-1R, IL-18R, and ST2 (5). IL-1 stimulation has been shown to increase GTP binding to cellular membranes and promote GTP hydrolysis (32). We have also seen that PTx can inhibit IL-1R-mediated signaling to Akt, but not the MAPKs (data not shown).

Our finding that genetic depletion of $G\alpha_{i2}$ is insufficient to abrogate signaling downstream of TLR4 suggests that the other Gαi isoforms, Gαi1 and Gαi3, may exhibit functional redundancy during LPS activation. Indeed, the highly homologous Gαi isoforms are functionally redundant downstream of α_2-adrenoreceptors in vivo (1), and Fan et al. have seen that transfection of a dominant negative for a single Gαi subunit is not sufficient to decrease ERK1/2 activation, but rather cotransfection of dominant negative constructs for at least two Gαi subunits is required for inhibition of the signal (13). Whether TLR4 signals through other Gαi isoforms in endothelial cells remains unclear. However, it has been shown that genetic deletion of the Gαi isoforms results in a decrease in the production of proinflammatory cytokines in macrophages and splenocytes, suggesting that all three isoforms may have signaling capacity downstream of TLR4 (14, 15).

There is emerging evidence that single transmembrane spanning receptors couple to heterotrimeric G proteins (37). Such nonclassical interactions have been demonstrated for receptor tyrosine kinases, epidermal growth factor receptor, and the insulin and insulin-like growth factor receptors (37). Insulin-dependent lipolysis and inhibition of glucose oxidation are blocked by PTx (19) and heterotrimeric G proteins have been shown to physically associate with, and be activated by, the insulin receptor (22, 36). Indeed, the intracellular domains of TLR2, TLR3, and TLR4 contain a consensus motif for binding of $G\alpha_{i/o}$, which consists of a basic residue at the NH_2 terminus and the COOH-terminal structure of BBXXB or BBXB (where B is any basic residue and X is any nonbasic residue) (35). It would be of interest to investigate whether this region of the intracellular TIR domain is responsible for binding and activation of Gαi.

In conclusion, we have shown for the first time that heterotrimeric $G\alpha_{i/o}$ proteins are activated downstream of multiple TLRs to contribute to endothelial activation of MAPK and Akt, as well as interferon activation downstream of TRIF-dependent receptors. These findings are important to our understanding of endothelial responses to bacterial and viral products in microbial infection.

GRANTS

This work was supported by grants to A. Karsan from the Canadian Institutes of Health Research (CIHR, MOP 97744). S. M. Dauphinee is the recipient of a studentship award from CIHR and the Michael Smith Foundation for Health Research (MSFHR). A. Karsan is a Senior Scholar of MSFHR.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Albarran-Juarez J, Gilsbach R, Piekorz RP, Pexa K, Beetz N, Schneider J, Nurnberg B, Birnbaumer L, Hein L.** Modulation of alpha2-adrenoceptor functions by heterotrimeric Galphai protein isoforms. *J Pharmacol Exp Ther* 331: 35–44, 2009.
2. **Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, Kubes P.** Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 111: 1011–1020, 2003.
3. **Andonegui G, Zhou H, Bullard D, Kelly MM, Mullaly SC, McDonald B, Long EM, Robbins SM, Kubes P.** Mice that exclusively express TLR4 on endothelial cells can efficiently clear a lethal systemic Gram-negative bacterial infection. *J Clin Invest* 119: 1921–1930, 2009.
4. **Bernot D, Peiretti F, Canault M, Juhan-Vague I, Nalbone G.** Upregulation of TNF-alpha-induced ICAM-1 surface expression by adenylate cyclase-dependent pathway in human endothelial cells. *J Cell Physiol* 202: 434–441, 2005.
5. **Boraschi D, Tagliabue A.** The interleukin-1 receptor family. *Vitam Horm* 74: 229–254, 2006.
6. **Camerer E, Regard JB, Cornelissen I, Srinivasan Y, Duong DN, Palmer D, Pham TH, Wong JS, Pappu R, Coughlin SR.** Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J Clin Invest* 119: 1871–1879, 2009.
7. **Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV.** TRAF6 is a signal transducer for interleukin-1. *Nature* 383: 443–446, 1996.
8. **Chi L, Li Y, Stehno-Bittel L, Gao J, Morrison DC, Stechschulte DJ, Dileepan KN.** Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplified by endotoxin and tumor necrosis factor-alpha. *J Interferon Cytokine Res* 21: 231–240, 2001.
9. **da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ.** Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex transfer from CD14 to TLR4 and MD-2. *J Biol Chem* 276: 21129–21135, 2001.
10. **Dauphinee SM, Karsan A.** Lipopolysaccharide signaling in endothelial cells. *Lab Invest* 86: 9–22, 2006.
11. **Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ.** Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103: 351–361, 2000.
12. **Fabian G, Szabo CA, Bozo B, Greenwood J, Adamson P, Deli MA, Joo F, Krizbai IA, Szucs M.** Expression of G-protein subtypes in cultured cerebral endothelial cells. *Neurochem Int* 33: 179–185, 1998.
13. **Fan H, Peck OM, Tempel GE, Halushka PV, Cook JA.** Toll-like receptor 4 coupled GI protein signaling pathways regulate extracellular signal-regulated kinase phosphorylation and AP-1 activation independent of NFkappaB activation. *Shock* 22: 57–62, 2004.
14. **Fan H, Williams DL, Zingarelli B, Breuel KF, Teti G, Tempel GE, Spicher K, Boulay G, Birnbaumer L, Halushka PV, Cook JA.** Differential regulation of lipopolysaccharide and Gram-positive bacteria induced cytokine and chemokine production in macrophages by $Galpha_i$ proteins. *Immunology* 122: 116–123, 2007.
15. **Fan H, Williams DL, Zingarelli B, Breuel KF, Teti G, Tempel GE, Spicher K, Boulay G, Birnbaumer L, Halushka PV, Cook JA.** Differential regulation of lipopolysaccharide and Gram-positive bacteria induced

cytokine and chemokine production in splenocytes by Galphai proteins. *Biochim Biophys Acta* 1763: 1051–1058, 2006.
16. **Fan H, Zingarelli B, Peck OM, Teti G, Tempel GE, Halushka PV, Spicher K, Boulay G, Birnbaumer L, Cook JA.** Lipopolysaccharide- and gram-positive bacteria-induced cellular inflammatory responses: role of heterotrimeric $G\alpha_i$ proteins. *Am J Physiol Cell Physiol* 289: C293–C301, 2005.
17. **Ferlito M, Romanenko OG, Guyton K, Ashton S, Squadrito F, Halushka PV, Cook JA.** Implication of $G\alpha_i$ proteins and Src tyrosine kinases in endotoxin-induced signal transduction events and mediator production. *J Endotoxin Res* 8: 427–435, 2002.
18. **Fitzner N, Clauberg S, Essmann F, Liebmann J, Kolb-Bachofen V.** Human skin endothelial cells can express all 10 TLR genes and respond to respective ligands. *Clin Vaccine Immunol* 15: 138–146, 2008.
19. **Goren HJ, Northup JK, Hollenberg MD.** Action of insulin modulated by pertussis toxin in rat adipocytes. *Can J Physiol Pharmacol* 63: 1017–1022, 1985.
20. **Hull C, McLean G, Wong F, Duriez PJ, Karsan A.** Lipopolysaccharide signals an endothelial apoptosis pathway through TNF receptor-associated factor 6-mediated activation of c-Jun NH_2-terminal kinase. *J Immunol* 169: 2611–2618, 2002.
21. **Into T, Dohkan J, Inomata M, Nakashima M, Shibata K, Matsushita K.** Synthesis and characterization of a dipalmitoylated lipopeptide derived from paralogous lipoproteins of Mycoplasma pneumoniae. *Infect Immun* 75: 2253–2259, 2007.
22. **Jo H, Cha BY, Davis HW, McDonald JM.** Identification, partial purification, and characterization of two guanosine triphosphate-binding proteins associated with insulin receptors. *Endocrinology* 131: 2855–2862, 1992.
23. **Kagan JC, Medzhitov R.** Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125: 943–955, 2006.
24. **Larrivee B, Karsan A.** Isolation and culture of primary endothelial cells. *Methods Mol Biol* 290: 315–329, 2005.
25. **Lee MT, Hooper LC, Kump L, Hayashi K, Nussenblatt R, Hooks JJ, Detrick B.** Interferon-beta and adhesion molecules (E-selectin and s-intracellular adhesion molecule-1) are detected in sera from patients with retinal vasculitis and are induced in retinal vascular endothelial cells by Toll-like receptor 3 signalling. *Clin Exp Immunol* 147: 71–80, 2007.
26. **Lentschat A, Karahashi H, Michelsen KS, Thomas LS, Zhang W, Vogel SN, Arditi M.** Mastoparan, a G protein agonist peptide, differentially modulates TLR4- and TLR2-mediated signaling in human endothelial cells and murine macrophages. *J Immunol* 174: 4252–4261, 2005.
27. **Magder S, Neculcea J, Neculcea V, Sladek R.** Lipopolysaccharide and TNF-alpha produce very similar changes in gene expression in human endothelial cells. *J Vasc Res* 43: 447–461, 2006.
28. **Maier U, Babich A, Nurnberg B.** Roles of noncatalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. *J Biol Chem* 274: 29311–29317, 1999.
29. **Mikami F, Lim JH, Ishinaga H, Ha UH, Gu H, Koga T, Jono H, Kai H, Li JD.** The transforming growth factor-beta-Smad3/4 signaling pathway acts as a positive regulator for TLR2 induction by bacteria via a dual mechanism involving functional cooperation with NF-kappaB and MAPK phosphatase 1-dependent negative cross-talk with p38 MAPK. *J Biol Chem* 281: 22397–22408, 2006.
30. **Murga C, Fukuhara S, Gutkind JS.** A novel role for phosphatidylinositol 3-kinase beta in signaling from G protein-coupled receptors to Akt. *J Biol Chem* 275: 12069–12073, 2000.
31. **Naiki Y, Michelsen KS, Zhang W, Chen S, Doherty TM, Arditi M.** Transforming growth factor-beta differentially inhibits MyD88-dependent, but not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling. *J Biol Chem* 280: 5491–5495, 2005.
32. **O′Neill LA, Bird TA, Gearing AJ, Saklatvala J.** Interleukin-1 signal transduction increased GTP binding and hydrolysis in membranes of a murine thymoma line (EL4). *J Biol Chem* 265: 3146–3152, 1990.
33. **O′Neill LA, Bowie AG.** The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol* 7: 353–364, 2007.
34. **Ogawa S, Lozach J, Benner C, Pascual G, Tangirala RK, Westin S, Hoffmann A, Subramaniam S, David M, Rosenfeld MG, Glass CK.** Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell* 122: 707–721, 2005.
35. **Okamoto T, Nishimoto I, Murayama Y, Ohkuni Y, Ogata E.** Insulin-like growth factor-II/mannose 6-phosphate receptor is incapable of activating GTP-binding proteins in response to mannose 6-phosphate, but capable in response to insulin-like growth factor-II. *Biochem Biophys Res Commun* 168: 1201–1210, 1990.
36. **Okamoto T, Okamoto T, Murayama Y, Hayashi Y, Ogata E, Nishimoto I.** GTP-binding protein-activator sequences in the insulin receptor. *FEBS Lett* 334: 143–148, 1993.
37. **Patel TB.** Single transmembrane spanning heterotrimeric g protein-coupled receptors and their signaling cascades. *Pharmacol Rev* 56: 371–385, 2004.
38. **Shin HS, Xu F, Bagchi A, Herrup E, Prakash A, Valentine C, Kulkarni H, Wilhelmsen K, Warren S, Hellman J.** Bacterial lipoprotein TLR2 agonists broadly modulate endothelial function and coagulation pathways in vitro and in vivo. *J Immunol* 186: 1119–1130.
39. **Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ.** TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412: 346–351, 2001.
40. **Watts VJ, Neve KA.** Sensitization of adenylate cyclase by Galpha i/o-coupled receptors. *Pharmacol Ther* 106: 405–421, 2005.
41. **Ye RD.** Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. *J Leukoc Biol* 70: 839–848, 2001.
42. **Yibin G, Jiang Z, Hong Z, Gengfa L, Liangxi W, Guo W, Yongling L.** A synthesized cationic tetradecapeptide from hornet venom kills bacteria and neutralizes lipopolysaccharide in vivo and in vitro. *Biochem Pharmacol* 70: 209–219, 2005.
43. **Zen K, Karsan A, Eunson T, Yee E, Harlan JM.** Lipopolysaccharide-induced NF-kappaB activation in human endothelial cells involves degradation of IkappaBalpha but not IkappaBbeta. *Exp Cell Res* 243: 425–433, 1998.
44. **Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, Mantovani A, Rothe M, Muzio M, Arditi M.** Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J Biol Chem* 274: 7611–7614, 1999.
45. **Zhou H, Andonegui G, Wong CH, Kubes P.** Role of endothelial TLR4 for neutrophil recruitment into central nervous system microvessels in systemic inflammation. *J Immunol* 183: 5244–5250, 2009.