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Endotoxin Tolerance Induced by Lipopolysaccharides Derived from *Porphyromonas gingivalis* and *Escherichia coli*: Alternations in Toll-Like Receptor 2 and 4 Signaling Pathway

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Abstract—Periodontitis is a chronic inflammatory disease induced by bacteria. Exposure of the host to periodontal pathogens and their virulence factors induces a hyporesponsive state to subsequent challenge, which is termed endotoxin tolerance. In this experiment, we studied the cytokine production in THP-1 cells upon single or repeated *Porphyromonas gingivalis* (*P. gingivalis*) lipopolysaccharide (LPS) or *Escherichia coli* (*E. coli*) LPS stimulation by ELISA. In addition, the protein expression profiles of Toll-like receptor 2 (TLR2), TLR4, IL-1 receptor-associated kinase 4 (IRAK4) and IRAK-M and the gene expression changes of Toll-interacting protein (Tollip) and suppressor of cytokine-signaling-1 (SOCS1) were explored to identify possible mechanisms for changes in cytokine secretion. After repeated stimulation with *P. gingivalis* LPS or *E. coli* LPS, secretions of TNF-α and IL-1β were decreased significantly compared with those following single challenge, while the levels of IL-10 were increased ($p<0.05$). Only comparable levels of IL-8 were confirmed in *P. gingivalis* LPS-tolerized cells ($p>0.05$). In addition, severe downregulation of TLR2 was detected in THP-1 cells retreated with *P. gingivalis* LPS, and the reduction of TLR4 expression was observed in cells restimulated with *E. coli* LPS ($p<0.05$). Precondition with *P. gingivalis* LPS or *E. coli* LPS also led to an enhancement of IRAK-M and SOCS1, while maintaining the expressions of IRAK4 and Tollip. This pattern of cytokine production indicates the different effects of endotoxin tolerance triggered by *P. gingivalis* LPS and *E. coli* LPS, which might contribute to limiting inflammatory damage. Moreover, TLR2, TLR4, IRAK-M, and SOCS1 might play important roles in developing tolerance.

KEY WORDS: endotoxin tolerance; lipopolysaccharide; *Porphyromonas gingivalis*; *Escherichia coli*; THP-1 cells.

INTRODUCTION

Periodontitis is a chronic inflammatory disease caused by bacteria, which is one of the two major oral diseases in humans. It is accompanied by the destruction of tooth-supporting structures and is difficult to treat [1]. *Porphyromonas gingivalis* (*P. gingivalis*), a gram-negative bacteria with black pigmentation, has been regarded as one of the most important periodontopathic bacteria [2]. As a major constituent of the outer membrane of *P. gingivalis*, lipopolysaccharide (LPS) is considered to be a potential inducer of proinflammatory cytokines, anti-inflammatory cytokines, and chemokines [3]. Appropriate host responses to *P. gingivalis* LPS are essential to resist invading bacteria, but excessive and prolonged immune responses can lead to the destruction of periodontal tissues [4].

Endotoxin tolerance is defined by a reduced capacity of the host to respond to subsequent LPS activation following an exposure to initial stimulus and is characterized by diminished release of proinflammatory cytokines, such as TNF-α and IL-1ß [5]. Hyporesponsiveness to a secondary challenge with the same LPS is referred to as homotolerance, while that with a different LPS is termed heterotolerance [6]. These two kinds of tolerances

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serve as important pathophysiological adaptations to regulate over-exuberant inflammation against bacteria, but they may also favor further infections [7]. Molecular mechanisms of endotoxin tolerance, especially for those in periodontitis, still need to be fully elucidated.

Toll-like receptors (TLRs) is a family of mammalian homologs of *Drosophlia* Toll [8]. Among them, TLR2 and 4 function as the principal innate sensors for virulence factors of periodontopathic bacteria and might be very important in endotoxin tolerance developed in periodontal tissues [9, 10].

TLR2 and 4 signaling pathways involve a cascade of adaptors, intermediates, and effectors, including myeloid differentiation factor-88 (MyD88), IL-1 receptor-associated kinases (IRAKs), and TNF receptor-associated factor-6 (TRAF6) [11]. The mammalian family of IRAK molecules contains four members (IRAK1, IRAK2, IRAK-M, and IRAK4). Among them, IRAK4 is essential for the phosphorylation of IRAK1. IRAK-M may act as a negative regulator by preventing the dissociation of IRAK1 and IRAK4 from MyD88 and the subsequent formation of its complex with TRAF6 [12].

Toll-interacting protein (Tollip) is identified initially as an inhibitory signaling molecule in interleukin-1 signaling. It functions through its ability to suppress potently the activity of IRAK after the activation of TLRs and therefore serve to limit the production of proinflammatory cytokines [13]. Suppressor of cytokine-signaling-1 (SOCS1) is another recently reported negative regulator in TLR2 and 4 signaling pathways, which is related with the inactivation of NF-κB [14].

In this present study, we hypothesized that TLR2, TLR4 and some of the signaling intermediates, including IRAK4, IRAK-M, Tollip and SOCS1, might be involved in endotoxin tolerance induced by *P. gingivalis* LPS and (or) *Escherichia coli* (*E. coli*) LPS, the most classic gram-negative bacterial LPS.

MATERIALS AND METHODS

Reagents

P. gingivalis ATCC 33277 LPS and *E. coli* O127:B8 LPS were purchased from InvivoGen (CA, USA) and Sigma Aldrich (MI, USA) separately. ELISA kits were provided by BioSource (CO, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-TLR2 antibody and PE-conjugated anti-TLR4 antibody were supplied by eBioscience (CA, USA). Antibodies to IRAK4 and IRAK-M were obtained from Cell Signaling (MA, USA).

Cell Culture and Tolerance Induction

THP-1 cells were cultured in 6-well plates (5×10^5 cells/ml) and divided into seven groups ($n=5$ per group). Group 1 was incubated in medium alone. Groups 2 and 5 were cultured in medium for 24 h, washed three times with fresh medium, and stimulated with 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS, respectively, for 4 h to perform western blot, 6 h for real-time PCR, and 24 h for ELISA and flow cytometry. Groups 3, 4, 6, and 7 were pretreated with 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for 24 h, washed, and challenged again with either 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E.coli* LPS for the subsequent detections as described in groups 2 and 5.

Cytokine Detection

At the end of the culture period, supernatants were harvested and stored at −20 °C. Levels of TNF-α, IL-1β, IL-8, and IL-10 in the culture supernatants were determined by commercial ELISA kits according to the manufacturer's instructions.

Flow Cytometry

THP-1 cells were collected and incubated with FITC-conjugated anti-TLR2 antibody and phycoerythrin (PE)-conjugated anti-TLR4 antibody for 30 min at 4 °C in the dark. FITC-conjugated IgG2b and PE-conjugated IgG1 were served as corresponding isotype controls for nonspecific binding of antibodies. After this incubation, cells were washed and then fixed in 1 % paraformaldehyde [15]. Expressions of TLR2 and 4 on 10,000 viable cells were gated and analyzed by a FACSCalibur (BD Biosciences, USA).

Western Blot

Cells were harvested in ice-cold lysis buffer consisting of 1 % Triton X-100, 20-mM Tris and inhibitors for proteinase. Protein extracts were then separated by electrophoresis in 10 % SDS-polyacrylamide gels, and transferred to a PVDF membrane. The membrane was blocked with PBS containing 0.1 % Tween-20 and 5 % nonfat milk, and incubated with antibodies against IRAK4, IRAK-M, and ß-actin overnight. After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h,

immunoreactive proteins were visualized using a chemiluminescent ECL assay kit (Thermo, USA) [16].

Real-Time PCR

Total RNA was prepared from THP-1 cells stimulated with or without LPS. cDNA was synthesized using a reverse transcription kit (Takara, China). Levels of ß-actin mRNA served as internal controls. The primer sequences were as follows (F/R): Tollip (CAGGCGTAGACTCTTTCTATCTC/GACTCCGAGATGGTGATGTG); SOCS1 (AACTGCTCTTTCGCCCTTAGC/CAGCTCGGAGAGGCAGTCG); and ß-actin (CCTGTACGCCAACACAGTGC/ATACTCCTGCTTGCTGATCC).

Real-time PCR analysis was performed in duplicates in an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, USA) with SYBR Premix EX Taq (Takara, China). The reaction product was quantified by the standard curve method [3].

Statistical Analysis

Statistical analysis of ELISA data was performed using ANOVA. The data of flow cytometry and real-time PCR were analyzed using the Kruskal–Wallis test, and subsequently, the Mann–Whitney test was performed as a post hoc test. The level of significance was set at $p<0.05$.

RESULTS

Cytokine Production in THP-1 Cells upon a Primary or Secondary Exposure to LPS

Stimulations with *P. gingivalis* LPS or *E. coli* LPS for 24 h resulted in an enhanced secretion of TNF-α, IL-1β, IL-10, and IL-8 ($p<0.05$), and the amounts of all cytokines induced by *P. gingivalis* were significantly lower than those induced by *E. coli* LPS ($p<0.05$) (Fig. 1).

After restimulation with the same LPS for additional 24 h, the levels of TNF-α and IL-1β were decreased markedly compared with those from THP-1 cells stimulated with *P. gingivalis* LPS or *E. coli* LPS only once ($p<0.05$), while the production of IL-10 was increased significantly ($p<0.05$). Interestingly, a serve reduction in the levels of IL-8 was only observed in cells challenged with repeated *E. coli* LPS but not *P. gingivalis* LPS, which demonstrated the differences in endotoxin tolerance drove by *P. gingivalis* LPS and *E. coli* LPS ($p<0.05$) (Fig. 1).

In heterotolerance groups (groups 4 and 7), a noticeable downregulation of TNF-α and IL-1β was also detected after retreatment with different LPS ($p<0.05$), while there were no significant differences at the levels of IL-10 and IL-8 ($p>0.05$). Moreover, the amounts of TNF-α and IL-1β in homotolerance groups were significantly lower than those in heterotolerance groups ($p<0.05$), except IL-1β production between groups 3 and 4 ($p>0.05$) (Fig. 1).

Expressions of TLR2 and 4 in THP-1 Cells after a Primary or Secondary LPS Exposure

Upregulation of TLR2 or TLR4 protein was observed in cells stimulated with *P. gingivalis* LPS or *E.coli* LPS, respectively ($p<0.05$), which demonstrated the different abilities of these two kinds of LPS to modulate expression levels of members of the TLR family (Fig. 2).

Furthermore, a significant downregulation of TLR2 protein was detected in THP-1 cells pretreated with either kind of LPS and challenged with *P. gingivalis* LPS again ($p<0.05$). A marked decrease in TLR4 expression was also observed in cells preconditioned with any kind of LPS and retreated with *E. coli* LPS ($p<0.05$). At the levels of both TLR2 and TLR4, there were no significant differences between homotolerance groups and heterotolerance groups ($p>0.05$) (Fig. 2).

Differential Expressions of IRAK-M but not IRAK4 in Endotoxin Tolerance

After the first *P. gingivalis* LPS or *E. coli* LPS stimulation, both IRAK4 and IRAK-M expressions were increased compared with those without LPS challenge. In addition, after *P. gingivalis* LPS or *E. coli* LPS retreatment, the expression levels of IRAK-M were increased in both homotolerance groups and heterotolerance groups, but no expression changes could be observed at the levels of IRAK4 (Fig. 3).

Enhanced Expressions of SOCS1 but not Tollip Contributed to the Induction of Endotoxin Tolerance

After the first 24-h challenge, both kinds of LPS led to the significant increases in Tollip and SOCS1 mRNA expressions ($p<0.05$). The levels of Tollip induced by *P. gingivalis* LPS were significantly higher than those induced by *E. coli* LPS ($p<0.05$), while the differences in SOCS1 expression levels

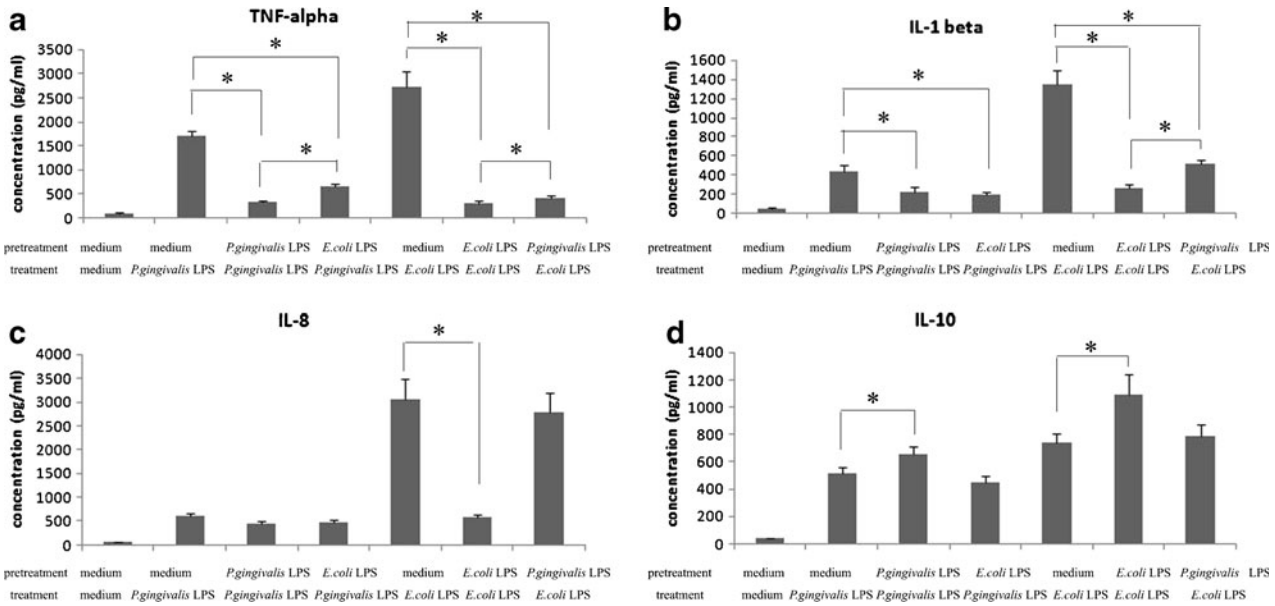

Fig. 1. Cytokine production in THP-1 cells stimulated with LPS. THP-1 cells were pretreated with medium, 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for 24 h, washed, and then incubated with medium, 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for another 24 h. The levels of TNF-α (**a**), IL-1β (**b**), IL-8 (**c**), and IL-10 (**d**) in the culture supernatants were measured by ELISA. Data are expressed as mean ± SD (n=5 per group). *p<0.05.

between these two groups weren't statistically significant (p>0.05) (Fig. 4).

After pretreatment with *P. gingivalis* LPS or *E. coli* LPS for 24 h and a subsequent restimulation for additional 6 h, the expressions of SOCS1 in both homotolerance groups and heterotolerance groups were significantly higher than those in cells stimulated only once (p<0.05), but no significant differences were observed between these two groups (p>0.05). In contrast, the expression levels of Tollip were only increased in THP-1 cells preconditioned with *E. coli* LPS and subsequently stimulated with *P. gingivalis* LPS (p<0.05) (Fig. 4).

DISCUSSION

In this present study, we evaluated the cytokine production in THP-1 cells upon repeated *P. gingivalis* LPS and (or) *E.coli* LPS challenges, indicating the differences in endotoxin tolerance triggered by these two kinds of LPS at the levels of several cytokines. In addition, we disclosed the involvement of TLR2 and 4 signaling pathways, especially the negative regulators, IRAK-M and SOCS1, in *P. gingivalis* LPS tolerization for the first time.

It is generally accepted that the primary etiologic factor of periodontitis is bacterial biofilm and specific microorganisms in subgingival plaque, in cluding *P. gingivalis*, *Prevotella intermedia* (*P. intermedia*), *Fusobacterium nucleatum* (*F. nucleatum*), and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), initiate the disease [17]. LPS is one of the most important virulence factors of gram-negative bacteria, and could cause direct destruction of periodontal tissues and stimulate the host to trigger a wide range of inflammatory responses [18, 19]. In comparison to the classic gram-negative bacterial LPS, *E. coli* LPS, *P. gingivalis* LPS is unique in its biological activities and pathogenicities. Its lipid A is a monophosphate type lacking a phosphate group in the 4′ position that does not consist of tetradecanoic acids, but rather long-chain fatty acids made up of only acyloxyacyl groups. Therefore, endotoxic activities of *P. gingivalis* LPS is relatively weaker, and some researchers believed that *P. gingivalis* LPS could act on host cells through TLR2, but not TLR4 just as what was found in *E. coli* LPS [10, 20]. Furthermore, accumulated evidences suggested that LPS from some other

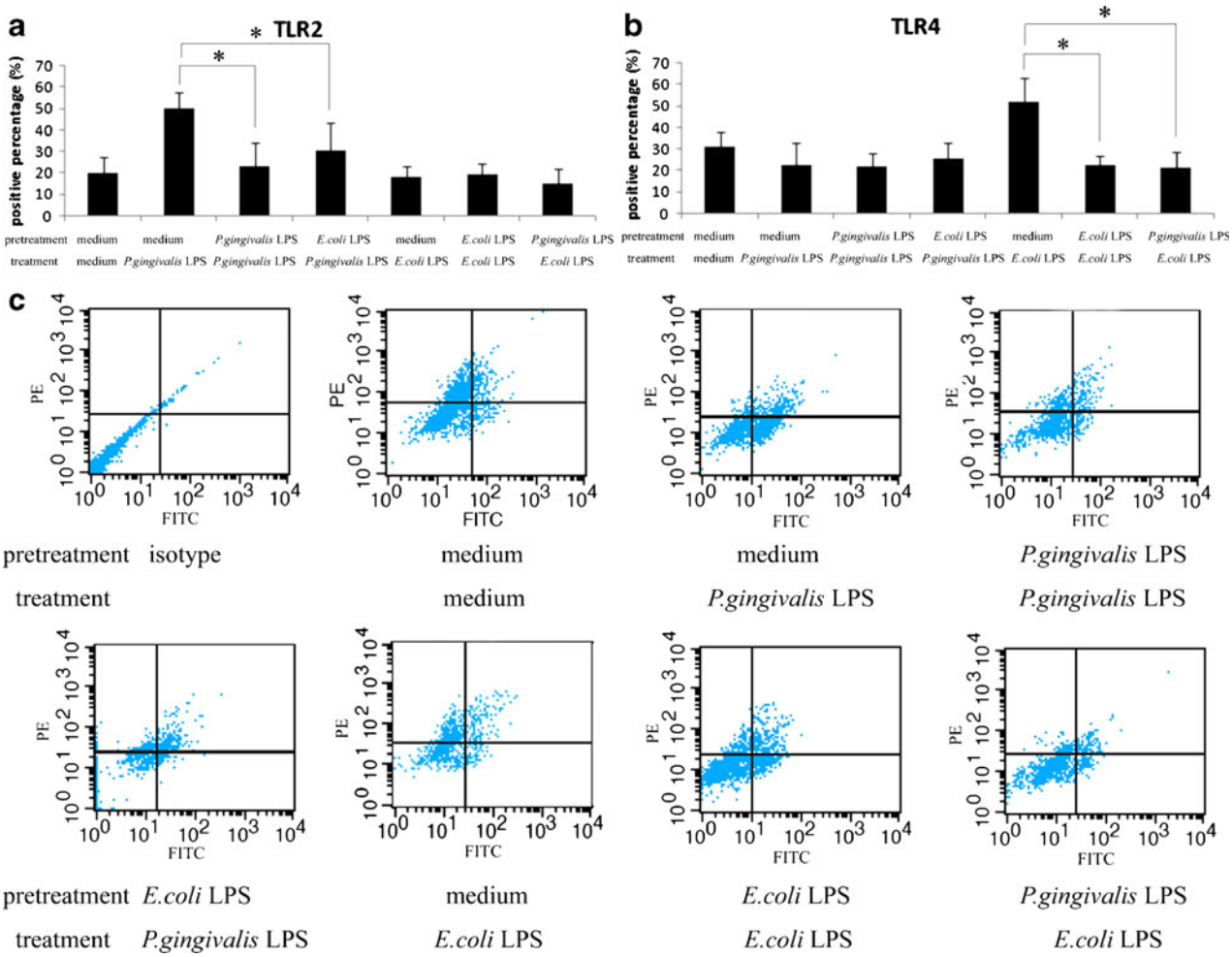

Fig. 2. Protein expression changes of TLR2 and 4 in THP-1 cells stimulated with LPS. THP-1 cells were stimulated with medium or LPS as described in the legends to Fig. 1. Protein expression levels of TLR2 (FITC-conjugated) (**a**) and TLR4 (PE-conjugated) (**b**) were detected by flow cytometry. Data are expressed as mean ± SD (n=5 per group). *p<0.05. One representative result of five independent experiments is shown (**c**).

periodontopathic bacteria, such as *P. intermedia*, *F. nucleatum*, and *A. actinomycetemcomitans*, could activate TLR4, and there were some similarities in potential for triggering inflammation between *E. coli* LPS and these periodontopathic bacterial LPS [21, 22]. Therefore, in this experiment, we chose two different kinds of LPS, *P. gingivalis* LPS and *E. coli* LPS, as the stimulators to explore the host responses to endotoxin tolerance, which might take place in the development of periodontal inflammation.

TNF-α is probably the best marker for endotoxin tolerance [23] and many researchers reported the reduced secretion of TNF-α in the cells upon repeated LPS challenge [24, 25]. However, endotoxin tolerance doesn't mean the global decline of all cytokines. Instead, it represents a selective reprogramming aimed at limiting inflammatory damage [26]. The reasons for differential cytokine expressions might be very complicated. We speculate it might be associated with different signaling pathways for different cytokine production.

Present results from our group also showed that the levels of TNF-α and IL-1β in the cells from homotolerance groups were much lower than those from heterotolerance groups. As for the production of IL-10, there was a significant change in homotolerance groups rather than heterotolerance groups. Therefore, hyporesponsiveness to a different subsequent stimulus might be usually weaker than that to the same LPS.

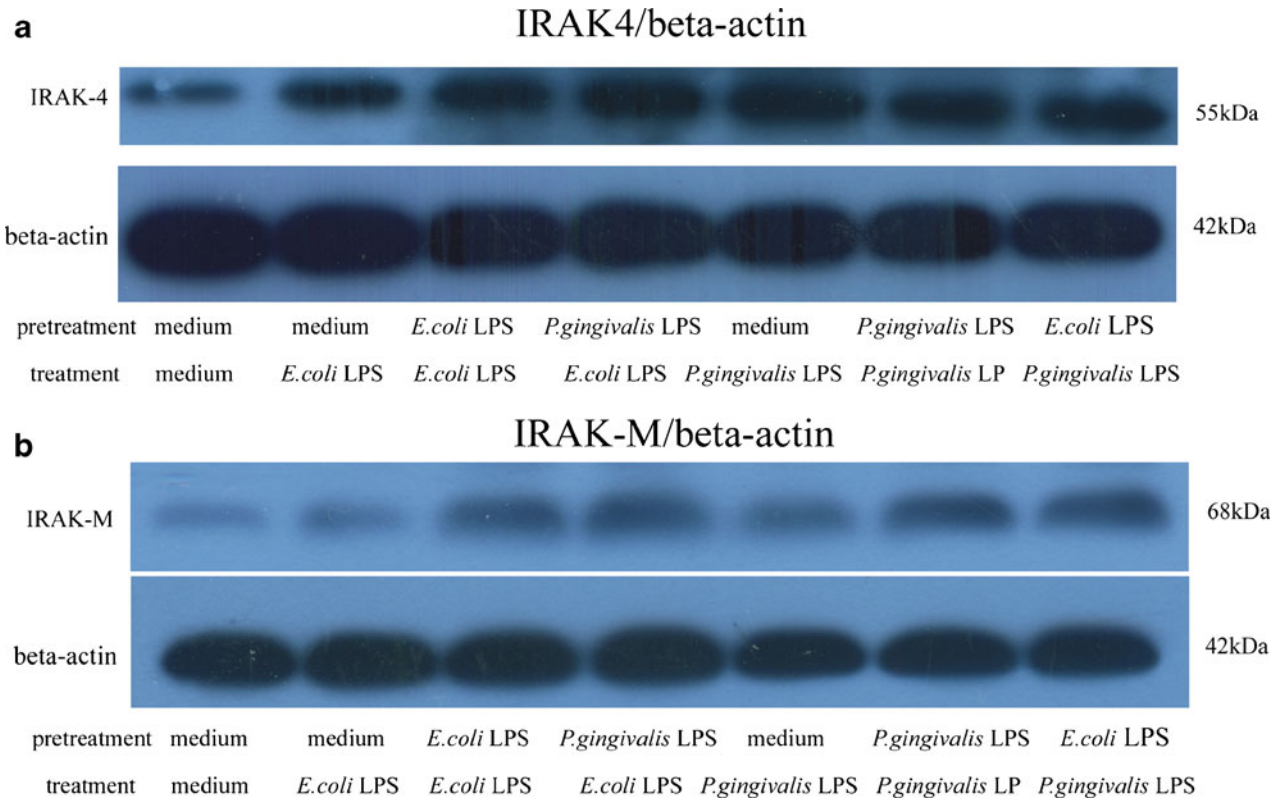

Fig. 3. Protein expressions of IRAK4 and IRAK-M in THP-1 cells stimulated with LPS detected by western blot. THP-1 cells were pretreated with medium, 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for 24 h, washed, and then challenged with medium, 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for another 4 h. Western blot was used to explore IRAK4 (**a**) and IRAK-M (**b**) protein expressions. Levels of ß-actin served as internal controls. One representative result of three independent experiments is shown.

According to the important roles of TLR2 and 4 in recognizing pathogen associated molecular patterns, we paid attention to their possible involvement in tolerance. Our results revealed that in response to repeated *P. gingivalis* LPS or *E. coli* LPS challenge, TLR2 or 4 expressions were down-regulated, which might inhibit excessive signaling transduction activated by persistent bacteria stimulations. Distinct expressions of TLR2 and 4 suggested the different regulatory mechanisms, which might be responsible for the different expression profiles of cytokines secreted by *P. gingivalis* LPS- or *E. coli* LPS-tolerized cells. *E. coli* LPS, as a TLR4 agonist, activates both MyD88-dependent and MyD88-independent pathways and down-regulates cytokine production in endotoxin-tolerant cells, while TLR2 signals only through MyD88-dependent pathway. Moreover, impaired and partial endotoxin tolerance induced in monocytes/macrophages by *P. gingivalis* LPS, characterized by persistent high secretion of IL-8 might be due to the lack of immunomodulatory effect of IFN-β [27].

Similar to our results, Li *et al.*'*s* research also confirmed that pretreatment of THP-1 cells with bacterial lipoprotein strongly inhibited TLR2 activation in response to subsequent stimulation [28]. Interestingly, Savidge *et al*. demonstrated that chronic exposure of intestinal epithelial cells (IEC) to LPS-induced tolerance at the levels of IL-8 independently of TLR4 signaling [29]. A possible explanation for this discrepancy might be the variation in TLR4 expression levels between THP-1 cells and IEC.

In this present research, we only explored protein expression levels of TLR2 and 4, but not activation states of these two receptors. Chen believed that LPS-induced NF-κB activation and cytokine gene expressions required tyrosine phosphorylation of TLR4 [30]. Tyrosine phosphorylation can regulate signal transducing functions of many receptors, including TLR2, TLR3, and TLR4,

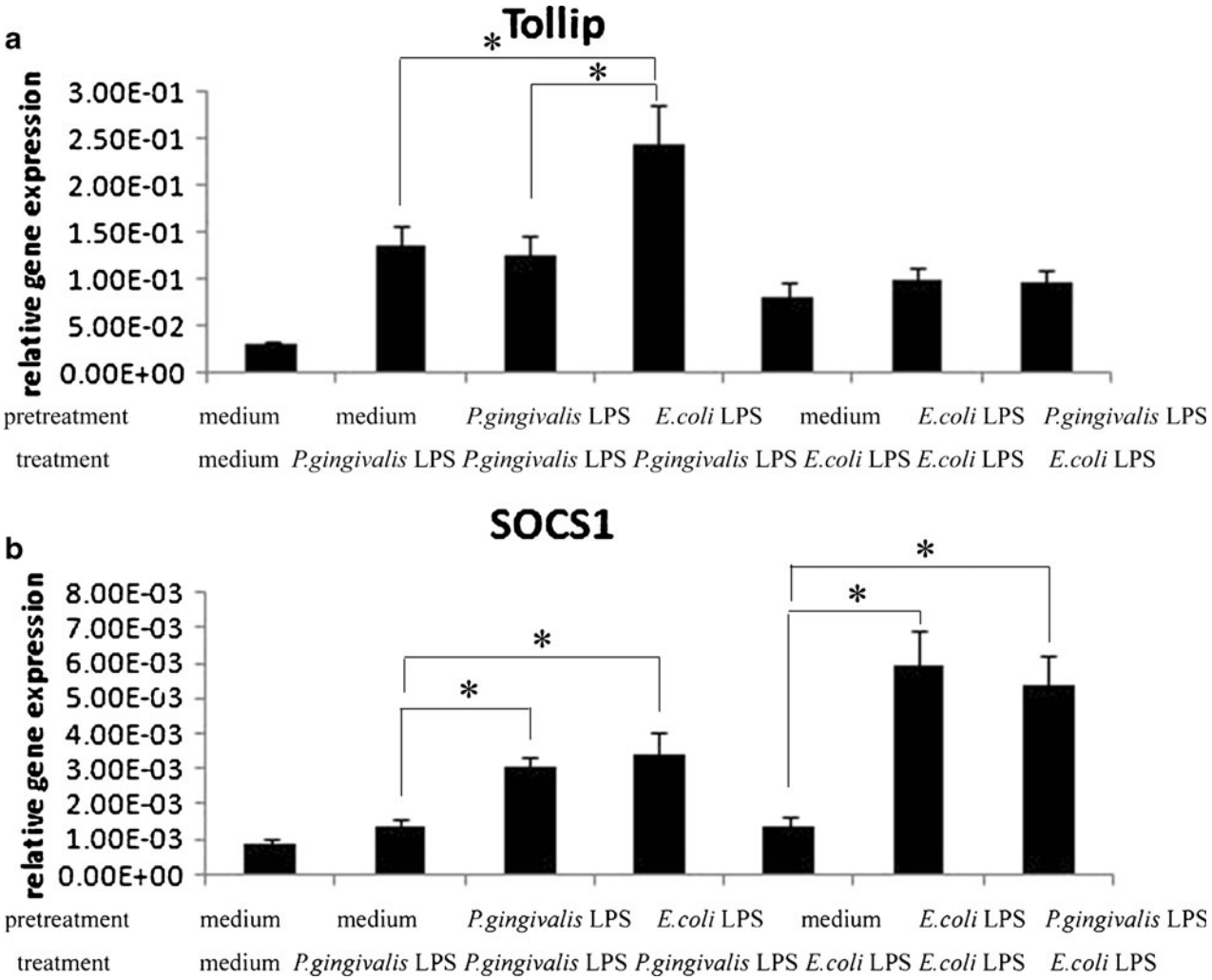

Fig. 4. Gene expression changes of Tollip and SOCS1 in THP-1 cells stimulated with LPS. THP-1 cells were pretreated with medium, 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for 24 h, washed, and then restimulated with medium, 1 μg/ml *P. gingivalis* LPS or 1 μg/ml *E. coli* LPS for 6 h. Real-time PCR was used to quantify Tollip (**a**) and SOCS1 (**b**) mRNA expression levels. The absolute mRNA levels of all the genes were normalized to ß-actin levels of individual samples. Data are expressed as mean ± SD (n=5 per group). *p<0.05.

which leads to post-translational modifications within the TIR domain and plays an important role in the recruiting of MyD88 and IRAKs [31, 32]. Therefore, further studies are needed to explore tyrosine phosphorylation of TLR2 and 4 in endotoxin-tolerant cells.

Mechanisms of heterotolerance might be more complex than those of homotolerance. Actually, periodontal infections are polymicrobial, and numerous virulence factors are involved in it. Endotoxin tolerance in periodontal tissues is a kind of heterotolerance rather than homotolerance. We presumed that there might be crosstalk between TLR2 and TLR4 pathways and some key signaling intermediates in the downstream of TLR2 and 4 might play a role in endotoxin tolerance, especially in heterotolerance.

Function of IRAKs is also highly regulated via multiple mechanisms, such as the positive regulator IRAK4 and the negative regulator IRAK-M [12]. In our present research, no significant differences were confirmed in IRAK4 protein expressions before and after *P. gingivalis* LPS retreatment. Xiong *et al*. believed that induction of endotoxin tolerance didn't suppress the total expression levels of IRAK4 in THP-1 cells and human monocytes. Instead, tolerance impaired recruitment of

IRAK4 to TLR4 and activation of IRAK4 [33]. In contrast to the comparable expressions of IRAK4, increased levels of IRAK-M in our experiment disclosed their contribution to the development of endotoxin tolerance. After LPS challenge, the complex formed by MyD88, IRAK1, IRAK4, and TRAF6 induces a series of phosphorylation events that lead to the activation of NF-κB and activator protein 1. It has been suggested that IRAK-M could block the signaling between MyD88 and IRAK1 by binding to the complex, and then inhibit the resulting cytokine production [34].

Tollip is an endogenously expressed molecule that limits proinflammatory signals from both innate and adaptive immune systems. It might potently suppress the activity of IRAK after the activation of TLRs, either by blocking IRAK autophosphorylation or by interfering with the binding of a crucial signaling molecule, such as a second kinase [35]. To our disappointment, we failed to confirm a distinct upregulation of Tollip mRNA expressions upon repeated *P. gingivalis* LPS or *E. coli* LPS treatment. Interestingly, there was an enhanced expression of Tollip in the cells pretreated with *E. coli* LPS and treated with *P. gingivalis* LPS. Advanced exploration is needed to disclose whether there is a synergetic effect or crosstalk between these two kinds of LPS signal transduction pathways.

SOCS1 was described as a suppressor factor involved in a coordinated negative regulation of immune responses [36]. Our study revealed that an increase in SCOC1 expressions was related with not only homotolerance but also heterotolerance. SOCS1 could regulate cytokine signaling pathways via inhibition of tyrosine phosphorylation on cytokine receptors and signaling molecules [37]. It also could inhibit NF-κB activation by IRAKs and TRAF6 but not by I-κB kinase-β (IKK-β), indicating that the inhibition by SOCS1 occurred downstream of TRAF6 and upstream of IKK-β [38]. In addition, SOCS1 could interact with Toll/IL-1 receptor-associated protein (TIRAP), which specifically mediates MyD88-dependent pathway via TLR2 and 4, and lead to its degradation. Degradation of signaling proteins, such as TIRAP, severely dampens LPS signaling and impairs cytokine production [39].

In summary, this present study demonstrated the quantitatively and qualitatively different cytokine production in *P. gingivalis* LPS- or *E. coli* LPS-tolerized THP-1 cells, and the involvement of TLR2, TLR4, IRAK-M, and SOCS1 in endotoxin tolerance. In addition, this pattern of immune response suggests that the above-mentioned receptors and inhibitory signaling molecules might be potential targets for controlling inflammation and maintaining homeostasis in periodontal tissues.

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REFERENCES

1. Oliver, R.C., L.J. Brown, and H. Loe. 1998. Periodontal diseases in the United States population. *Journal of Periodontology* 69(2): 269–278.
2. Komiya Ito, A., K. Ishihara, S. Tomita, T. Kato, and S. Yamada. 2010. Investigation of subgingival profile of periodontopathic bacteria using polymerase chain reaction. *The Bulletin of Tokyo Dental College* 51(3): 139–144.
3. Sun, Y., R. Shu, C.L. Li, and M.Z. Zhang. 2010. Gram-negative periodontal bacteria induce the activation of toll-like receptors 2 and 4, and cytokine production in human periodontal ligament cells. *Journal of Periodontology* 81(10): 1488–1496.
4. Garlet, G.P. 2010. Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints. *Journal of Dental Research* 89(12): 1349–1363.
5. Kopanakis, K., I.M. Tzepi, A. Pistiki, D.P. Carrer, M.G. Netea, M. Georgitsi, *et al*. 2013. Pre-treatment with low-dose endotoxin prolongs survival from experimental lethal endotoxic shock: benefit for lethal peritonitis by *Escherichia coli*. *Cytokine* 62(3): 382–388.
6. Ruud, T.E., Y. Gundersen, J.E. Wang, S.J. Foster, C. Thiemermann, and A.O. Aasen. 2007. Activation of cytokine synthesis by systemic infusions of lipopolysaccharide and peptidoglycan in a porcine model in vivo and in vitro. *Surgical Infections* 8(5): 495–503.
7. Broad, A., D.E. Jones, and J.A. Kirby. 2006. Toll-like receptor (TLR) response tolerance: a key physiological "damage limitation" effect and an important potential opportunity for therapy. *Current Medicinal Chemistry* 13(21): 2487–2502.
8. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway Jr. 1997. A human homologue of the drosophila toll protein signals activation of adaptive immunity. *Nature* 388(6640): 394–397.
9. Tateishi, F., K. Hasegawa-Nakamura, T. Nakamura, Y. Oogai, H. Komatsuzawa, K. Kawamata, *et al*. 2012. Detection of *Fusobacterium nucleatum* in chorionic tissues of high-risk pregnant women. *Journal of Clinical Periodontology* 39(5): 417–424.
10. Wara-Aswapati, N., A. Chayasadom, R. Surarit, W. Pitiphat, J.A. Boch, T. Nagasawa, *et al.* 2013. Induction of toll-like receptor expression by *Porphyromonas gingivalis*. *Journal of Periodontology* 84(7): 1010–1018.
11. Brown, J., H. Wang, G.N. Hajishengallis, and M. Martin. 2011. TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *Journal of Dental Research* 90(4): 417–427.
12. Gottipati, S., N.L. Rao, and W.P. Fung-Leung. 2008. IRAK1: a critical signaling mediator of innate immunity. *Cellular Signalling* 20(2): 269–276.
13. Ankem, G., S. Mitra, F. Sun, A.C. Moreno, B. Chutvirasakul, H.F. Azurmendi, *et al.* 2011. The C2 domain of tollip, a toll-like receptor signalling regulator, exhibits broad preference for phosphoinositides. *Biochemical Journal* 435(3): 597–608.
14. Liu, Z.J., X.L. Liu, J. Zhao, Y.J. Shi, L.N. Yan, and X.F. Chen. 2008. The Effects of SOCS-1 on liver endotoxin tolerance development induced by a low dose of lipopolysaccharide are related to dampen

NF-kappaB-mediated pathway. *Digestive and Liver Disease* 40(7): 568–577.
15. Liu, Q.Y., Y.M. Yao, S.W. Zhang, Y.H. Yan, and X. Wu. 2011. Naturally Existing CD11c (low) CD45RB (high) dendritic cells protect mice from acute severe inflammatory response induced by thermal injury. *Immunobiology* 216(1–2): 47–53.
16. Park, S.Y., J. da Park, Y.H. Kim, Y. Kim, Y.W. Choi, and S.J. Lee. 2011. *Schisandra chinensis* α-iso-cubebenol induces heme oxygenase-1 expression through PI3K/Akt and Nrf2 signaling and has anti-inflammatory activity in *Porphyromonas gingivalis* lipopolysaccharide-stimulated macrophages. *International Immunopharmacology* 11(11): 1907–1915.
17. Haffajee, A.D., and S.S. Socransky. 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000* 2000(5): 78–111.
18. Chiang, C.Y., E. Fu, E.C. Shen, and H.C. Chiu. 2003. Effects of CD14 receptors on tissue reactions induced by local injection of two gram-negative bacterial lipopolysaccharides. *Journal of Periodontal Research* 38(1): 36–43.
19. Posch, G., O. Andrukhov, E. Vinogradov, B. Lindner, P. Messner, O. Holst, *et al.* 2013. Structure and immunogenicity of the rough-type lipopolysaccharide from the periodontal pathogen *Tannerella forsythia*. *Clinical and Vaccine Immunology* 20(6): 945–953.
20. Choi, S., J.E. Baik, J.H. Jeon, K. Cho, D.G. Seo, K. Ky, *et al.* 2011. Identification of *Porphyromonas gingivalis* lipopolysaccharide-binding proteins in human saliva. *Molecular Immunology* 48(15–16): 2207–2213.
21. Yoshimura, A., Y. Hara, T. Kaneko, and I. Kato. 1997. Secretion of IL-1 Beta, TNF-Alpha, IL-8 and IL-1ra by human polymorphonuclear leukocytes in response to lipopolysaccharides from periodontopathic bacteria. *Journal of Periodontal Research* 32(3): 279–286.
22. Gutiérrez-Venegas, G., P. Kawasaki-Cárdenas, S.R. Cruz-Arroyo, M. Pérez-Garzón, and S. Maldonado-Frías. 2006. *Actinobacillus Actinomycetemcomitans* lipopolysaccharide stimulates the phosphorylation of p44 and p42 MAP kinases through CD14 and TLR-4 receptor activation in human gingival fibroblasts. *Life Sciences* 78(22): 2577–2583.
23. Mathison, J.C., G.D. Virca, E. Wolfson, P.S. Tobias, K. Glaser, and R.J. Ulevitch. 1990. Adaptation to bacterial lipopolysaccharide controls lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages. *Journal of Clinical Investigation* 85(4): 1108–1118.
24. Zingarelli, B., H. Fan, S. Ashton, G. Piraino, P. Mangeshkar, and J.A. Cook. 2008. Peroxisome proliferator-activated receptor gamma is not necessary for the development of LPS-induced tolerance in macrophages. *Immunology* 124(1): 51–77.
25. Zhu, M.F., J. Zhang, J.M. Qu, H.J. Zhang, S.C. Zhou, S.F. Dong, *et al.* 2010. Up-regulation of growth factor independence 1 in endotoxin tolerant macrophages with low secretion of TNF-Alpha and IL-6. *Inflammation Research* 59(10): 855–860.
26. Melo, E.S., D.F. Barbeiro, R. Gorjão, E.C. Rios, D. Vasconcelos, I.T. Velasco, *et al.* 2010. Gene expression reprogramming protects macrophage from septic-induced cell death. *Molecular Immunology* 47(16): 2587–2593.
27. Zaric, S.S., W.A. Coulter, C.E. Shelburne, C.R. Fulton, M.S. Zaric, A. Scott, *et al.* 2011. Altered toll-like receptor 2-mediated endotoxin tolerance is related to diminished interferon beta production. *Journal of Biological Chemistry* 286(34): 29492–29500.
28. Li, C.H., J.H. Wang, and H.P. Redmond. 2006. Bacterial lipoprotein-induced self-tolerance and cross-tolerance to LPS are associated with reduced IRAK-1 expression and MyD88-IRAK complex formation. *Journal of Leukocyte Biology* 79(4): 867–875.
29. Savidge, T.C., P.G. Newman, W.H. Pan, M.Q. Weng, H.N. Shi, M.C. Ba, *et al.* 2006. Lipopolysaccharide-induced human enterocyte tolerance to cytokine-mediated interleukin-8 production may occur independently of TLR-4/MD-2 signaling. *Pediatric Research* 59(1): 89–95.
30. Chen, L.Y., B.L. Zuraw, M. Zhao, F.T. Liu, S. Huang, and Z.K. Pan. 2003. Involvement of protein tyrosine kinase in toll-like receptor 4-mediated NF-kappa B activation in human peripheral blood monocytes. *American Journal of Physiology - Lung Cellular and Molecular Physiology* 284(4): L607–L613.
31. Medvedev, A.E., W. Piao, J. Shoenfelt, S.H. Rhee, H. Chen, S. Basu, *et al.* 2006. Role of TLR4 tyrosine phosphorylation in signal transduction and endotoxin tolerance. *Journal of Biological Chemistry* 282(22): 16042–16053.
32. Arbibe, L., J.P. Mira, N. Teusch, L. Kline, M. Guha, N. Mackman, *et al.* 2000. Toll-like receptor 2-mediated NF-Kappa B activation requires a Rac1-dependent pathway. *Nature Immunology* 1(6): 533–540.
33. Xiong, Y., F. Qiu, W. Piao, C. Song, L.M. Wahl, and A.E. Medvedev. 2011. Endotoxin tolerance impairs IL-1 receptor-associated kinase (IRAK) 4 and TGF-beta-activated kinase 1 activation, K63-linked polyubiquitination and assembly of IRAK1, TNF receptor-associated factor 6, and Ikappa B kinase gamma and increases A20 expression. *Journal of Biological Chemistry* 286(10): 7905–7916.
34. Harada, K., K. Isse, Y. Sato, S. Ozaki, and Y. Nakanuma. 2006. Endotoxin tolerance in human intrahepatic biliary epithelial cells is induced by upregulation of IRAK-M. *Liver International* 26(8): 935–942.
35. Bulut, Y., E. Faure, L. Thomas, O. Equils, and M. Arditi. 2001. 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. *Journal of Immunology* 167(2): 987–994.
36. Dimitriou, I.D., L. Clemenza, A.J. Scotter, G. Chen, F.M. Guerra, and R. Rottapel. 2008. Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins. *Immunological Reviews* 224: 265–283.
37. Alexander, W.S., and D.J. Hilton. 2004. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annual Review of Immunology* 22: 503–529.
38. Mun, H.S., F. Aosai, K. Norose, L.X. Piao, H. Fang, S. Akira, *et al.* 2005. Toll-like receptor 4 mediates tolerance in macrophages stimulated with toxoplasma gondii-derived heat shock protein 70. *Infection and Immunity* 73(8): 4634–4642.
39. Scott, M.J., S. Liu, R.A. Shapiro, Y. Vodovotz, and T.R. Billiar. 2009. Endotoxin uptake in mouse liver is blocked by endotoxin pretreatment through a suppressor of cytokine signaling-1-dependent mechanism. *Hepatology* 49(5): 1695–1708.