SMAD4 Is Required for Development of Maximal Endotoxin Tolerance

Hongjie Pan, Enyu Ding, Mai Hu, Anand S. Lagoo, Michael B. Datto and Sandhya A. Lagoo-Deenadayalan

*J Immunol* 2010; 184:5502-5509; Prepublished online 19 April 2010;
doi: 10.4049/jimmunol.0901601
http://www.jimmunol.org/content/184/10/5502

Why *The JI*?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References  This article *cites 36 articles*, 14 of which you can access for free at:
http://www.jimmunol.org/content/184/10/5502.full#ref-list-1

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
SMAD4 Is Required for Development of Maximal Endotoxin Tolerance

Hongjie Pan,* Enyu Ding,* Mai Hu,* Anand S. Lagoo, † Michael B. Datto, † and Sandhya A. Lagoo-Deenadayalan*

Initial exposure of monocytes/macrophages to LPS induces hyporesponsiveness to a second challenge with LPS, a phenomenon termed LPS tolerance. Molecular mechanisms responsible for endotoxin tolerance are not well defined. We and others have shown that IL-1R–associated kinase (IRAK)-M and SHIP-1 proteins, negative regulators of TLR4 signaling, increase in tolerized cells. TGF-β1, an anti-inflammatory cytokine, is upregulated following LPS stimulation, mediating its effect through SMAD family proteins. Using a monocytic cell line, THP1, we show that LPS activates endogenous SMAD4, inducing its migration into the nucleus and increasing its expression. Secondary challenge with high dose LPS following initial low-dose LPS exposure does not increase IRAK-M or SHIP1 protein expression in small hairpin (sh)SMAD4 THP-1 cells compared with control shLUC THP1 cells. TNF-α concentrations in culture supernatants after second LPS challenge are higher in shSMAD4 THP-1 cells than shLUC THP1 cells, indicating failure to induce maximal tolerance in absence of SMAD4 signaling. Identical results are seen in primary murine macrophages and mouse embryonic fibroblasts, demonstrating the biological significance of our findings. TGF-β1 regulates IRAK-M and SHIP1 expression through a SMAD4-dependent pathway. Knockdown of endogenous SHIP1 by shSHIP1 RNA decreases native and inducible IRAK-M protein expression and prevents development of endotoxin tolerance in THP1 cells. We conclude that in THP-1 cells and primary murine cells, SMAD4 signaling is required for maximal induction of endotoxin tolerance via modulation of SHIP1 and IRAK-M. The Journal of Immunology, 2010, 184: 5502–5509.

The endotoxin LPS is a major component of the outer cell wall of Gram-negative bacteria and is a potent inducer of inflammation. It stimulates monocytes and macrophages/histiocytes and induces them to release proinflammatory cytokines and other mediators. This results in augmentation of the host immune defense system and helps eliminate bacterial infection (1, 2). Following infection with Gram-negative bacteria, LPS, which forms a complex with LPS-binding protein, binds to the TLR4 receptor complex consisting of the adaptor molecules MyD88 and Toll-IL-1R domain-containing adaptor protein (TIRAP), which then recruits cytoplasmic proteins IL-1R–associated kinase (IRAK)1, IRAK4, and TNFR-associated factor 6. Phosphorylation and degradation of IRAK1 induces ubiquitination of TNFR-associated factor 6 and releases the adaptor protein complex into the cytoplasm, resulting in activation of downstream kinases such as TGF-β–activated kinase 1 (TAK1), inhibitor of κB kinase (IKK), and JNK (3, 4). This leads to activation of NF-κB, resulting in downstream induction of TNF-α, IL-6, and NO. TAK1, which was originally identified as a member of MAPK kinase kinase family mediating TGF-β/bone morphogenetic protein (BMP) signaling, is involved in LPS-induced activation of NF-κB and JNK (5, 6). In the TGF-β signaling pathway, TAK1 deletion leads to impaired NF-κB and JNK activation without impacting SMAD2 activation or TGF-β–induced gene expression (7).

SMAD4 is the common SMAD (co-SMAD) and is the common mediator of signal transduction by TGF-β/BMP superfamily. Signaling by TGF-β family members occurs through type I and type II receptors. The activated type I receptor kinase propagates the signal inside the cell through phosphorylation of receptor-regulated SMADs (R-SMADs): SMAD2/SMAD3 for TGF-β, SMAD1/SMAD5/SMAD8 for BMP). Activated receptor-regulated SMADs form heteromeric complexes with SMAD4. These complexes migrate into the nucleus, where they interact with transcription factors, activators, and corepressors to induce targeted gene expression (8). TGF-β1 is now well established as a potent anti-inflammatory cytokine, which plays a pivotal role in maintaining balanced host responses in immune and nonimmune inflammatory conditions (9, 10). TGF-β1 null mice as well as mice lacking the TGF-β transcription factor SMAD3 show increased TLR4 mRNA expression and increased expression of inflammatory cytokines and NO following LPS stimulation (11).

Endotoxin tolerance is a phenomenon in which previous exposure to a low level of LPS induces a transient period of hyporesponsiveness to subsequent challenge with high-dose LPS. Endotoxin tolerance has been also called deactivation, adaptation, desensitization, anergy, refractoriness, or reprogramming (12–14). The underlying molecular mechanisms for endotoxin tolerance are not clearly resolved. IRAK-M (15), SHIP1 (16), and suppressor of cytokine signaling 1 (SOCS1) (17) participate in negative regulation of LPS response. The expression of all three proteins increases following LPS restimulation in LPS tolerant cells. SHIP1 inhibits LPS–induced activation of MAPKs and cytokine production primarily by its phosphatase activity and in a PI3K-independent mechanism (18).
TGF-β expression is also increased by stimulation with LPS. TGF-β is an anti-inflammatory cytokine and plays a critical role in LPS-induced tolerance to repeat LPS stimulation. Interestingly, SHIP1 protein increase is mediated by the autocrine activity of LPS-induced production of TGF-β (16). In this study, we examine the regulatory role of SMAD4 in modifying cell signaling molecules SHIP1 and IRAK involved in induction of endotoxin tolerance.

Materials and Methods

Cell culture and reagents

THP1 and 293T cells were obtained from American Type Culture Collection (Manassas, VA). THP1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA). 293T cells were cultured in low glucose DMEM supplemented with 10% FBS and antibiotics (Invitrogen). Mouse bone marrow derived macrophages and mouse embryonic fibroblast (MEF) cells were gifts from Dr. X. Zhong (Department of Pediatrics, Duke Medical Center, Duke University, Durham, NC). Bone marrow cells from femurs and tibias in the C57BL/6 mice were plated into petri dishes containing RPMI 1640 medium supplemented with 10% FBS, 15% L929 cell-conditioned medium, and antibiotics. After 2–3 d of culture at 37°C in 5% CO2 incubator, different cells were collected and cultured in the medium for another 3–5 d. More than 95% of cells were CD11b+ using flow cytometry analysis. These cells were used in experiments to evaluate the role of Smad4 in endotoxin tolerance. MEF cells are grown in DMEM supplemented with 10% FBS and antibiotics.

For MEF cells, mouse embryos 12.5–14.5 d postcoitum were obtained and after removing limbs, brain, and internal organs, they were minced with a sterile razor blade. The tissue was placed in a 15 ml screw-cap tube containing 10 ml 0.05% trypsin/0.02% EDTA in PBS, and the tube was rotated end-over-end at 37°C for 10 min. A 5 ml aliquot from the incubated tube was removed, and another 5 ml fresh trypsin/EDTA was added to the original tube for 10 min. The procedure was repeated at least five times. The cell pellet was resuspended in 50 ml DMEM with 10% FBS, and the cells collected on 10-cm sterile plastic tissue culture dishes. The next day, the medium was changed, and the cells maintained in culture until confluent. The cells were split 1:10 and allowed to grow to confluency. MEF cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 0.1 mmol/l nonessential amino acids, 0.1 mmol/l 2-ME, and antibiotics at 37°C in an atmosphere of 95% air and 5% CO2.

Endotoxin tolerance induction and TNF-α ELISA

THP1 cells were grown on chamber slides (BD Discovery Labware, Bedford, MA) in growth medium supplemented with PMA (1 μg/ml) for 24 h at 37°C. The adherent cells were cultured in serum-free medium overnight, followed by adding LPS for 3–6 h. After three washes with PBS, cells were fixed in 4% paraformaldehyde/PBS for 15 min, and permeabilized with 0.2% Triton X-100/PBS for 20 min and preblocked in 5% BSA/PBS for 1 h. The slides were then incubated with mouse anti-human SMAD4 Ab (diluted in 1/100 in blocking solution) for 1 h, washed three times with PBS, and incubated with FITC-conjugated anti-mouse Ab (Jackson ImmunoResearch Laboratories) for 1 h. After additional washes, the slides were incubated with DAPI for 3 min, and mounted with antifade solution. Cells were examined under the Zeiss LSM510 inverted confocal microscope (Light Microscopy Core Facility, Duke University).

Results

SMAD4 participates in LPS signaling in THP1 cells

In this study, we used THP1 cells as a model to define the role of SMAD4 in the development of endotoxin tolerance. THP1 cells were treated with low-dose LPS (10 ng/ml) overnight and subsequently stimulated with 100 ng/ml LPS for 0, 3, 6, and 24 h. This activation protocol is referred to as “100 ng/ml.” Control cells were treated with 100 ng/ml LPS for similar time points but without prior stimulation with low dose LPS. This activation protocol is referred to as “100 ng/ml.”

Downloaded from http://www.jimmunol.org/ By guest on November 8, 2017
FIGURE 1. SMAD4 participates in endotoxin tolerance development. A, THP1 cells were treated with 100 or 10–100 ng/ml of LPS for 0, 1, 3, 6, and 24 h. After a specified period, cells were harvested for Western blot analysis and culture supernatants assessed for TNF-α concentration. Each treatment was executed in triplicates. Results are shown as mean ± SD. B, Cytosolic and nuclear proteins were isolated from the above treated cells and subjected to Western blot analysis with indicated Abs. All blots were reprobed with anti-β-actin to show equal loading. All results are representative of three separate experiments. C, THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 2, and 10 min. After a specified period, cells were harvested for Western blot analysis against p-SMAD2, SMAD2, or β-actin.

FIGURE 2. THP1 cells were treated with 100 ng/ml LPS for 3 h. Cells were fixed, permeabilized, and blocked in 5% BSA/PBS for 1 h. The slides were incubated with mouse anti-human SMAD4 Ab and FITC-conjugated anti-mouse Ig and DAPI. Cells were examined under the Zeiss LSM510 inverted confocal microscope (original magnification ×400). Representative nuclear staining by DAPI (left column), SMAD4 immunostaining (cytoplasmic in control cells and nuclear in LPS stimulated cells, middle column), and a combined image (right column) show the nuclear translocation of SMAD4 after LPS stimulation.

p-AKT and p-IkBα levels are retained following second exposure to LPS in shSMAD4 THP1 cells

Both PI3K and NF-κB pathways play important roles in LPS-induced endotoxin tolerance development (18). To explore the role SMAD4 plays in cell signaling following LPS stimulation or restimulation, we knocked down endogenous SMAD4 by using shSMAD4 RNA technology. Western blot analysis showed that SMAD4 shRNA successfully knocked down endogenous SMAD4 compared with control shLUC (Fig. 3A). SHIP-1 and IRAK-M levels were also decreased in shSMAD4 THP-1 cells. To investigate p-AKT and p-IκBα phosphorylation activities, both shLUC and shSMAD4 THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 10, 30 and 60 min. Both p-AKT and p-IκBα levels were sustained or slightly decreased in shSMAD4 THP1 following second exposure to LPS, whereas they decreased considerably with second LPS treatment in shLUC THP1 cells (Fig. 3B). LPS-induced degradation of total IκB-α protein was enhanced in shSMAD4 THP1 compared with shLUC THP1 cells following second exposure to LPS. These results demonstrate that both p-AKT and p-IκB-α continue to be activated in shSMAD4 THP1 cells following repeated exposure to LPS.

Knockdown of SMAD4 by shSMAD4 RNA results in failure to induce endotoxin tolerance in THP1 cells

To investigate the role SMAD4 plays in endotoxin tolerance, both shLUC and shSMAD4 THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 1, 3, 6, and 24 h. TNF-α concentrations, measured by ELISA, were significantly higher in the shSMAD4 THP1 cells than in the shLUC THP1 cells at the same time points (Fig. 4A). The abrogation of SMAD4 expression by shRNA in THP1 cells resulted in higher levels of TNF-α release following 100 and 10–100 ng/ml LPS stimulation compared with control, which indicates a partial abrogation of tolerance.

Upregulation of SHIP1 and IRAK-M is dependent on SMAD4 in the course of LPS-induced endotoxin tolerance

We investigated whether SMAD4, the co-SMAD, which mediates signal transduction by TGF-β/BMP superfamily (8) also regulates SHIP1 and IRAK-M in tolerized cells. Endogenous SMAD4 was knocked down with small hairpin (sh)SMAD4 RNA. We found that both SHIP1 and IRAK-M protein expressions were reduced in SMAD4 knockdown THP1 cells compared with control shLUC THP1 cells (Fig. 3A). Further studies showed that both SHIP1 and IRAK-M were not induced upon second exposure to LPS (Fig. 4B), whereas they were strongly induced in shLUC following similar stimulation with LPS. These data demonstrate that SMAD4 plays an important role in endotoxin tolerance development through regulation of SHIP1 and IRAK-M.

As shown above, abrogating SMAD4 signaling with shSMAD4 in THP1 cells prevented the development of LPS-induced endotoxin...
tolerance. To test whether TGF-β-induced upregulation of SHIP1 and IRAK-M is dependent on SMAD4, shSMAD4 and shLUC THP1 cells were treated with TGF-β1 (1 ng/ml) for 0, 3, 6, and 24 h. Western blot analysis showed decreased induction of both SHIP1 and IRAK-M protein expressions after TGF-β treatment in shSMAD4 THP1 cells compared with shLUC THP1 cells (Fig. 5). The above results indicate that TGF-β-induced upregulation of SHIP-1 and IRAK-M is dependent on SMAD4.

SMAD4 participates in LPS signaling in primary cells
To confirm the induction of SMAD4, SHIP1 and IRAK-M by LPS or TGF-β in primary cells, mouse bone marrow-derived macrophages were treated with 10 ng/ml LPS for 0, 6, and 24 h. For TGF-β stimulation assay, the mouse bone marrow-derived macrophages were starved in serum-free medium overnight and subsequently stimulated with 10 ng/ml TGF-β for 0, 6, and 24 h. Western blot analysis was used to measure the above indicated protein expressions. The results showed that SMAD4, IRAK-M, and SHIP1 protein expressions increase upon LPS or TGF-β stimulation in primary mouse macrophages (Fig. 6A). Furthermore, the SMAD4 gene of the mouse bone marrow-derived macrophages and mouse embryonic fibroblasts was knocked down with SMAD4 small interfering RNA (siRNA), and control siRNA was used as control. The cells were treated with low-dose LPS (10 ng/ml) overnight and subsequently stimulated with 100 ng/ml LPS for 0, 6, and 24 h. The cells were also treated with 100 ng/ml LPS for 0, 6, and 24 h as control. The indicated protein levels were analyzed by Western blot (Fig. 6B). Induced IRAK-M and SHIP1 protein expression levels were increased following priming with low dose LPS and subsequent stimulation with high dose LPS in control siRNA murine bone macrophages but unchanged upon LPS stimulation in the SMAD4 siRNA murine bone marrow macrophages. Similar results were obtained in control siRNA MEF cells and SMAD4 siRNA MEF cells (data not shown).

FIGURE 3. Knockdown of SMAD4 by shSMAD4 RNA results in increased p-AKT and p-IκBα levels in THP1 cells. A, Isolated proteins from shSMAD4 and shLUC THP1 cells were subjected to Western blot analysis with the indicated Abs. Blots were reprobed with anti-β-actin to show equal loading. B, shSMAD4 and shLUC THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 10, 30, and 60 min. After the indicated periods, cells were harvested for Western blot analysis with indicated Abs. Blots were reprobed with anti-β-actin to show equal loading. All results are representative of three separate experiments. shLUC, control shRNA; shSMAD4, shSMAD4 RNA.

FIGURE 4. SMAD4 regulates SHIP1 and IRAK-M during development of endotoxin tolerance. shSMAD4 and shLUC THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 1, 3, 6, and 24 h. After the indicated periods, some cells were harvested and lysed for Western blot analysis. A, Culture supernatants assessed for TNF-α concentration. Each treatment was executed in triplicates. Each experiment was repeated at least three times. Results shown are the mean ± SD. B, The cell lysates from the above treatments (A) were subjected to Western analysis with indicated Abs. The blots were reprobed with anti-β-actin to show equal loading. shLUC, control shRNA; shSMAD4, shSMAD4 RNA.
To study the role SMAD4 plays in endotoxin tolerance in primary cells, subconfluent MEF cells were transfected with siSMAD4 or control siRNA. The transfected MEF cells were respectively treated with 100 and 10–100 ng/ml LPS for 0, 6, and 24 h. LPS-induced TNF-α release from primary cells was measured by ELISA. Western blot analysis showed that endogenous SMAD4 was knocked down in MEF cells (data not shown). TNF-α secretion was significantly higher in the SMAD4 siRNA MEF cells than in the control siRNA MEF cells in both nontolerized (100 ng/ml) and tolerized (10–100 ng/ml) MEF cells (Fig. 6C). This result further demonstrates that SMAD4 is a negative regulator of proinflammatory cytokines, not only in THP-1 cells but also in primary murine cells.

Knockdown of SHIP1 by shSHIP1 RNA prevents induction of endotoxin tolerance in THP1 cells

LPS-induced upregulation of SHIP1 is essential for endotoxin tolerance (16). SHIP1 is a phosphatase that hydrolyzes phosphatidylinositol-3,4,5-triphosphate to phosphatidylinositol-3,4-bisphosphate. To verify whether this occurs in THP1 cells (data not shown), TNF-α secretion was significantly higher in the SMAD4 siRNA MEF cells than in the control siRNA MEF cells in both nontolerized (100 ng/ml) and tolerized (10–100 ng/ml) MEF cells (Fig. 6C). This result further demonstrates that SMAD4 is a negative regulator of proinflammatory cytokines, not only in THP-1 cells but also in primary murine cells.

Knockdown of SHIP1 by shSHIP1 RNA reduces IRAK-M expression in THP1 cells

Expression of both SHIP1 and IRAK-M expressions increases in tolerized cells. To explore whether SHIP1 regulates IRAK-M expression in monocytes, we blotted cell lysates from both shSHIP1 and shLUC THP1 cells with IRAK-M Abs. Western blot analysis showed that IRAK-M expression was not induced following LPS stimulation in shSHIP1 cells compared with control cells (Fig. 8A). TGF-β stimulation in THP1 cells increased both SHIP1 and IRAK-M expression. To further determine whether the induced IRAK-M expression is dependent on shSHIP1 expression, we treated both shSHIP1 and shLUC cells with TGF-β. Induced IRAK-M expression is more marked in shLUC cells following treatment with TGF-β than in shSHIP1 cells (Fig. 8B). These data indicate that SHIP1 regulates IRAK-M expression in endotoxin tolerance development and that this is enhanced via a TGF-β-dependant mechanism.

Discussion

Endotoxin tolerance has also been termed hyporesponsiveness, deactivation or desensitization. Tolerized cells produce less proinflammatory cytokines and NO in response to a second dose of LPS. However, LPS tolerance is not a global downregulation of
signaling proteins and mediators. LPS-tolerant animals and cells can still respond to further LPS challenge and express anti-inflammatory proteins, including IL-10, IRAK-M, SHIP1, SOCS1, and TGF-β (9, 15–17, 21, 22). TGF-β1 inhibits LPS-induced NF-κB activation and TNF-α release in mouse RAW264.7 cells and microglial cells (23, 24). In vitro tolerance of human monocytes can be partially mimicked by IL-10 and TGF-β, and the use of anti–IL-10 and anti–TGF-β Abs during the step of tolerization can prevent the phenomenon of endotoxin tolerance (25). Clearly TGF-β is an anti-inflammatory cytokine. However, LPS activates TAK1, which can be activated by TGF-β. SMAD4 is the co-SMAD mediating signal transduction by TGF-β/BMP superfamily. We aimed to determine whether upregulation of SHIP1 and IRAK-M are dependent on SMAD4.

In this study, we demonstrate that upon LPS stimulation, SMAD4 is translocated from the cytosol into the nucleus within 3 h. Also, SHIP1 regulates IRAK-M expression during endotoxin tolerance development in THP1 cells. A, shSHIP1 and shLUC THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 3, 6, and 24 h. After indicated periods, cells were harvested for Western blot analysis with indicated Abs. The blots were reprobed with anti–β-actin to show equal loading. B, shSHIP1 and shLUC THP1 cells were grown in serum free media overnight and stimulated with TGF-β1 (10 ng/ml) for 0, 3, 6, and 24 h. Cell lysates were subjected to Western blot analysis with indicated Abs. Blots were reprobed with anti–β-actin to show equal loading. shSHIP1, shSHIP1 RNA; shLUC, control shRNA.

**FIGURE 7.** Knockdown of SHIP1 by shSHIP1 RNA abrogates the development of endotoxin tolerance in THP1 cells. A, The isolated proteins from shSHIP1 and shLUC THP1 were subjected to Western blot analysis with the indicated Abs. The blots were reprobed with anti–β-actin to show equal loading. B, shSHIP1 and shLUC THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 3, 6, and 24 h. After the indicated periods, cells were harvested for Western blot analysis for p-AKT. C, shSHIP1 and shLUC THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 1, 3, 6, and 24 h. After indicated periods, culture supernatants were harvested and assessed for TNF-α. Each treatment was executed in triplicates. Each experiment was repeated at least three times. Results shown as mean ± SD. The blots were reprobed with anti–β-actin to show equal loading. shLUC, control shRNA; shSHIP1, shSHIP1 RNA.

**FIGURE 8.** SHIP1 regulates IRAK-M expression during endotoxin tolerance development in THP1 cells. A, shSHIP1 and shLUC THP1 cells were treated with 100 or 10–100 ng/ml LPS for 0, 3, 6, and 24 h. After indicated periods, cells were harvested for Western blot analysis with indicated Abs. The blots were reprobed with anti–β-actin to show equal loading. B, shSHIP1 and shLUC THP1 cells were grown in serum free media overnight and stimulated with TGF-β1 (10 ng/ml) for 0, 3, 6, and 24 h. Cell lysates were subjected to Western blot analysis with indicated Abs. Blots were reprobed with anti–β-actin to show equal loading. shSHIP1, shSHIP1 RNA; shLUC, control shRNA. Each experiment was repeated at least three times.
there is an upregulation of SMAD4 expression upon stimulation with 100 or 10–100 ng/ml LPS within a period of 24 h. The abrogation of SMAD4 expression resulted in higher level of TNF-α release following 100 or 10–100 ng/ml LPS stimulation compared with control cells, which indicates a partial failure of induction of endotoxic tolerance and highlights the critical role of SMAD4 signaling in this phenomenon. LPS-induced increase in SHIP1 is mediated by autocrine-activity of TGF-β (16). Our studies show that both SHIP1 and IRAK-M expression are reduced in the quiescent shSMAD4 cells and showed decreased induction following 100 or 10–100 ng/ml LPS stimulation in shSMAD4 THP1 compared with shLUC THP1 cells. SHIP1 is a negative mediator of AKT activities. The second exposure to LPS leads to the reduced phosphorylation of AKT and IκBα in control shLUC cells, but not in shSMAD4 THP1 cells (Fig. 3B), because of reduced SHIP1 in shSMAD4 cells. Total IκBα degradation is much faster in shSMAD4 THP1 cells than in shLUC THP-1 cells. In vitro TGF-β upregulation of SHIP1 and IRAK-M takes place in shLUC but not in shSMAD4 THP1 cells following TGF-β treatment (Fig. 5). Induction of IRAK-M by TGF-β is a novel finding as is the fact that it is partially through a SMAD4-dependent pathway. Thus, SMAD4 negatively regulates LPS signaling through upregulation of both SHIP1 and IRAK-M expression.

Taken together, AKT is activated in THP1 cells (nontolerized) upon the first exposure to LPS and AKT is inactivated in LPS restimulated cells (tolerized). AKT activation is retained in both non-tolerized and tolerated shSMAD4 cells, along with higher TNF-α production. It has been reported that AKT promotes NF-κB activation and inhibition of PI3K decreases LPS-induced transcriptional activity of NF-κB (26–28). This is in contrast to other reported data (29, 30). In their studies, AKT dampens NF-κB activation and subsequent production of proinflammatory cytokines. It is unclear how AKT can mediate these distinctly opposing effects on NF-κB activation. Perhaps different cell types and LPS origins or doses may contribute to these differences. Clearly, AKT activation, p38 phosphorylation, and NF-κB activation are diminished in tolerized cells. As a result, tolerized cells produce less proinflammatory cytokines and NO in response to a second dose of LPS. In the meantime, anti-inflammatory proteins, including SHIP1, IRAK-M, and SOCS1, are produced by tolerized cells. It is known that bone marrow-derived macrophages and mast cells show increased TGF-β expression in response to LPS, which increases SHIP1, IRAK-M, and SOCS1 expression (15–17). SHIP1 negatively regulates PI3K and therefore inactivates AKT.

Consistent with data by Sly et al. (16), we observe that THP1 cells with knockdown of SHIP1 expression do not develop endotoxin tolerance following a second exposure to LPS. shSHIP1 cells have reduced IRAK-M expression. IRAK-M expression is much less induced following 100 or 10–100 ng/ml LPS in shSHIP1 cells than in shLUC THP1 cells. In vitro TGF-β upregulation of IRAK-M takes place in shLUC but not in shSHIP THP1 cells following TGF-β treatment (Fig. 8B). These findings indicate that SMAD4 regulates SHIP1 expression, which in turn controls IRAK-M expression.

IRAK-M expression is upregulated in both nontolerized and tolerized cells (15, 31). In the nontolerized cells, IRAK-M expression, at least in part, depends on the activation of Tpl2/ERK and PI3K/AKT1 signaling pathways (32). In tolerized cells, both ERK1/2 and AKT activation are dramatically reduced, but IRAK-M expression is retained and elevated. Other pathways involving SHIP1, SOCS1, or other anti-inflammatory proteins might play important roles. Knockdown of SMAD4 by shRNA reduces both SHIP1 and IRAK-M expression. Interestingly, knockdown of SHIP1 by shRNA results in significant reduction of IRAK-M expression induced by TGF-β and LPS. How SHIP1 regulates IRAK-M expression needs to be explored. The levels of IRAK-M expression in PI3K inhibitor pretreated cells rose more rapidly and reached higher levels after 6 h compared with levels in cells that were not pretreated (31). SHIP1 induction takes place in both nontolerized and tolerized cells. Overexpressed SHIP1 inactivates AKT and increases IRAK-M protein level. In contrast, activated AKT contributes to IRAK-M reduction. Hence, reduced SHIP1 by shRNA results in diminished IRAK-M expression, increasing proinflammatory cytokine secretion.

SMAD4 is probably induced both by TLR4 signaling as well as by autocrine production of TGF-β. We show that stimulation of THP-1 cells with LPS (Fig. 4B) or with TGF-β (Fig. 5) results in induction of SMAD4. We also show that primary murine cells, stimulated with either LPS or TGF-β, show induction of SMAD4 (Fig. 6A). To study the role SMAD4 plays in endotoxin tolerance in primary cells, LPS-induced TNF-α release from primary cells was measured by ELISA. TNF-α secretion was significantly higher in the SMAD4 siRNA MEF cells than in the control siRNA MEF cells in both non-tolerized (100 ng/ml) and tolerized (10–100 ng/ml) cells (Fig. 6C). This result further demonstrates that SMAD4 is a negative regulator of proinflammatory cytokines, not only in THP-1 cells but also in primary mouse cells.

On the basis of the above studies, we demonstrate that TGF-β, through SMAD4, negatively regulates LPS signaling during endotoxin tolerance development. In contrast, there are reports of elevated levels of circulating TGF-β in patients with sepsis syndrome (33). Hepatic overexpression of TGF-β1 promotes LPS-induced inflammatory cytokine secretion by liver cells and endotoxin-induced shock (34). Furthermore, there have been reports of increased mortality, blunted production of NO, and increased production of TNF-α in endotoxemic TGF-β1 transgenic mice (35). These results indicate that TGF-β1 promotes LPS-signaling and releases various cytokines that can lead to septic shock in patients. TAK1, which mediates TGF-β/BMP signaling, is phosphorylated concomitantly with its activation in LPS-stimulated macrophages and its activity is necessary for the activation of NF-κB (3, 36). The effects of TGF-β on macrophages can be either stimulatory or inhibitory, depending on the other cytokines present and the state of differentiation or tissue origin of the cells (14). Therefore, the clinical administration of exogenous TGF-β1 to induce endogenous production of TGF-β1 may require careful in vivo studies to evaluate the utility of this cytokine in the clinical management of septic shock.

In conclusion, we provide the first evidence that SMAD4 regulates SHIP1 and IRAK-M expression during LPS-induced endotoxin tolerance development. In addition to inhibiting LPS-induced PI3K activation, SHIP1 regulates IRAK-M expression, which controls TNF-α release. These results suggest that manipulation of SMAD4 expression or properly targeting TGF-β pathway might provide novel therapeutic strategies to modulate response to repeated in vivo exposure to LPS in patients with sepsis.

Disclosures
The authors have no financial conflicts of interest.

References


