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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 monocyctic 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 treatment does not increase IRAK-M or SHIP1 protein expression in shSMAD4 THP-1 cells, whereas it does so in shLUC THP1 cells, indicating that 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.

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Abbreviations used in this paper: BMP, bone morphogenetic protein; co-SMAD, common SMAD; IKK, inhibitor of NF-κB kinase; IRAK, IL-1R–associated kinase; MEF, mouse embryonic fibroblast; sh, small hairpin; shLUC, control shRNA; shSHIP1, shSHIP1 RNA; shSMAD4, shSMAD4 RNA; siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; TAK1, TGF-β–activated kinase 1; TIRAP, Toll-IL-1R domain-containing adaptor protein.

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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 a 5% CO2 incubator, nonadherent cells were collected and cultured in fresh medium for another 3–5 d. More than 95% of cells were CD11b+ using fluency. MEF cells were maintained 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 plated on 10-cm sterile plastic tissue culture dishes. The next day, the medium was changed, and the cells maintained in culture 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 MEFs, 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 plated 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 confluence. MEF cells were maintained in DMEM supplemented with 10% heat inactivated FBS, 0.1 mmol/l nonessential amino acids, 0.1 mmol/l 2-mercaptoethanol, and antibiotics at 37°C in an atmosphere of 95% air and 5% CO2.

Rabbit anti-human IRAK-M and IRAK-1 Abs were purchased from ProSci (Poway, CA). Mouse anti-human SHIP1 or SMAD4 Abs and BLOCK-iT RNAi Designer from Invitrogen. Oligo sequences used were as follows: shSMAD4-RA, 5′-GGAATTGGTATGTCCATAG-3′; shSHIP1-RA, 5′-GCGATATCAATGTGCAACA-3′; and shSHIP1-RA, 5′-GGCATACTGATGACAAGC-3′.

Double-stranded oligos were subcloned into pLKOpurol (20). pLKOpurol-shLUC was used as a control. pLKOpurol-shLUC and pLKOpurol plasmids were a gift from Drs. J. Yang and R.A. Weinberg (Whitehead Institute for Biomedical Research, Cambridge, MA). pLKOpurol-shLUC and pLKOpurol1 targeted shRNA were cotransfected with pCMV-VSVG and pHSA.2VAR (Weinberg) into 293T cells. After 48 h, medium was collected and centrifuged for 10 min at 2000 rpm/min. Supernatants were transferred into targeted cell lines, and these infected cell lines were selected with 1 µg/ml puromycin for 2 wk.

Endotoxin tolerance induction and TNF-α ELISA

A total of 1 × 10⁶ THP1 cells from a 75-cm² flask were split into two 175-cm² flasks with 50 ml fresh medium and cultured for 2 additional days. On the afternoon of the second day, LPS at a final concentration of 10 ng/ml was added into one of two flasks. On the morning of the third day, LPS at a final concentration of 100 ng/ml was added into each of two flasks. In this study, we specify some terminologies: 100 ng/ml LPS stimulation stands for one-time stimulation without low dose of LPS priming; 10–100 ng/ml stands for 100 ng/ml treatment following 10 ng/ml LPS priming overnight. Cells were harvested at a specified period, and supernatants were stored for ELISA analysis. TNF-α concentrations in cultured medium were determined by using human TNF-α/TNFSF1A kit (R&D Systems). The OD of each well was read by using a microplate reader at 450 nm with 540-nm correction (Molecular Devices, Sunnyvale, CA). Each sample was executed in triplicates. Each experiment was repeated at least three times.

Immunofluorescence staining

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 microscopy (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 “10–100 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.” Assessment of TNF-α secretion by ELISA showed that TNF-α did not increase over time in low-dose LPS–primed cells compared with unprimed cells, indicating induction of tolerance by pretreatment with low-dose LPS (Fig. 1A).

Cytosolic SMAD4 protein level decreased at 3 and 6 h and increased at 24 h after 100 or 10–100 ng/ml LPS stimulation. At similar time points, nuclear SMAD4 protein level, like p65, increased with the above treatments (Fig. 1B). To further confirm SMAD4 activation, we evaluated the levels of phospho-SMAD2, which forms heteromeric activation complex with SMAD4 during TGF-β signaling. We found that phospho-SMAD2 increased following LPS treatment (Fig. 1C). Confocal microscopy confirmed nuclear localization of SMAD4 after LPS stimulation (Fig. 2). Endotoxin tolerance in THP1 cells can be induced by pretreatment with low-dose LPS and SMAD4 is activated following LPS stimulation. When cells pretreated with low-dose LPS (10 ng/ml tolerance model) were restimulated with higher dose of LPS, there was...
SMAD4 plays an important role in endotoxin tolerance development. 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. Cytoplasmic 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. Knockdown of SMAD4 by shSMAD4 RNA results in failure to induce endotoxin tolerance in THP1 cells compared with control shLUC (Fig. 3A). SHIP-1 and IRAK-M protein expressions were reduced in shSMAD4 THP1 following second exposure to LPS, whereas they were strongly induced in shLUC THP1 cells following second exposure to LPS. These results demonstrate that both SHIP1 and IRAK-M cells following repeated exposure to LPS.

p-AKT and p-IκBα levels are retained following second exposure to LPS in shSMAD4 THP1 cells
Both P13K 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-β (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 subject to Western analysis with indicated Abs. The blots were reprobed with anti–β-actin to show equal loading. shLUC, control shRNA; shSMAD4, shSMAD4 RNA.

**FIGURE 5.** TGF-β–mediated upregulation of SHIP1 and IRAK-M is dependent on SMAD4. shSMAD4 and shLUC THP1 cells were grown in serum-free media overnight and stimulated with TGF-β1 (1 ng/ml) for 0, 3, 6, and 24 h. Cell lysates were subjected to Western blot analysis with indicated Abs. The 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 6A.** SMAD4 regulates SHIP1 and IRAK-M during development of endotoxin tolerance. 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.
To study the role SMAD4 plays in endotoxin tolerance in primary cells, subconfluent MEF cells were transfected with siS-MAD4 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-biphosphate. To verify whether this occurs in THP1 cells, we knocked down the endogenous SHIP1 by using shSHIP1 RNA. Western blot analysis showed that shSHIP1 RNA successfully knocked down SHIP1 protein expression in THP1 cells (Fig. 7A). p-AKT level is upregulated in shSHIP1 cells following LPS stimulation (Fig. 7B). Consistent with others (16), TNF-α secretion significantly increases in shSHIP1 cells after restimulation with LPS than in shLuc cells at similar time points (Fig. 7C). These results confirm that SMAD4 regulated SHIP1 plays an essential role in endotoxin tolerance development.

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 Ab. Western blot analysis shows that IRAK-M expression is reduced in shSHIP1 cells (Fig. 8A). 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-dependent 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

![FIGURE 6. The induction of SMAD4, IRAK-M and SHIP1 by LPS or TGF-β in primary cells. A. Mouse bone marrow-derived macrophages were treated with 10 ng/ml LPS for 0, 6, and 24 h. For TGF-β stimulation, the mouse bone marrow-derived macrophages were starved overnight in the serum-free medium and then stimulated with 10 ng/ml TGF-β for 0, 6, and 24 h. After the indicated periods, the above cells were harvested for Western blot analysis with the indicated Abs. The blots were reprobed with anti-β-actin to show equal loading. Results are representative of the three experiments. B, SMAD4 gene of the mouse bone marrow-derived macrophages was knocked down with SMAD4 siRNA, and control siRNA was taken as control. Furthermore, the above cells were respectively treated with 100 or 10–100 ng/ml LPS for 0, 6, and 24 h. After the indicated periods, the cells were harvested for Western blot analysis with the indicated Abs. The blots were reprobed with anti-β-actin to show equal loading. Results are representative of the three experiments. C, SMAD4 gene of the MEF cells was knocked down with SMAD4 siRNA, and control siRNA was taken as control. Then, the above cells were treated with 100 or 10–100 ng/ml LPS for 0, 6, and 24 h. The culture supernatants were assessed by ELISA for TNF-α concentration. Each treatment was executed in triplicates. Each experiment was repeated at least three times. Results shown are the mean ± SD.](http://www.jimmunol.org/)
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,
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 endotoxin tolerance and highlights the critical role of SMAD4 signaling in this phenomenon. LPS-induced increase in SHP1 is mediated by autocrine-activity of TGF-β (16). Our studies show that both SHP1 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. SHP1 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 SHP1 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 SHP1 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 SHP1 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 SHP1, 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 SHP1, IRAK-M, and SOCS1 expression (15–17). SHP1 negatively regulates PI3K and therefore inactivates AKT.

Consistent with data by Sly et al. (16), we observe that THP1 cells with knockdown of SHP1 expression do not develop endotoxin tolerance following a second exposure to LPS. shSHP1 cells have reduced IRAK-M expression. IRAK-M expression is much less induced following 100 or 10–100 ng/ml LPS in shSHP1 cells than in shLUC THP1 cells. In vitro TGF-β upregulation of IRAK-M takes place in shLUC but not in shSHP1 THP1 cells following TGF-β treatment (Fig. 8B). These findings indicate that SMAD4 regulates SHP1 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 SHP1, SOCS1, or other anti-inflammatory proteins might play important roles. Knockdown of SMAD4 by shRNA reduces both SHP1 and IRAK-M expression. Interestingly, knockdown of SHP1 by shRNA results in significant reduction of IRAK-M expression induced by TGF-β and LPS. How SHP1 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). SHP1 induction takes place in both nontolerized and tolerized cells. Overexpressed SHP1 inactivates AKT and increases IRAK-M protein level. In contrast, activated AKT contributes to IRAK-M reduction. Hence, reduced SHP1 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 shows 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 shRNA 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 concomitant 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 SHP1 and IRAK-M expression during LPS-induced endotoxin tolerance development. In addition to inhibiting LPS-induced PI3K activation, SHP1 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.

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