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SMAD4 is required for development of maximal endotoxin tolerance

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Abstract

Initial exposure of monocytes/macrophages to lipopolysaccharide (LPS) induces hypo-responsiveness 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 IRAK-M and SHIP-1 proteins, negative regulators of TLR4 signaling, increase in tolerized cells. TGFβ1, an anti-inflammatory cytokine, is up-regulated 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 shSMAD4 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 murine 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 while 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.

Keywords

SMAD4; SHIP1; IRAK-M; LPS; TNF-α; THP1; endotoxin tolerance

Introduction

The endotoxin, Lipopolysaccharide (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

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eliminate bacterial infection (1,2). Following infection with Gram-negative bacteria, LPS, which forms a complex with LPS-binding protein (LBP), 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 IRAK1, IRAK4 and TRAF6. Phosphorylation and degradation of IRAK1 induces ubiquitination of TRAF6 and releases the adaptor protein complex into the cytoplasm, resulting in activation of downstream kinases such as TAK1, IKK and JNK (3,4). This leads to activation of NF-κB, resulting in downstream induction of TNFα, IL-6 and nitric oxide. TAK1 (TGF-β activated kinase 1), which was originally identified as a member of MAP kinase kinase kinase (MAPKKK) family mediating TGF-β/bone morphogenetic protein (BMP) signaling, is involved in LPS-induced activation of NF-κB and c-Jun NH2-terminal protein kinase (JNK) (5,6). In the TGF-β signaling pathway, TAK1 deletion leads to impaired NF-κB and C-Jun N-terminal kinase (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 TGFβ, SMAD1/SMAD5/ SMAD8 for BMP). Activated R-SMADs form heteromeric complexes with SMAD4. These complexes migrate into the nucleus, where they interact with transcription factors, activators and co-repressors to induce targeted gene expression (8). TGFβ1 is now well established as a potent anti-inflammatory cytokine which plays a pivotal role in maintaining balanced host responses in immune and non-immune inflammatory conditions (9,10). TGFβ1 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 nitric oxide following LPS stimulation (11).

Endotoxin tolerance is a phenomenon in which previous exposure to a low level of lipopolysaccharide (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 (12,13, and 14). The underlying molecular mechanisms for endotoxin tolerance are not clearly resolved. IRAK-M (15), SHIP1 (16) and SOCS-1 (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 PI-3K-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). Here we examine the regulatory role of SMAD4 in modifying cell signaling molecules SHIP1 and IRAKM involved in induction of endotoxin tolerance.

Materials and Methods

Cell Culture and Reagents

THP1 and 293T cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). THP1 cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, 10mM HEPES, 1mM sodium pyruvate, 4.5g/L glucose, penicillin (100 units/mL), and streptomycin (100ug/mL) (Invitrogen, Carlsbad, CA, USA). 293T cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). Mouse bone marrow derived macrophages and mouse embryonic fibroblast cells (MEF) were gifts from Dr. Xiaoping Zhong (Department of Pediatrics, Duke Medical

Center). 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 days of culture at 37°C in CO_2 incubator, nonadherent cells were collected and cultured in fresh medium for another 3-5 days. 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 murine embryonic fibroblast (MEF) cells, mouse embryos 12.5-14.5 days p.c. 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 10ml of 0.05% trypsin/0.02% EDTA in PBS and the tube rotated end-over-end at 37°C for 10 minutes. A 5ml-aliquot from the incubated tube was removed and another 5ml of fresh trypsin/EDTA was added to the original tube for 10 minutes. The procedure was repeated at least five times. The cell pellet was resuspended in 50ml of 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 confluency. MEF cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal bovine serum, 0.1 mmol/L non-essential amino acids, 0.1 μmol/L 2-mercaptoethanol, and antibiotics at 37 °C in an atmosphere of 95% air and 5% CO_2.

Rabbit anti-human IRAK-M and IRAK-1 antibodies were purchased from ProSci Inc (Poway, CA, USA). Mouse anti-human SHIP1 or SMAD4 antibodies and SMAD4 siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit $(Fab)_2$ and anti-mouse $(Fab)_2$ antibodies conjugated with horseradish peroxidase were obtained from Jackson ImmunoResearch Labs (West Grove, PA). The sources of other antibodies are as follows: rabbit anti-phospho-AKT and SMAD2 antibodies; mouse anti-AKT and SMAD2 antibodies (Cell Signaling Technology; Danvers, MA), mouse anti-β-actin antibody (Sigma; Milwaukee, WI). Lipopolysaccharide (LPS) was obtained from Sigma (Cat# L6529). TNF-α ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN). Western Lightning Chemiluminescence Reagent was from PerkinElmer (Boston, MA).

Western Blot Analysis and Nuclear Protein Preparation

Cells were lysed in cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1%SDS, pH 7.5) containing proteinase inhibitor cocktail (Roche) on ice for 30 min. The lysates were clarified at 4°C for 30 min. at 14,000 rpm/min. Protein concentrations were determined using Bio-Rad Protein Assay at OD595nm (Bio-Rad Laboratories, Inc, Hercules, CA). Equivalent amounts of protein for each sample were subjected to SDS-polyacrylamide gel electrophoresis and transferred to the PVDF membrane (BIO-RAD Laboratories). After blocking with 5% nonfat dry milk/PBS, the membranes were incubated with a primary antibody for 2 hours, washed with TTBS (100mM Tris, pH 7.5, NaCl 0.9%, Tween 0.1%) three times, reacted with a secondary antibody for 45 min, and washed with TTBS three times. Protein bands were visualized by ECL (PerkinElmerLife Sciences, Waltham, MA). For nuclear protein extraction, we used the procedure described by Xu et al. (19). For densitometric analysis, the ImageJ software from NIH was employed.

Small hairpin RNAs (shRNA)

The complementary oligonucleotides to generate the shRNA against human SMAD4, IRAK-M and SHIP1 were designed using BLOCK-iT™ RNAi Designer from Invitrogen (Carlsbad, CA). Oligo sequences used were as follows: shSMAD4-RNA=GCATAGTTTGATGTGCCATAG; shIRAK-

RNA=GCAGTATATCAAGTGCAAACA; shSHIP1-RNA=GCCACATCTGTACTGACAACG Double stranded (ds) oligos were subcloned into pLKOpuro1 (20). pLKOpuro1-shLUC was used as a control. pLKOpuro1-shLUC and pLKOpuro1 plasmids were a generous gift from Drs. J.Yang and R.A.Weinberg (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts). pLKOpuro1-shLUC and pLKOpuro1 targeted shRNA were co-transfected with pCMV-VSVG and pHR'8.2ΔR (Dr. Weinberg) into 293T cells. After 48 hours, media 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 1ug/ml puromycin for two weeks.

Endotoxin Tolerance Induction and TNF-α ELISA

1×10^7 THP1 cells from a 75-cm^2 flask were split into two 175-cm^2 flasks with 50 ml of fresh media and cultured for two additional days. On the afternoon of the 2nd day, LPS at a final concentration of 10ng/ml was added into one of two flasks. On the morning of the 3rd day, LPS at a final concentration of 100ng/ml was added into each of two flasks. In this study, we specify some terminologies: 100ng/ml LPS stimulation stands for one-time stimulation without low dose of LPS priming; 10-100ng/ml stands for 100ng/ml treatment following 10ng/ml LPS priming overnight. Cells were harvested at a specified period and supernatants were stored for ELISA analysis. TNF-α concentrations in cultured media were determined by using human TNF-α/TNFSF1A kit (R&D Systems, Inc). The optical density of each well was read by using a microplate reader at 450 nm with 540nm 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 Bioscience Discovery Labware, Two Oak Park, Bedford, MA) in growth media supplemented with PMA (1ug/ml) for 24 hours at 37°C. The adherent cells were cultured in serum-free media overnight, followed by adding LPS for 3-6 hours. 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 pre-blocked in 5% Bovine Serum Albumin/PBS (BSA) for 1 hour. The slides were then incubated with mouse anti-human SMAD4 antibody (diluted in 1:100 in blocking solution) for 1 hour, washed three times with PBS, and incubated with FITC-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 hour. After additional washes, the slides were incubated with DAPI for 3 min, and mounted with anti-fade 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 use 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 (10ng/ml) overnight and subsequently stimulated with 100ng/ml LPS for 0, 3, 6 and 24 hours. This activation protocol is referred to as “10-100ng/ml”. Control cells were treated with 100ng/ml of LPS for similar time points but without prior stimulation with low dose LPS. This activation protocol is referred to as “100ng/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 hours and increased at 24 hours after 100ng/ml or 10-100ng/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 TGFß signaling. We found that phospho-SMAD2 increased following LPS treatments (Fig. 1C). Confocal microscopy confirmed nuclear localization of SMAD4 after LPS stimulation (Fig. 2). Endotoxin tolerance in THP1 cells can be induced by pre-treatment with low dose LPS and SMAD4 is activated following LPS stimulation. When cells pre-treated with low dose LPS (10 ng/ml - tolerance model) were re-stimulated with higher dose of LPS, there was no increase in nuclear SMAD4, but rather SMAD4 was already in the nucleus. Similarly, p65 is already in the nucleus when cells are pre-treated with 10ng/ml of LPS. These findings suggest an important role of SMAD4 in tolerance. In the tolerance model (10ng pretreatment) SMAD4 and p65 are already in the nucleus, and activation of SMAD4 and p65 is sustained following 100ng/ml restimulation.

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

Both the PI-3K pathway 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 100ng/ml or 10-100ng/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 while 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 100ng/ml or 10-100ng/ml of LPS for 0, 1, 3, 6 and 24 hours. 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 100ng/ml and 10-100ng/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 common SMAD (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) wheras 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 if TGF-β-induced upregulation of SHIP1 and IRAK-M is dependent on SMAD4, shSMAD4 and shLUC THP1 cells were treated with TGF-β1 (1ng/ml) for 0, 3, 6 and 24 hours. 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 ß in primary cells, mouse bone marrow-derived macrophages were treated with 10ng/ml LPS for 0, 6 and 24 hours. For TGF-β stimulation assay, the mouse bone marrow-derived macrophages were starved in serum free medium overnight and subsequently stimulated with 10ng/ml TGF ß for 0, 6 and 24 hours. The 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 ß 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 siRNA, and control siRNA was used as control. The cells were treated with low dose LPS (10ng/ml) overnight and subsequently stimulated with 100ng/ml LPS for 0, 6 and 24 hours. The cells were also treated with 100ng/ml LPS for 0, 6 and 24 hours as control. The indicated protein levels were analyzed by Western blot (Fig. 6 B). 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 macropgaes. Similar results were obtained in control siRNA MEF cells and SMAD4 siRNA MEF cells (data not shown).

To study the role SMAD4 plays in endotoxin tolerance in primary cells, sub confluent mouse embryonic fibroblast (MEF) cells were transfected with siSMAD4 or control siRNA. The transfected mouse embryonic fibroblast (MEF) cells were respectively treated with 100ng/ml LPS and 10-100ng/ml LPS for 0, 6 and 24 hours. LPS-induced TNF α release from primary cells was measured by ELISA. Western 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 non-tolerized (100ng/ml) and tolerized (10-100ng/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 if 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 re-stimulation 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 if SHIP1 regulates IRAK-M expression in monocytes, we blotted cell lysates from both shSHIP1 and shLUC THP1 cells with IRAK-M antibody. 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 if 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, de-activation or desensitization. Tolerized cells produce less proinflammatory cytokines and nitric oxide in response to a second dose of LPS. However, LPS tolerance is not a global down- regulation 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, 16, 17, 21, and 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 antibodies 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 (TGF-β activated kinase 1), which can be activated by TGF-β. SMAD4 is the common SMAD (co-SMAD) mediating signal transduction by TGF-β/BMP superfamily. We aimed to determine if 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 three hours. Also, there is an upregulation of SMAD4 expression upon stimulation with 100ng/ml or 10-100ng/ml LPS within a period of 24 hours. The abrogation of SMAD4 expression resulted in higher level of TNF-α release following 100ng/ml or 10-100ng/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 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 100ng/ml or 10-100LPS 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) due to 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 (non-tolerized) upon the first exposure to LPS and AKT is inactivated in LPS re-stimulated cells (tolerized). AKT activation is retained in both non-tolerized and tolerized shSMAD4 cells, along with higher TNFα

production. It has been reported that AKT promotes NF-κB activation and inhibition of PI 3-kinase decreases LPS-induced transcriptional activity of NF-κB (26, 27, and 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, the 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, 16, and 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. **s**hSHIP1 cells have reduced IRAK-M expression. IRAK-M expression is much less induced following 100ng/ml or 10-100ng/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 non-tolerized and tolerized cells (15,31). In the non-tolerized 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 to levels in cells that were not pretreated (31). SHIP1 induction takes place in both non-tolerized and tolerized cells. Over expressed SHIP1 inactivates AKT and increases IRAK-M protein level. On the other hand, 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-I cells with LPS (Figure 4B) or with TGF-β (Figure 5) results in induction of SMAD4. We also shows that primary murine cells, stimulated with either LPS or TGF-β, show induction of SMAD4 (Figure 6 A). 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 (100ng/ml) and tolerized (10-100ng/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.

Based on the above studies, we demonstrate that TGF-β, through SMAD4, negatively regulates LPS-signaling during endotoxin tolerance development. On the other hand, there are reports of elevated levels of circulating TGF-β in patients with sepsis syndrome (33). Hepatic over-expression 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 nitric oxide, 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 SHIP1 and IRAK-M expression during LPS-induced endotoxin tolerance development. In addition to inhibiting LPS-induced PI-3K 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.

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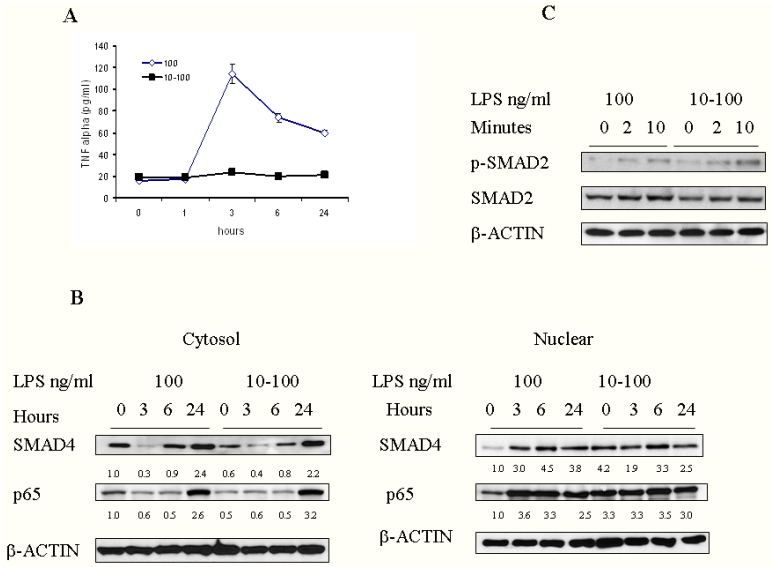

Figure 1. SMAD4 participates in endotoxin tolerance development

A. THP1 cells were treated with 100ng/ml or 10-100ng/ml of LPS for 0, 1, 3, 6 and 24 hours. 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 antibodies. 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 100ng/ml or 10-100ng/ml of 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.

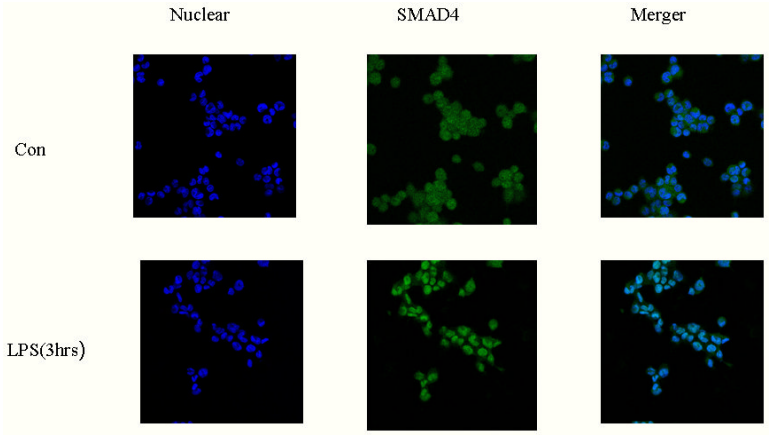

Figure 2.

THP1 cells were treated with 100ng/ml of LPS for 3 hours. Cells were fixed, permeabilized and blocked in 5% Bovine Serum Albumin/PBS (BSA) for 1 hour. The slides were incubated with mouse anti-human SMAD4 antibody and FITC-conjugated anti-mouse Ig and DAPI. Cells were examined under the Zeiss LSM510 inverted confocal microscope (magnification 400x). 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.

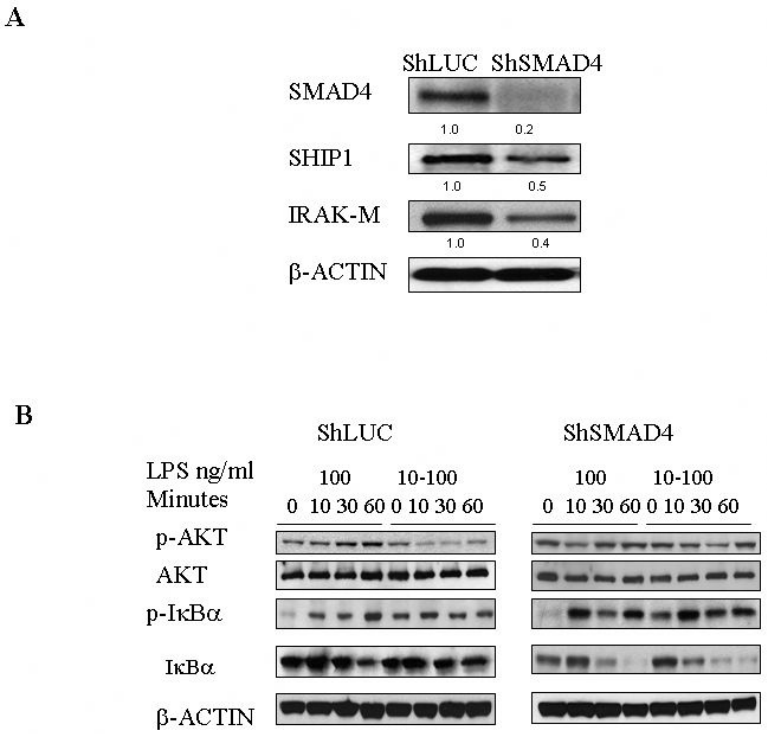

Figure 3. Knockdown of SMAD4 by shSMAD4 RNA results in increased p-AKT and p-IκBα levels in THP1 cells

A. Isolated proteins from shSMAD4 and shLUC THP1 cells were subjected to Western blot analysis with the indicated antibodies. Blots were reprobed with anti-β-Actin to show equal loading. B. shSMAD4 and shLUC THP1 cells were treated with 100ng/ml or 10-100ng/ml of LPS for 0, 10, 30 and 60 minutes. After the indicated periods, cells were harvested for Western blot analysis with indicated antibodies. Blots were reprobed with anti-β-Actin to show equal loading. shSMAD4: shSMAD4 RNA; shLUC: control shRNA. All results are representative of three separate experiments.

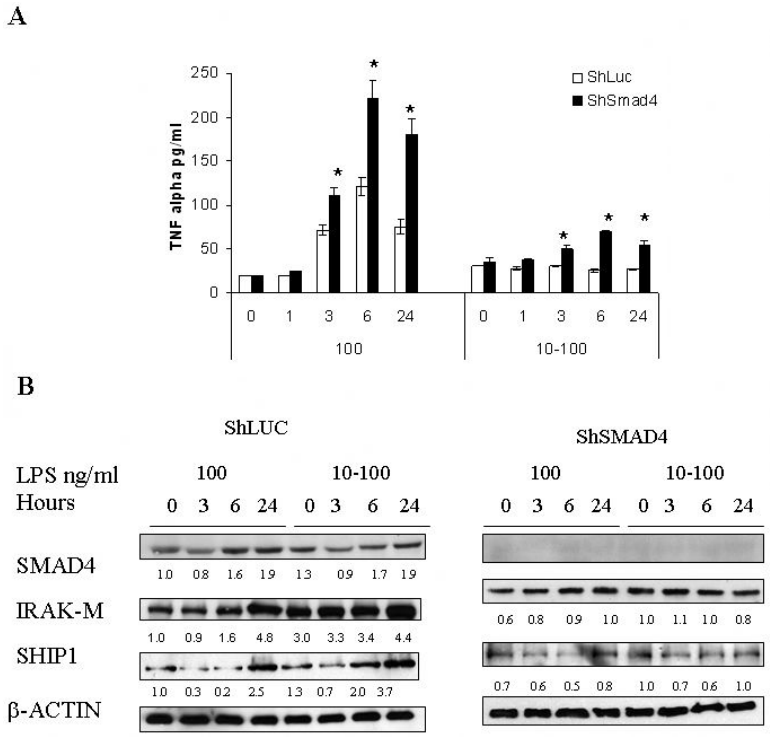

Figure 4. SMAD4 regulates SHIP1 and IRAK-M during development of endotoxin tolerance

shSMAD4 and shLUC THP1 cells were treated with 100ng/ml or 10-100ng/ml of LPS for 0, 1, 3, 6 and 24 hours. 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 antibodies. The blots were reprobed with anti-β-Actin to show equal loading. shSMAD4: shSMAD4 RNA; shLUC: control shRNA.

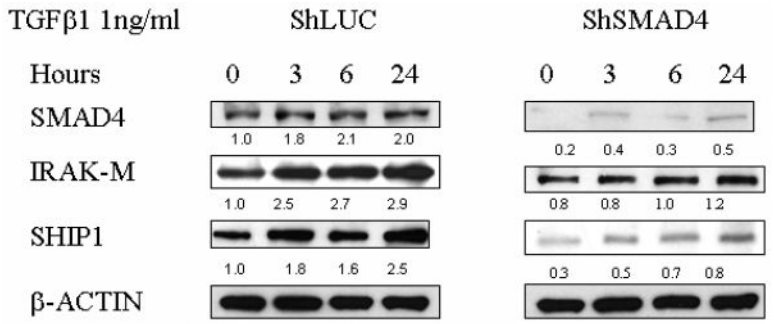

Figure 5.

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 (1ng/ml) for 0, 3 6 and 24 hours. Cell lysates were subjected to Western blot analysis with indicated antibodies. The blots were reprobed with anti-β-Actin to show equal loading. All results are representative of three separate experiments. shSMAD4: shSMAD4 RNA; shLUC: control shRNA.

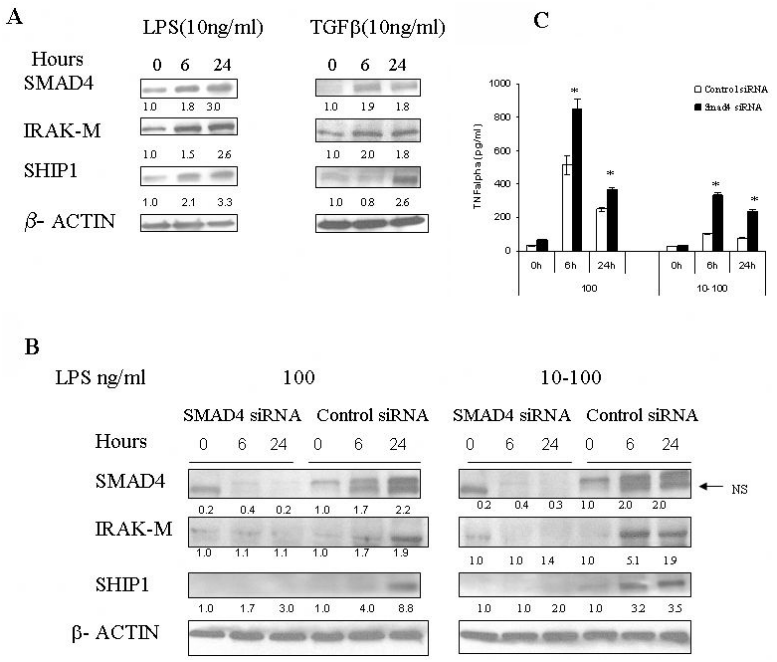

Figure 6. The induction of SMAD4, IRAK-M and SHIP1 by LPS or TGF ß in primary cells

A. Mouse bone marrow- derived macrophages were treated with 10ng/ml of LPS for 0, 6 and 24 hours. For TGFβ stimulation, the mouse bone marrow- derived macrophages were starved overnight in the serum-free media and then stimulated with 10ng/ml TGF ß for 0, 6 and 24 hours. After the indicated periods, the above cells were harvested for Western blot analysis with the indicated antibodies. The blots were reprobed with anti- ß – 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 100ng/ml LPS or 10-100ng/ml LPS for 0, 6 and 24 hours. After the indicated periods, the cells were harvested for Western blot analysis with the indicated antibodies. The blots were reprobed with anti- ß- actin to show equal loading. Results are representative of the three experiments. C. SMAD4 gene of the mouse embryonic fibroblast (MEF) cells was knocked down with SMAD4 siRNA, and control siRNA was taken as control. Then, the above cells were treated with 100ng/ml LPS or 10-100ng/ml LPS for 0, 6 and 24 hours. 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.

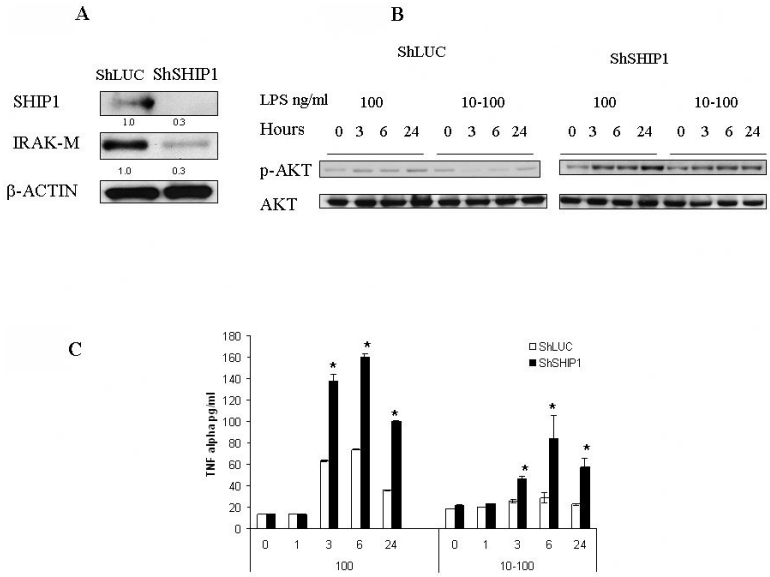

Figure 7. Knockdown of SHIP1 by shSHIP1 RNA abrogates the development of endotoxin tolerance in THP1 cells

A. The isolated proteins from shSHIP1 and shLUC THP1 were subjected to Western analysis with the indicated antibodies. The blots were reprobed with anti-β-Actin to show equal loading. B. shSHIP1 and shLUC THP1 cells were treated with 100ng/ml or 10-100ng/ml of LPS for 0, 3, 6 and 24 hours. After the indicated periods, cells were harvested for Western analysis for p-AKT. C. shSHIP1 and shLUC THP1 cells were treated with 100ng/ml or 10-100ng/ml of LPS for 0, 1, 3, 6 and 24 hours. 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. shSHIP1: shSHIP1 RNA; shLUC: control shRNA.

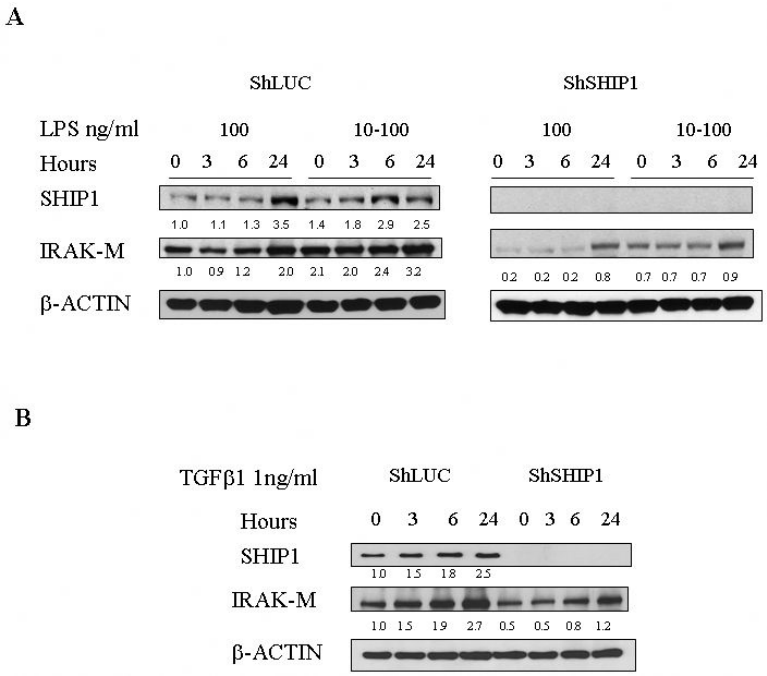

Figure 8. SHIP1 regulates IRAK-M expression during endotoxin tolerance development in THP1 cells

A. shSHIP1 and shLUC THP1 cells were treated with 100ng/ml or 10-100ng/ml of LPS for 0, 3, 6 and 24 hours. After indicated periods, cells were harvested for Western analysis with indicated antibodies. 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 (10ng/ml) for 0, 3 6 and 24 hours. Cell lysates were subjected to Western analysis with indicated antibodies. 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. Each experiment was repeated at least three times.