Published in final edited form as:

J Neuroimmunol. 2018 January 15; 314: 30–41. doi:10.1016/j.jneuroim.2017.11.005.

TGF- β 1/Smad2/3/Foxp3 signaling is required for chronic stress-induced immune suppression

Haiju Zhang^{a,b}, Yi Caudle^b, Clay Wheeler^b, Yu Zhou^b, Charles Stuart^b, Baozhen Yao^a, and Deling Yin^b

^aDepartment of Pediatrics, Renmin Hospital of Wuhan University, Wuhan 430063, China

^bDepartment of Internal Medicine, College of Medicine, East Tennessee State University, Johnson City, TN 37614

Abstract

Depending on the duration and severity, psychological tension and physical stress can enhance or suppress the immune system in both humans and animals. Although it has been established that chronic stress exerts a significant suppressive effect on immune function, the mechanisms by which affects immune responses remain elusive. By employing an *in vivo* murine system, we revealed that TGF- β 1/Smad2/3/Foxp3 axis was remarkably activated following chronic stress. Furthermore, TLR9 and p38 MAPK played a critical role in the activation of TGF- β 1/Smad2/3/Foxp3 signaling cascade. Moreover, inhibition of TGF- β 1/Smad2/3/Foxp3 or p38 significantly attenuated chronic stress-induced lymphocyte apoptosis and apoptosis-related proteins, as well as the differentiation of T regulatory cells in spleen. Interestingly, disequilibrium of pro-inflammatory and anti-inflammatory cytokines balance caused by chronic stress was also rescued by blocking TGF- β 1/Smad2/3/Foxp3 axis. These findings yield insight into a novel mechanism by which chronic stress modulates immune functions and identifies new targets for the development of novel anti-immune suppressant medications.

Keywords

TGF- β 1; Smad2/3; Foxp3; TLR9; Chronic stress; Immune suppression

1. Introduction

It has been shown that stress can either increase or decrease immune function depending on a variety of factors including duration of stressful situation (Dhabhar, 2014; Hu et al., 2014; Li et al., 2014). Acute stress enhances immune response, however, chronic stress induces a

Address correspondence and reprint requests to: Deling Yin, M.D., Ph.D., Professor of Immunology, Department of Internal Medicine, College of Medicine, East Tennessee State University, Johnson City, TN37614, Phone: 423 439 8826 (o), yin@etsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures

The authors have no financial conflicts of interest.

Author Manuscript

suppressive effect on the bodily defense system (Dhabhar, 2014; Hu et al., 2014). Our previous studies have shown that reduction of immune responses occurs in chronic restraint stress (Hu et al., 2014; Li et al., 2011). Chronic stress conditions can disrupt immune cell responses particularly by altering patterns of cytokine secretion (Hu et al., 2013; Hu et al., 2014; Li et al., 2011). This process results in the suppression of Type 1 helper T cells (Th1) with a subsequent increase in Th2 response, also increases the apoptosis of T cell and dendritic cell (Li et al., 2011; Li et al., 2014). And it also enhances the differentiation of suppressor cells, such as T regulatory (Treg) cells, rendering the defense system vulnerable to pathogenic infections and autoimmune diseases (Hou et al., 2016; Jin et al., 2013). Elucidation of the cell signal regulatory mechanism is fundamental to understand how the immune suppression has been triggered in chronic stress.

Author Manuscript

Toll-like receptors (TLRs) are an ancient and evolutionarily conserved receptor family that plays a critical role in the innate immune and inflammatory responses (Babdor et al., 2017; Li et al., 2014). Engagement of ligands to respective TLRs initiates series of intracellular signaling events, and then activates multiple downstream pathways to regulate the apoptosis of lymphocytes or the secretion of pro-inflammatory and anti-inflammatory factors subsequently (Hu et al., 2014; Takeuchi and Akira, 2010). TLR9 is subcellular localized in endosomal, and can be activated by circulation mtDNA (Medzhitov, 2001). After recognition of their ligands, TLR9 activates intracellular signaling pathways such as MAPKs (p38, ERK, and JNK) resulting in the transcription of inflammatory and immunomodulatory genes including chemokines, cytokines and co-stimulatory molecules (Gu et al., 2015). Previous reports demonstrated that the activation TLR9 could induce production of TGF- β 1 and was related to autoimmunity and fibrosis (Fang et al., 2016; Li et al., 2014). The activation of p38 MAPK (Kamaraju and Roberts, 2005) and ERK MAPK (Hayashida et al., 2003) may augment Smad signaling, and activation of p38 MAPK may act downstream of TGF- β 1, mediating TGF- β 1-dependent apoptosis. Confirmation of these phenotypes of TLR9 and TGF- β 1 signal led us to our hypothesis that TLR9/MAPK pathway activated TGF- β 1/Smad2/3 signal to mediate immune responses during chronic stress.

Author Manuscript

TGF- β 1 belongs to a family of regulatory cytokines that has pleiotropic functions involved in numerous physiological and pathological processes. TGF- β 1 synthesis can be regulated at the transcriptional level by multiple stress factors (Lee et al., 2006). Active TGF- β 1 phosphorylates downstream targets and activate different signaling pathway, including Smad2 and Smad3 (Gu et al., 2012). TGF- β 1 plays an important role on T cells in immune-regulatory functions, such as suppression of cell proliferation, cytokine production, as well as inducing apoptosis (Das et al., 2015; Li and Flavell, 2008). Smad2 and Smad3 play essential roles in forkhead box P3 (Foxp3) induction and cytokine suppression (Johnston et al., 2016). Foxp3 has been shown to be associated with the development of regulatory T cells (Tregs), which can inhibit activation of other T cells and hinder the induction of immune responses against cancer and infectious agents (Curiel et al., 2004; Luan et al., 2015). Meanwhile most Foxp3⁺ regulatory T cells develop in the thymus as a functionally mature T cell subpopulation specialized for immune suppression. However, it hasn't been known yet whether TGF- β 1 plays a role in chronic stress-induced immune suppression.

The objective of this study is to evaluate whether TGF- β 1/Smad2/3 signaling pathway plays a role in the chronic stress-induced immune suppression. We identified the regulatory role of TLR9/p38 in the activation of TGF- β 1/Smad2/3. We also demonstrated the activation of TGF- β 1/Smad2/3 contributed to the apoptosis of lymphocytes and the activation of Foxp3 to promote the regulation of Treg cells in spleen to mediate immune-suppression following chronic stress.

2. Materials and Methods

2.1. Animals

Wild Type (WT) male BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). TLR9 knockout (KO) mice on Balb/c background were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan) via Dr. Dennis Klinman (National Cancer Institute, Frederick, MD). Pairs of TLR9 KO were bred and littermates were utilized as controls. TLR2 KO mice were obtained from the Jackson Laboratory. All mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal protocols were approved by ETSU Committee on Animal Care.

2.2. Chronic restraint stress model

Seven to eight-week-old male mice were subjected to an established chronic restraint protocol by us and others (Hu et al., 2014; Li et al., 2011). Briefly, mice were placed in a 50-ml conical centrifuge tube with multiple punctures to allow ventilation. Mice were held horizontally in the tubes for 12-h from 9:00 pm to 9:00 am without food or water, while food and water were provided when they were liberated. This schedule was repeated the following night. Mice were thus subjected to restraint stress on two consecutive days.

Control littermates were kept in their original cage with food and water provided on the same schedule as for the stressed mice. Mice were physically restraint regimen for one or two cycles as specified. After physical restraint, mice were anesthetized via inhalation with 2.5% isoflurane (Abbott Laboratory, Abbott Park, IL). Blood and serum samples were collected for cytokines and corticosterone measurements. Spleens were harvested.

2.3. Animals treatment protocol

P38 inhibitor SB203580 (1mg/kg body weight, Calbiochem, La Jolla, CA), TGF- β inhibitor SB431542 (4.2mg/kg body weight, Sigma-Aldrich), Smad3 inhibitor ISI3 (2.5mg/kg body weight, Cayman Chemical, Michigan), and Foxp3 inhibitor peptide P60 (50 nmol/mouse (i.p., Abbtotec, San Diego, CA) were administered via i.p. 1 h (for SB203580) or 2 h (for SB431542, ISI3 and peptide P60) before the initiation of stress (Caraci et al., 2015; Casares et al., 2010; Hu et al., 2014; Li et al., 2010; Wang et al., 2013). The control (non-inhibitor) group also received PBS injection to avoid the different stressful response. Parts of the two-cycle stressed mice were challenged with 10 μ g of Gram-negative bacterial LPS (i.p., Sigma-Aldrich, St. Louis, MO). After 6 h injection, the mice were sacrificed. The specific details of experimental group were shown in supplemental material (Tables of experimental group).

2.4. Isolation of RNA, and real-time quantitative PCR

RNA in spleen was isolated with Qiagen RNeasy kit (Qiagen). Quantitative real-time RT-PCR was carried out to quantify the expression levels of cytokines, TNF- α , IL-10, IL-17, and TGF- β 1 as described previously (Hu et al., 2013; Li et al., 2011). Briefly, one microgram of RNA from each sample was used for reverse transcription and synthesis of cDNA using a Reaction Ready™ first strand cDNA synthesis kit (SABiosciences, Frederick, MD). PCR was performed using RT² real-time™ SYBR Green Fluorescein PCR Master Mix (SABiosciences). GAPDH expression was used as internal control. The primer sequences used were as follows: TNF- α forward 5'-CAT GAG CAC AGA AAG CAT GAT CCG-3', TNF- α reverse 5'-AAG CAG GAA TGA GAA GAG GCT GAG-3', IL-17 forward 5'-GCC CTC AGA CTA CCT CAA-3', IL-17 reverse 5'-GAA TTC ATG TGG TGG TCC AG-3', TGF- β 1 forward 5'-CAA CAA TTC CTG GCG TTA CCT TGG-3', TGF- β 1 reverse 5'-GAA AGC CCT GTA TTC CGT CTC CTT-3', IL-10 forward 5'-TGC TAA CCG ACT CCT TAA TGC AGG AC-3', IL-10 reverse 5'-CCT TGA TTT CTG GGC CAT GCT TCT C-3'. GAPDH forward 5'-TGA CCA CAG TCC ATG CCA TC-3', GAPDH reverse 5'-GAT GGG GGT TAC ACA GGC AG-3'.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear protein was isolated from spleens and thymuses with NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manual. Smad2 and Smad3 binding activity was performed using a light shift chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) as previously described (Gao et al., 2015) in a 20 fmol double-stranded Smad2 and Smad3 consensus oligonucleotide, which was 5'-end-labeled with biotin and 15ug nuclear proteins. The binding reaction mixture was incubated at room temperature for 20 min and analyzed by electrophoresis and transferred to a nylon membrane. The biotin end-labeled DNA was detected using the streptavidin-HRP conjugate and chemiluminescent substrate.

2.6. Immunohistochemistry and apoptosis detection

Immunohistochemistry (ICH) was performed with a Vector® M.O.M.™ Immunodetection Kit (Vector Laboratories, Burlingame, CA). Paraffin spleen sections (5µm thick) were cut, dewaxed and hydrated. The sections were incubated in 1% Blocking Reagent solution for 1 h at room temperature and then labeled with anti-Smad3 antibody, phospho-specific (Ser425) (Sigma-Aldrich) diluted 1:50 and incubated for 30 min. After washing, sections were incubated with M.O.M.™ biotinylated anti-mouse IgG reagent for 10 min and subsequently with VECTASTAIN® ABC Reagent. Finally, the signal was with DAB substrate and counterstained with hematoxylin.

Nucleosomal DNA fragmentation in spleens was determined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay with an In Situ Cell Death Detection kit (Roche Diagnostic, Indianapolis, IN) according to the manufacturer's instructions as described previously by us (Yin et al., 2000). Sections were examined with the light microscope by a 40× objective. The percentage of apoptotic cells was calculated (TUNEL-positive cells/total cells) and averaged across at least five randomly chosen microscopic fields for each slide.

2.7. Isolation of CD4⁺ T cells and flow cytometry

Half of the spleen was excised for isolation and flow cytometric analysis after 2 cycles of 12-h restraint stress. Single splenocyte suspension was prepared and CD4⁺ T cells were negatively selected and purified with a mouse CD4⁺ T cell column system (R&D Systems) (Chen et al., 2003).

CD25⁺Foxp3⁺ cells from the isolated and purified CD4⁺ T cells were identified as Tregs. CD25⁺Foxp3⁺ cells were analyzed by flow cytometry. They were labeled by incubation for 30 min on ice in staining buffer (PBS plus 2% FBS) with the appropriated fluorochrome-conjugated antibodies. After washing, the samples were analyzed by flow cytometry on FACSscan (BD Biosciences). The following antibodies were used: Anti-mouse CD25 conjugated to fluorescein isothiocyanate (FITC) (Abcam, Cambridge, MA) (1:50), anti-mouse-foxp3 conjugated to phycoerythrin (PE) (Abcam, Cambridge, MA) (1:50). An appropriate isotype-matched control was used for each antibody.

2.8. Western blot analysis

Western blotting was performed as described previously (Zhang et al., 2008). In some experiments, nuclear extracts from mouse spleens were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo). The membrane was incubated overnight at 4°C with the primary antibody. The signals were detected with the ECL system (Amersham Biosciences) and quantified by scanning densitometry using a Bio-Image Analysis System (Bio-Rad). Anti-phospho-Smad2 (Ser465/467)/Smad3(Ser423/425) (1:500), anti-Smad2/3(1:1000), anti-phospho-p38 MAPK (1:500), anti-p38 MAPK (1:1000), anti-phospho-JNK (1:500), anti-JNK (1:1000), anti-phospho-ERK (1:500), anti-ERK (1:1000), anti-cleaved-caspase-3(1:500), anti-caspase-3(1:1000), anti-Bcl-2 (1:1000), anti-Bax (1:1000), anti-Foxp3 (1:500), anti-phospho-ASK1 (1:500), ASK1 (1:1000), and anti-GAPDH (1:1000) were obtained from Cell Signaling Technology (Beverly, MA). Anti-TLR2 (1:200), anti-TLR4 (1:100) and anti-TLR9 (1:200) were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.9. Enzyme linked immunosorbent assay (ELISA) for cytokines and corticosterone

The levels of proinflammatory cytokines TNF- α , IL-17 and anti-inflammatory factors IL-10, TGF- β 1 in serum were analyzed with mouse Quantikine ELISA immunoassay kits (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. The serum corticosterone levels were determined with a corticosterone ELISA kit (IBL America, Minneapolis, MN) according to the manufacturer's instruction.

2.10. Statistical analysis

Data were analyzed using software Graphpad Prism 6. Data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA as appropriate. One-way ANOVA with Newman-Keuls multiple comparison test for intergroup comparison was performed. Significant two-way ANOVA results were further analyzed using Bonferroni's multiple comparison tests. Student's t-test was used for two-group comparison. All values are expressed as mean and standard deviations (SD). A value of $p < 0.05$ is considered statistically significant.

3. Results

3.1. Chronic restraint stress induces Smad2/3 phosphorylation

To examine whether chronic restraint stress can activate Smad2/3/signaling, the levels of phosphorylated Smad2/3 in the spleens were determined with or without restraint stress at different time points. The levels of phospho-Smad2/3 in the stressed mice significantly increased and reached the peak at 12 h compared with unstressed mice (Fig. 1A). Similar results were observed for phospho-Smad3 in the spleens (Fig. 1B). Therefore, it can be concluded that chronic stress promotes Smad2/3 phosphorylation.

3.2. TLR9 is required for chronic stress-induced Smad2/3 phosphorylation

It has been shown that a deficiency of TLR9 blocks chronic stress-induced lymphocyte reduction (Li et al., 2014). In this study, the expression of TLR9 significantly increased in spleens following chronic stress (Fig. 2A). In contrast, TLR2 expression in spleens was considerably decreased. To evaluate whether TLR2 or TLR9 contributes to chronic restraint stress-induced Smad2/3 phosphorylation, we subjected TLR2 KO, TLR9 KO mice to chronic stress. Notably, a deficiency of TLR9 eliminated the level of Smad2/3 phosphorylation induced by chronic stress (Fig. 2B). Chronic stress induced the phosphorylation of Smad2/3 in TLR2 deficient mice, especially comparing with the TLR9 deficient mice, but less than in WT mice (Fig. 2B). Taken together, our data suggest that TLR9, but not TLR2, is required for chronic stress-induced Smad2/3 phosphorylation.

3.3. A deficiency of TLR9 attenuates chronic stress-induced Smad2/3 activation via p38 MAPK

To investigate the mechanisms responsible for TLR9-regulated p38 MAPK response to chronic stress, we first examined the effect of chronic stress on activation of MAPKs. Here, chronic stress dramatically enhanced the activation of p38 MAPK in spleens, but significantly diminished the activation of both phospho-JNK and phospho-ERK. We then determined whether TLR9 regulated p38 MAPK following chronic stress. Chronic stress-induced phosphorylation of p38 MAPK was abrogated by TLR9 deficiency (Fig. 3B) but not TLR2 deficiency (data not shown), suggesting that chronic stress induced p38 activation through TLR9. Next, we evaluated whether p38 played an essential role in chronic stress-induced Smad2/3 activation. Interestingly, inhibition of p38 reduced chronic stress-induced Smad2/3 phosphorylation (Fig. 3C). Taken together, p38, a target of TLR9, is crucial for chronic stress-induced Smad2/3 phosphorylation.

3.4. Critical role of TLR9/p38 signaling on TGF- β 1 mediates Smad2/3 phosphorylation following chronic stress

Our previous studies demonstrated that chronic stress increased endogenous corticosterone release depending on TLR9 (Li et al., 2014). In this study, chronic stress significantly enhanced corticosterone serum levels in WT mice, and the deficiency of TLR9 attenuated stress-enhanced corticosterone levels (Fig. 4A). Larger amounts of TGF- β 1 were observed in the serum from stressed mice than those from control mice (Fig. 4B). The mRNA levels of TGF- β 1 in spleens also significantly increased in stressed mice (Fig. 4C). Therefore,

chronic stress dramatically enhances TGF- β 1 production. The effect of TLR9 on TGF- β 1 production induced by chronic stress was also tested. As shown in Fig. 4B, 4C, chronic stress-induced production of TGF- β 1 dramatically diminished by TLR9 deficiency both at the transcriptional and protein levels, suggesting that chronic stress induced TGF- β 1 production, at least partially, through TLR9.

The modulations of p38 on TGF- β 1 production were examined. Interestingly, administration of the p38 selective inhibitor SB203580 dramatically attenuated the TGF- β 1 production both in protein and mRNA levels in WT mice (Fig. 4B, C). To identify whether the phosphor-Smad2/3 initiated by TGF- β activation following chronic stress, the mice were administrated with TGF- β inhibitor SB431542 before the initiation of stress. As shown in Fig. 4D, pretreatment with SB431542 attenuated chronic stress-induced nuclear Smad2/3 phosphorylation in spleens. Inhibition of Smad3 by ISI3 significantly blocked chronic stress-induced phosphorylation of Smad2/3 (Fig. 4D). Taken together, these results suggest that activation of TLR9/p38 signaling induces the production of TGF- β 1, which in turn increases Smad2/3 phosphorylation following chronic stress.

We wondered whether the activation of TGF- β 1/Smad2/3 contributed to the release of corticosterone after stress, the levels of corticosterone in serum were determined after pretreatment with p38 inhibitor SB203580, TGF- β 1 inhibitor SB431542 and Smad2/3 inhibitor ISI3 before the initiation of stress. As shown in Fig. 4E, blocking the activation of TGF- β /Smad2/3 couldn't diminish chronic stress-induced corticosterone production. These results suggest that the effectiveness of TGF- β /Smad2/3 signal in chronic stress plays in a specific way, independent from the mediation of corticosterone.

3.5. Smad2/3 function is regulated via TLR9/p-38/TGF- β signaling during chronic stress

To confirm that Smad2/3 was activated by chronic stress, Smad2/3 DNA-binding activity was determined by EMSA. The levels of phosphorylated Smad2/3 enhanced in a time dependent manner following chronic stress. The expression of phospho-Smad2/3 started to increase at 3 h of stress (Fig. 5A, upper panel). Interestingly, Smad3-DNA-binding activity in spleen significantly enhanced at 6 h of stress as shown by EMSA (Fig. 5A, bottom panel), but no significant alteration of Smad2-DNA-binding activity was observed (data not shown), revealed that functional change of Smad2/3 happened during chronic stress. Moreover, Smad3-DNA-binding activity began to decrease at 12 h, although the accumulation of phospho-Smad2/3 in the nucleus was significantly induced at this time point by chronic stress (Fig. 5A). Collectively, our data suggest that Smad2/3 is functionally activated transiently but not persistently during chronic stress.

Since TLR9 deficiency, SB203580, SB431542 and ISI3 all exhibited inhibitory effects on the phosphorylation of Smad2/3 in spleens and thymuses, we then investigated if they had similar effects on the DNA binding activity of Smad2/3. WT and TLR9 KO mice were subjected to chronic stress for 6 h. Moreover, WT mice were pre-treated with or without p38 inhibitor SB203580, TGF- β inhibitor SB431542 or Smad3 inhibitor ISI3 before the initiation of stress. Inhibition of p38 and TGF- β , dramatically suppressed stress-induced enhancement of Smad2/3 phosphorylation in spleens (Fig. 5B, upper panel), and Smad3 DNA-binding activity was also significantly repressed (Fig. 5B, bottom panel). TLR9

deficiency also dramatically attenuated the phosphorylation and binding activity of Smad2/3. In addition, pretreatment of Smad3 inhibitor ISI3, was also exhibited an inhibitory effect on the phosphorylation of Smad3, and inhibited the binding activity of Smad3 (Fig. 5B, bottom panel). Taken together, Smad2/3 is functionally regulated through TLR9/P38/TGF- β 1 signaling during chronic stress.

3.6. Transcription factor Foxp3 is activated by TGF- β 1/Smad2/3 pathway and stimulates Treg cells following chronic stress

Whether chronic stress contributed to Foxp3 expression was determined. As shown in Fig. 6A, chronic stress induced the expression of Foxp3 in a time dependent manner. Whether TGF- β 1/Smad2/3 signaling played a role in stress-induced Foxp3 expression was defined. The results showed that stress-induced Foxp3 expression, which could be abolished by TGF- β 1 and Foxp3 inhibitors, but partially blocked by p38 and Smad3 inhibitors (Fig. 6B). Thus, these findings suggest that the activation of Foxp3 induced by chronic stress is mediated by TGF- β 1/Smad2/3 signal.

Foxp3 is required for the production and maintenance of Tregs (Johnston et al., 2016; Luan et al., 2015). CD25+FOXP3+ cells isolated and purified CD4+ T cells were evaluated by flow cytometry after 2 cycles of 12 h-restraint stress. The percentage of CD25+FOXP3+ cells from the total isolated CD4+ T cells was significantly increased after stress (Fig. 6C). Interestingly, inhibition of p38 with SB203580, TGF- β 1 with SB431542, Smad2/3 with ISI3, as well as blockage of Foxp3 with P60 dramatically blocked this enhancement of Tregs in spleens (Fig. 6C). Collectively, these results suggest that Smad2/3 is important for TGF- β -mediated Foxp3 induction and Tregs differentiation following chronic stress.

3.7. Effect of p38/TGF- β 1/Smad2/3/ASK1 on splenocyte apoptosis induced by chronic stress

We investigated whether phosphorylation of Smad2/3 had effect on apoptosis following chronic stress. As shown in Fig. 7A, chronic stress significantly enhanced splenocyte apoptosis. Inhibition of p38, TGF- β 1, Smad2/3, or Foxp3 by their inhibitors dramatically attenuated the number of splenocyte apoptosis induced by chronic stress (Fig. 7A). We then evaluated the role of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. The expression of Bcl-2 in the stressed mice decreased. While Bax and cleaved caspase-3 increased, comparing with the control group (Fig. 7B). Importantly, these changes were significantly attenuated by inhibition of p38, TGF- β , Smad2/3, or Foxp3. The similar results were obtained when we examined apoptosis signal-regulating kinase 1 (ASK1) following chronic stress (Fig. 7C). Collectively, these results suggest that chronic stress induces splenocyte apoptosis, at least partially through p38, TGF- β , Smad2/3 and ASK1.

3.8. TGF- β 1/Smad2/3 mediates immune suppression induced by chronic stress

To evaluate the effect of TGF- β 1/Smad2/3 signaling in chronic stress-induced immune suppression, the gene expressions of pro-inflammatory (TNF- α and IL-17) and anti-inflammatory cytokines (IL-10 and TGF- β 1) in spleens were examined by quantitative RT-PCR (Fig. 8A) and the protein levels in serum were detected by ELISA (Fig. 8B). The mRNA level and protein level of TNF- α decreased, as well as the alterations of IL-17.

Whereas the mRNA and protein levels of IL-10 and TGF- β 1 increased significantly (Fig. 8A, B). As expected, administration of SB431542, ISI3 and peptide P60 reversed the disequilibrium of pro-inflammatory and anti-inflammatory cytokines both in transcriptional level (Fig. 8A) and serum concentration (Fig. 8B). These data imply that chronic stress induces immunosuppression through mechanisms dependent on TGF- β 1/Smad2/3 signaling.

4. Discussion

In the current study, we found a new mechanism by which chronic stress induced immune suppression. The production of TGF- β 1, a critical anti-inflammatory cytokine and a crucial regulator of T cell regulation during the immune responses, augmented both at transcriptional and protein levels in chronic stressed mice. The activity of Smad2/3, the key effector molecule of TGF- β 1 signal, also markedly enhanced during chronic stress. Moreover, TLR9/p38 played a fundamental role in the activation of TGF- β 1/Smad2/3 pathway. Of great importance was that inhibition of TGF- β 1/Smad2/3 blocked splenocyte apoptosis and regulation of Treg cells, and restored the disequilibrium of pro-inflammatory (TNF- α and IL-17) and anti-inflammatory (IL-10 and TGF- β 1) cytokines, the notable consequences of chronic stress. Our discovery supports that TLR9/p38/TGF- β 1/Smad2/3 signaling pathway mediates immune suppression induced by chronic stress.

Previous studies have revealed that chronic stress suppresses immune functions (Dhabhar, 2014; Hu et al., 2014; Li et al., 2011). However, the mechanisms that chronic stress modulates the regulatory effects remained to be elucidated. In the current study, we observed that the nuclear phosphor-Smad2/3 expression displayed an increase in stressed mice, suggesting that Smad2/3 was activated in response to chronic stress. Additionally, the results of western blot showed that the activation of Smad3 was more dominant than Smad2, and a remarkably accumulation of phosphor-Smad3-positive cells was also examined by ICH, further verifying the activation of Smad2/3 by chronic stress. Smad2 and Smad3 are highly similar proteins, and Smad3 can bind DNA directly, while Smad2 is dependent on co-activators to associate with DNA. This difference between Smad2 and Smad3 is caused by an additional exon that is inserted in front of the DNA binding domain in Smad2 (Dennler et al., 1999).

Our previous studies have shown that a deficiency of TLR9 attenuates the reduction of splenocytes and alters cytokine secretion in response to chronic stress (Li et al., 2014). In the current study, we found that chronic stress significantly increased the expression of TLR9, suggesting that TLR9 signaling pathway was positively modulated during chronic stress. Our previous studies have demonstrated that TLR2 plays an important role in stress-induced immune responses (Hu et al., 2013; Li et al., 2014), and the reduced expression of TLR2 was further verified in stressed mice in current study. The phosphorylation of Smad2/3 in TLR2 deficient mice was less than in WT mice after chronic stress, which implied that the protection of TLR2 targeted other signal pathway but not TGF- β 1/Smad2/3 signal. Activation of TLR2 diminishes chronic stress-induced up-regulation of corticosterone production (Hu et al., 2013), which is consistent with result that the effectiveness of TGF- β 1/Smad2/3 signal in chronic stress plays in a specific way, independent from the mediation of corticosterone. MAPKs are important mediators for TLR-mediated signaling pathways

(Anur et al., 2012; Gu et al., 2015). We found that the level of phosphorylated p38 MAPK dramatically enhanced, whereas JNK and ERK both attenuated in stressed mice, suggesting that p38 played an essential role that was distinct from that of JNK and ERK during chronic stress. Previous studies have confirmed that the inhibition of JNK1 activation prevents the TGF- β 1-induced Smad3 activation and nuclear translocation (Liu et al., 2012), and the upregulation of the TGF- β 1 receptor on hepatic stellate cells is in a JNK-dependent manner (Thomas et al., 2015). ERK-mediated pathways are mostly involved in proliferation and differentiation and generally considered anti-apoptotic. ERK activation is associated with phosphorylation of the Smad outside the C-terminal-SSMS motif and leads to enhanced nuclear translocation of Smad2 (Javelaud et al., 2005). Inhibiting ERK1/2 activation attenuates TGF- β 1 tumor promoting effect in intrahepatic cholangiocarcinoma (ICC) cells (Phaijit et al., 2017). These experiments suggest that the phosphorylation of JNK and ERK contributes to the activation of smad2/3 in different tissues and cells. The contradictory findings of diminished phosphorylation of JNK and ERK accompanying with activation of smad2/3 in this study suggest that other signal promotes the production of smad2/3 induced by chronic stress. The p38 activity was notably abrogated by TLR9 deficiency. These data verified that TLR9 was the upstream activator of p38 during chronic stress. TLR9 deficiency and p38 inhibitor blocked the up-regulation of TGF- β 1, which demonstrated that TLR9/p38 signaling contributed to the production of TGF- β 1 in response to chronic stress.

Many types of cells can secrete TGF- β 1, including lymphocytes, neutrophils and macrophages (Massague, 2012). It has been reported that the production of TGF- β 1 by TLR-activated T regulatory cells was significantly decreased in Foxp3 $^{-/-}$ mice compared with the control mice (Mikulic et al., 2016). Our previous and current studies also demonstrated that splenocyte apoptosis occurred after chronic stress (Hu et al., 2014; Li et al., 2014). Therefore, apoptosis and regulatory T cells might be responsible for the production of TGF- β 1 following chronic stress. Smad2/3 protein, as the downstream effect molecules of TGF- β 1 signal, threonine or serine phosphorylation is required for Smad2/3, triggering their translocation into the nucleus in complex with the proteins Smad4 or transcription intermediary factor 1 gamma (TIF1 γ). In our current study, a deficiency of TLR9 and inhibition of p38 attenuated chronic stress-increased Smad2/3 phosphorylation and DNA-binding activity. These findings support that the link between TLR9/p38 and Smad2/3 in chronic stress. Corticosterone and TGF- β 1 were up-regulated following chronic stress. Moreover, inhibition of TGF- β 1 signal remarkably alleviated stress-induced augmentation of Smad2/3 phosphorylation, as well as DNA-binding activity, but the release of endogenous corticosterone couldn't be blocked, which implied that TGF- β 1/Smad2/3 played the role in a specific way independent from the mediation of corticosterone. Collectively, TLR9/p38/TGF- β 1 mediates Smad2/3 activation following chronic stress (Fig. 9). Which specific cell type is located for the activated Smad2/3 will be studied in our further research.

TGF- β 1 plays an essential role in establishing immunological tolerance (Regateiro et al., 2011). However, little is known about the molecular mechanisms involved in TGF- β 1-mediated immune suppression. In the current study, elevated cleaved caspase-3 activity and apoptotic cells were observed following chronic stress. Furthermore, the level of the pro-apoptotic protein Bax increased while the level of anti-apoptotic protein Bcl-2 decreased.

Pre-treatment with TGF- β 1 inhibitor or Smad3 inhibitor reversed chronic stress-induced apoptotic cells, and restored the expression of cleavage of caspase-3, Bax, and Bcl-2. Phosphorylated Smad2/3 clearly reduced after inhibition of p38, TGF- β 1, or Smad3. Consistent with these results, chronic stress-induced apoptosis attenuated after inhibition of p38, TGF- β 1, or Smad3, supporting that chronic stress induced apoptosis through TGF- β 1/Smad2/3 signal. Moreover, inhibition of Foxp3 also alleviated chronic stress-induced apoptosis. Recent studies showed that Treg promotes T lymphocyte apoptosis and its regulatory mechanism through TGF- β 1 (Luan et al., 2015). We also found that phospho-ASK1 increased obviously after chronic stress. Inhibition of TGF- β 1/Smad2/3 signal abrogated chronic stress-induced activation of ASK1, as well as the alterations of Bax, Bcl-2, and cleaved caspase-3. These data suggest the activation of TGF- β 1/Smad2/3 mediates stress-induced splenocyte apoptosis by modulating the phosphorylation of ASK1. However, the molecular mechanisms how TGF- β 1/Smad2/3 signal modulates the activation of ASK1 need the further investigations in our future study.

TGF- β induces Foxp3-positive regulatory T cells and inhibits the proliferation of T cells as well as cytokine production via Foxp3-dependent and independent mechanisms (Wan and Flavell, 2007). In the present study, Foxp3 was activated by chronic stress, and inhibition of TGF- β 1 signal and Smad3 restored the expression of Foxp3. Simultaneously, the ratio of regulatory T cells in spleen remarkably increased following 2 cycles of 12-h chronic stress. Consistent with the alteration of Foxp3, inhibition of TGF- β 1 and Foxp3 by their inhibitors blocked the regulation of Tregs after chronic stress. TGF- β 1-Foxp3 axis drives the development of Treg cells. These results reveal that chronic stress induced the activation of foxp3 and promoted the modification of Tregs mediated by TGF β 1/Smad2/3 signal pathway. Tregs exert strong suppressive activity on Th1, but much lower on Th2 cells (Chyi, 2012). Over-expression of Foxp3 in CD4+T cells suppresses the production of proinflammatory cytokines, and promotes the production of anti-inflammatory cytokines, including IL-10, IL-4, and IL-6 (Killebrew et al., 2011). In our study, the results also demonstrated the disequilibrium of pro-inflammatory and anti-inflammatory cytokines in stressed mice, which is consistent with the promoted differentiation of Treg after induced-stress. Our results also showed the transcriptional levels and serum concentrations of pro-inflammatory (TNF- α and IL-17) cytokines decreased dramatically in stressed mice, but inhibiting the activation of TGF- β 1/Smad2/3 and Foxp3 restored the cytokine levels. On the other hand, anti-inflammatory cytokines remarkably increased in stressed mice, but alterable tendencies were inverted after inhibiting TGF- β 1/Smad2/3/Foxp3 signal. The activation of TGF- β 1/Smad2/3/foxp3 induced the differentiation of Treg may be partially responsible for the immune suppression after chronic stress, which also further demonstrated Treg cells exert higher suppressive activity on Th1 than on Th2 cells. Apoptosis can lead to the production of TGF- β 1 intrathymically (Konkel et al., 2014). In the current study, TGF- β 1/Smad2/3 signal promoted splenocyte apoptosis during chronic stress. Importantly, we show that enhanced levels of apoptosis in the spleens after chronic stress are consistent with an augmented Treg population. Moreover, that decreasing splenocyte apoptosis accompanied reduced Tregs after inhibiting TGF- β 1/Smad2/3/foxp3 signal. Taken together, our results indicate that the activation of TGF- β 1/Smad2/3 is a key event in mediating the apoptosis and Treg generation.

Collective findings, we reveal that chronic stress-induced activation of TGF- β 1/Smad2/3/foxp3 signal promotes the spenocyte apoptosis and the differentiation of Treg. Apoptosis and increased Tregs mediate the immune suppression by contributing to the production of anti-inflammatory cytokines and suppressing the pro-inflammatory cytokines following chronic stress. Inhibiting the activation of TGF- β 1/Smad2/3/Foxp3 restored the disequilibrium of pro-inflammatory and anti-inflammatory cytokines.

5. Conclusions

In summary, our studies demonstrate that TGF- β /Smad2/3/Foxp3 axis is activated by TLR9/p38 pathway following chronic stress. TGF- β /Smad2/3/Foxp3 triggers lymphocyte apoptosis, promotes the regulation of Tregs and production of anti-inflammatory (IL-10 and TGF- β 1) cytokines, and suppresses the pro-inflammatory (TNF- α and IL-17) cytokines. These findings provide evidence that TGF- β 1/Smad2/3/Foxp3 mediates immune suppression induced by chronic stress and identifies new targets for the development of novel anti-immune suppressant medications.

Acknowledgments

This work was supported in part by NIH grants NIGM114716 and NIGM094740 to D. Yin. This research was also supported in part by NIH grant C06RR0306551. This work was also supported in part by NSFC81401051. We thank Dr. Shizuo Akira, Osaka University, Osaka, Japan and Dr. Dennis Klinman, National Cancer Institute, Frederick of MD, for providing breeding pairs of TLR9 KO mice. We also thank Dr. Theo Hagg (Department of Biomedical Sciences, ETSU College of Medicine) for the use of laboratory equipment. We also thank Dr. Tammy Ozment (Department of Surgery, ETSU College of Medicine) for the flow cytometric assistance and advice. We also thank all lab members for technical assistance and advice.

Abbreviations

TLR9	Toll-like receptor 9
WT	wild-type
KO	knockout
IHC	immunohistochemistry
Treg	T regulatory
Foxp3	forkhead box P3

References

- Babdor J, Descamps D, Adiko AC, Tohme M, Maschalidi S, Evnouchidou I, Vasconcellos LR, De Luca M, Mauvais FX, Garfa-Traore M, Brinkmann MM, Chignard M, Manoury B, Saveanu L. IRAP+ endosomes restrict TLR9 activation and signaling. *Nat Immunol.* 2017; 18:509–518. [PubMed: 28319098]
- Caraci F, Gulisano W, Guida CA, Impellizzeri AA, Drago F, Puzzo D, Palmeri A. A key role for TGF- β 1 in hippocampal synaptic plasticity and memory. *Sci Rep.* 2015; 5:11252. [PubMed: 26059637]
- Casares N, Rudilla F, Arribillaga L, Llopiz D, Riezu-Boj JI, Lozano T, Lopez-Sagaseta J, Guembe L, Sarobe P, Prieto J, Borrás-Cuesta F, Lasarte JJ. A peptide inhibitor of FOXP3 impairs regulatory T

cell activity and improves vaccine efficacy in mice. *J Immunol.* 2010; 185:5150–5159. [PubMed: 20870946]

Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25– naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med.* 2003; 198:1875–1886. [PubMed: 14676299]

Chyi SH, Hyang ML, Chan Wang JL. Selection of regulatory T cells in the thymus. *Nat Rev Immunol.* 2012; 12:157–167. [PubMed: 22322317]

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med.* 2004; 10:942–949. [PubMed: 15322536]

Das R, Xu S, Nguyen TT, Quan X, Choi SK, Kim SJ, Lee EY, Cha SK, Park KS. Transforming Growth Factor beta1-induced Apoptosis in Podocytes via the Extracellular Signal-regulated Kinase-Mammalian Target of Rapamycin Complex 1-NADPH Oxidase 4 Axis. *J Biol Chem.* 2015; 290:30830–30842. [PubMed: 26565025]

Dennler S, Huet S, Gauthier JM. A short amino-acid sequence in MH1 domain is responsible for functional differences between Smad2 and Smad3. *Oncogene.* 1999; 18:1643–1648. [PubMed: 10102636]

Dhabhar FS. Effects of stress on immune function: the good, the bad, and the beautiful. *Immunol Res.* 2014; 58:193–210. [PubMed: 24798553]

Fang F, Marangoni RG, Zhou X, Yang Y, Ye B, Shangguang A, Qin W, Wang W, Bhattacharyya S, Wei J, Tourtellotte WG, Varga J. Toll-like Receptor 9 Signaling Is Augmented in Systemic Sclerosis and Elicits Transforming Growth Factor beta-Dependent Fibroblast Activation. *Arthritis Rheumatol.* 2016; 68:1989–2002. [PubMed: 26946325]

Gao M, Wang X, Zhang X, Ha T, Ma H, Liu L, Kalbfleisch JH, Gao X, Kao RL, Williams DL, Li C. Attenuation of Cardiac Dysfunction in Polymicrobial Sepsis by MicroRNA-146a Is Mediated via Targeting of IRAK1 and TRAF6 Expression. *J Immunol.* 2015; 195:672–682. [PubMed: 26048146]

Gu AD, Wang Y, Lin L, Zhang SS, Wan YY. Requirements of transcription factor Smad-dependent and -independent TGF-beta signaling to control discrete T-cell functions. *Proc Natl Acad Sci U S A.* 2012; 109:905–910. [PubMed: 22219364]

Gu X, Wu G, Yao Y, Zeng J, Shi D, Lv T, Luo L, Song Y. Intratracheal administration of mitochondrial DNA directly provokes lung inflammation through the TLR9-p38 MAPK pathway. *Free Radic Biol Med.* 2015; 83:149–158. [PubMed: 25772007]

Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. *Faseb J.* 2003; 17:1576–1578. [PubMed: 12824291]

Hou DD, Zhu RZ, Sun Z, Ma XD, Wang DC, Timothy H, Chen WN, Yan F, Lei P, Han XW, Chen DX, Cai LP, Guan HQ. Serum proteomics analysis in rats of immuno-suppression induced by chronic stress. *Scand J Immunol.* 2016; 84:165–173. [PubMed: 27384769]

Hu D, Denney J, Liang M, Javer A, Yang X, Zhu R, Yin D. Stimulatory Toll-like receptor 2 suppresses restraint stress-induced immune suppression. *Cell Immunol.* 2013; 283:18–24. [PubMed: 23850672]

Hu D, Wan L, Chen M, Caudle Y, LeSage G, Li Q, Yin D. Essential role of IL-10/STAT3 in chronic stress-induced immune suppression. *Brain Behav Immun.* 2014; 36:118–127. [PubMed: 24513872]

Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene.* 2005; 24:5742–50. [PubMed: 16123807]

Jin J, Wang X, Wang Q, Guo X, Cao J, Zhang X, Zhu T, Zhang D, Wang W, Wang J, Shen B, Gao X, Shi Y, Zhang J. Chronic psychological stress induces the accumulation of myeloid-derived suppressor cells in mice. *Plos One.* 2013; 8:e74497. [PubMed: 24058577]

Johnston CJ, Smyth DJ, Dresser DW, Maizels RM. TGF-beta in tolerance, development and regulation of immunity. *Cell Immunol.* 2016; 299:14–22. [PubMed: 26617281]

- Kamaraju AK, Roberts AB. Role of Rho/ROCK and p38 MAP kinase pathways in transforming growth factor-beta-mediated Smad-dependent growth inhibition of human breast carcinoma cells in vivo. *J Biol Chem.* 2005; 280:1024–1036. [PubMed: 15520018]
- Killebrew JR, Perdue N, Kwan A, Thornton AM, Shevach EM, Campbell DJ. A self-reactive TCR drives the development of Foxp3+ regulatory T cells that prevent autoimmune disease. *J Immunol.* 2011; 187:861–869. [PubMed: 21690323]
- Konkela J, Jina W, Abbatielloa B, Graingerb JR, Chena WJ. Thymocyte apoptosis drives the intrathymic generation of regulatory T cells. *Proc Natl Acad Sci USA.* 2014; 111:E465–E473. [PubMed: 24474796]
- Lee KY, Ito K, Hayashi R, Jazrawi EP, Barnes PJ, Adcock IM. NF-kappaB and activator protein 1 response elements and the role of histone modifications in IL-1beta-induced TGF-beta1 gene transcription. *J Immunol.* 2006; 176:603–615. [PubMed: 16365456]
- Li C, Li H, Jiang K, Li J, Gai X. TLR4 signaling pathway in mouse Lewis lung cancer cells promotes the expression of TGF-beta1 and IL-10 and tumor cells migration. *Biomed Mater Eng.* 2014; 24:869–875. [PubMed: 24211974]
- Li H, Chen L, Zhang Y, Lesage G, Zhang Y, Wu Y, Hanley G, Sun S, Yin D. Chronic stress promotes lymphocyte reduction through TLR2 mediated PI3K signaling in a beta-arrestin 2 dependent manner. *J Neuroimmunol.* 2011; 233:73–79. [PubMed: 21183229]
- Li H, Hu D, Fan H, Zhang Y, LeSage GD, Caudle Y, Stuart C, Liu Z, Yin D. beta-Arrestin 2 negatively regulates Toll-like receptor 4 (TLR4)-triggered inflammatory signaling via targeting p38 MAPK and interleukin 10. *J Biol Chem.* 2014; 289:23075–23085. [PubMed: 25012660]
- Li H, Zhao J, Chen M, Tan Y, Yang X, Caudle Y, Yin D. Toll-like receptor 9 is required for chronic stress-induced immune suppression. *Neuroimmunomodulat.* 2014; 21:1–7.
- Li J, Qu X, Yao J, Caruana G, Ricardo SD, Yamamoto Y, Yamamoto H, Bertram JF. Blockade of endothelial-mesenchymal transition by a Smad3 inhibitor delays the early development of streptozotocin-induced diabetic nephropathy. *Diabetes.* 2010; 59:2612–2624. [PubMed: 20682692]
- Li MO, Flavell RA. TGF-beta: a master of all T cell trades. *Cell.* 2008; 134:392–404. [PubMed: 18692464]
- Liu X, Hubchak SC, Browne JA, Schnaper HW. Epidermal growth factor inhibits transforming growth factor-beta-induced fibrogenic differentiation marker expression through ERK activation. *Cell Signal.* 2014; 10:2276–2283.
- Luan YY, Yin CF, Qin QH, Dong N, Zhu XM, Sheng ZY, Zhang QH, Yao YM. Effect of Regulatory T Cells on Promoting Apoptosis of T Lymphocyte and Its Regulatory Mechanism in Sepsis. *J Interferon Cytokine Res.* 2015; 35:969–980. [PubMed: 26309018]
- Massague J. TGFbeta signalling in context. *Nat Rev Mol Cell Biol.* 2012; 13:616–630. [PubMed: 22992590]
- Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol.* 2001; 1:135–145. [PubMed: 11905821]
- Mikulic J, Longet S, Favre L, Benyacoub J, Corthesy B. Secretory IgA in complex with Lactobacillus rhamnosus potentiates mucosal dendritic cell-mediated Treg cell differentiation via TLR regulatory proteins, RALDH2 and secretion of IL-10 and TGF-beta. *Cell Mol Immunol.* 2016
- Phaijit S, Natthaporn S, Parichut T, Kittiya I, Tuangporn S. Blocking ERK1/2 signaling impairs TGF-beta1 tumor promoting function but enhances its tumor suppressing role in intrahepatic cholangiocarcinoma cells. *Cancer Cell Int.* 2017; 17:85. [PubMed: 28959141]
- Regateiro FS, Howie D, Cobbold SP, Waldmann H. TGF-beta in transplantation tolerance. *Curr Opin Immunol.* 2011; 23:660–669. [PubMed: 21839624]
- Shi Y, Devadas S, Greeneltch KM, Yin D, Allan MR, Zhou JN. Stressed to death: implication of lymphocyte apoptosis for psychoneuroimmunology. *Brain Behav Immun.* 2003; 17(Suppl 1):S18–S26. [PubMed: 12615182]
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell.* 2010; 140:805–820. [PubMed: 20303872]
- Thomas F, Hassen K, Scott LF, Naglaa HS. IL-17A enhances the expression of pro-fibrotic genes through upregulation of the TGF-beta receptor on hepatic stellate cells in a JNK-dependent manner. *J Immunol.* 2014; 193:3925–3933. [PubMed: 25210118]

- Wan YY, Flavell RA. Regulatory T cells, transforming growth factor-beta, and immune suppression. *Proc Am Thorac Soc.* 2007; 4:271–276. [PubMed: 17607012]
- Wang D, Warner GM, Yin P, Knudsen BE, Cheng J, Butters KA, Lien KR, Gray CE, Garovic VD, Lerman LO, Textor SC, Nath KA, Simari RD, Grande JP. Inhibition of p38 MAPK attenuates renal atrophy and fibrosis in a murine renal artery stenosis model. *Am J Physiol Renal Physiol.* 2013; 304:F938–F947. [PubMed: 23364805]
- Wang KX, Shi YF, Ron Y, Kazanecki CC, Denhardt DT. Plasma osteopontin modulates chronic restraint stress-induced thymus atrophy by regulating stress hormones: inhibition by an anti-osteopontin monoclonal antibody. *J Immunol.* 2009; 182:2485–2491. [PubMed: 19201904]
- Yin D, Tuthill D, Mufson RA, Shi Y. Chronic restraint stress promotes lymphocyte apoptosis by modulating CD95 expression. *J Exp Med.* 2000; 191:1423–1428. [PubMed: 10770807]
- Zhang Y, Zhang Y, Miao J, Hanley G, Stuart C, Sun X, Chen T, Yin D. Chronic restraint stress promotes immune suppression through toll-like receptor 4-mediated phosphoinositide 3-kinase signaling. *J Neuroimmunol.* 2008; 204:13–19. [PubMed: 18814920]

Highlights

- Define the effect of TGF- β 1/Smad2/3/Foxp3 axis on chronic stress-induced immune responses.
- TLR9/p38 MAPK mediates the activation of TGF- β 1/Smad2/3/Foxp3 axis in chronic stress.
- TGF- β 1/Smad2/3/Foxp3 axis contributes to lymphocyte apoptosis induced by chronic stress in mice.
- TGF- β 1/Smad2/3/Foxp3 axis promotes the differentiation of Treg cells to mediate immunosuppression induced by chronic stress in mice.

Fig. 1.

Smad2/3 phosphorylation is induced by chronic stress. We subjected WT BALB/c mice aged 6–8 weeks ($n=5$ per group) to physical stress. (A) After stress of the indicated time periods, spleens were harvested. Expression levels of total and phosphorylated Smad2/3 in the nucleus were examined by Western blot analysis. Each sample protein was from the spleen of one mouse. The result of the statistic panel represented five experiments per group ($N=5$ per group). (B) At 12 h of stress, WT mouse spleens were harvested and fixed in 10% buffered formalin. Sections ($5\ \mu\text{m}$ thick) were stained with hematoxylin and eosin ($100\times$) (upper panels). Five sections from each spleen of one mouse was examined. The quantitative analysis was performed by the mean value in every group ($N=5$ per group). IHC (lower panels) was performed on adjacent sections with anti-phospho-Smad3 (Ser 465) Ab and hematoxylin was used as a counterstain (full panels, $100\times$; insets, $400\times$). Arrows indicate cells with positive-phospho-Smad3 staining in the nucleus. $*p < 0.05$, $***p < 0.001$ compared with indicated groups.

Fig. 2.

TLR9 activation mediates chronic stress-induced Smad2/3 phosphorylation. (A) After 12 h of stress, WT mouse spleens were harvested and cellular lysates were extracted. The expressions of TLR9, and TLR2 were examined by Western blot analysis. (B) Age matched WT, TLR9 KO and TLR2 KO mice were experienced chronic stress for 12h. Expression of phosphor-Smad2/3 in the nucleus was examined by Western blot analysis. N = 5 per group ** $p < 0.01$, *** $p < 0.001$ compared with indicated groups.

Fig. 3. p38 MAPK activity is required for Smad2/3 phosphorylation induced by chronic stress. (A) WT mice were subjected to chronic restraint stress for 12 h, spleens were harvested and cellular lysates were isolated. Immunoblotting was performed to detect the expression levels of phosphor-p38, phosphor-JNK and phosphor-ERK with specific Abs. (B) Age-matched WT and TLR9 KO mice were subjected to chronic restraint stress for 12 h, the levels of phosphor-p38 in spleens were examined by Western blot. (C) WT mice were pretreatment with or without p38 MAPK inhibitor SB203580 (1mg/kg body weight, i.p.) 1 h before the mice were performed to chronic restraint stress for 12 h. Mouse spleens were harvested and nuclear proteins were extracted. The levels of total and phosphor-Smad2/3 in the nuclear were determined by Western blot analysis. N = 5 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with indicated groups.

Fig. 4.

TGF- β 1 mediates chronic stress-induced Smad2/3 phosphorylation, independent from the activation of corticosterone. (A) Age-matched WT and TLR9 KO mice were subjected to restraint stress for 12 h. Corticosterone levels in serum were determined by ELISA. (B) TGF- β 1 production in serum was examined in serum by ELISA utilized the protocol in (A). TGF- β 1 mRNA in spleens (C) was detected by quantitative RT-PCR. (D) WT mice were pretreated with or without TGF- β 1 inhibitor SB431542 (4.2mg/kg body weight, i.p.) and Smad3 inhibitor ISI3 (2.5mg/kg body weight, i.p.) 2 h respectively before the initiation of restraint stress. The levels of total and phosphor-Smad2/3 in spleens were determined by Western blot analysis after 12 h stress. (E) Corticosterone levels in serum were examined by ELISA as the same protocol in (D). N = 5 per group. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with indicated groups.

Fig. 5.

Smad2/3 is activated by chronic restraint stress via the TLR9/p38/TGF- β 1 pathway. (A) WT mice were subjected to chronic stress for different time periods. Nuclear proteins were extracted from the spleens. The levels of phospho-Smad2/3 and total Smad2/3 were detected by immunoblotting (upper panel) and DNA-binding activity of Smad2/3 was examined by EMSA (middle panel) in the spleens. Each sample from the spleen of one mouse in EMSA was performed, and five experiments per group were performed (N=5 per group). (B) Age-matched WT and TLR9 KO mice were subjected to stress for 6 h. WT mice were administrated with SB203580 (1mg/Kg body weight, i.p.), SB431542 (4.2mg/kg, i.p.) and ISI3 (2.5mg/kg body weight, i.p.) for 1 h, 2 h, and 2 h, respectively, before the initiation of restraint stress. The levels of phospho-Smad2/3 and total Smad2/3 (upper panel) and DNA-binding activity of Smad3 (bottom panel) in spleens were detected. N = 5 per group. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with indicated groups.

Fig. 6.

Critical role of TGF- β 1/Smad2/3 signaling on chronic stress-induced FoxP3 and the numbers of CD25⁺Foxp3⁺ cells. (A) After stress of the indicated time periods, spleens were harvested. The expression of Foxp3 in the nucleus was examined by Western blot analysis. (B) WT mice were administrated with SB203580 (1mg/kg body weight, i.p.), SB431542 (4.2mg/kg body weight, i.p.), ISI3(2.5mg/kg body weight, i.p.), peptide P60 (4.8mg/kg body weight, i.p.) for 1 h, 2 h, 2 h and 2 h respectively, before the initiation of stress. Foxp3 expression in spleens was determined by Western blot analysis. (C) After treated the mice as protocol (B), the mice were subjected to chronic stress for 2 days of 12 h. The spleens were harvested and isolated CD4⁺ cells by CD4⁺ isolation kit, stained the isolated cells with FICT-Foxp3 and PE-CD25 and determined the cells gated on CD25⁺Foxp3⁺ staining cells by flow cytometry. (N = 5 per group. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with indicated groups.

Fig. 7.

Inhibition of TGF- β 1/Smad2/3 signal prevents splenocyte apoptosis induced by chronic stress. WT mice were administrated SB203580, SB431542, ISI3, and P60 2 h before the mice were subjected to chronic stress for 2 days of 12 h. (A) Apoptotic cells in spleens were determined by TUNEL assay. (B) Cellular lysates were isolated from mouse spleens and the levels of Bax, Bcl-2, and total and cleaved caspase-3 were determined by western blot analysis. (C) Apoptosis regulated kinase1 (ASK1) expression levels were detected in the mouse spleens by western blot analysis. N = 5 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with indicated groups.

Fig. 8.

Inhibition of TGF- β 1/Smad2/3 restores chronic-induced immune suppression. WT mice were pretreated with SB203580, SB431542, ISI3 and peptide P60 as Fig. 7. After 2 days of 12 h chronic stress, the mice were challenged with 10 μ g of bacterial LPS (i.p.). After 6 h, blood and spleens were harvested. (A) The transcriptional levels of TNF- α , IL-17, TGF- β 1, and IL-10 cytokines were determined by quantitative RT-PCR. (B) The circulating levels of TNF- α , IL-17, TGF- β 1, and IL-10 cytokines were examined by ELISA. N = 5 per group. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with indicated groups.

Fig. 9.