Smad2 and Smad3 are redundantly essential for the suppression of iNOS synthesis in macrophages by regulating IRF3 and STAT1 pathways

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Abstract

Although transforming growth factor (TGF)-β1 is a well-known immunosuppressive cytokine, little is known about the role of its downstream transcription factors, Smad2 and Smad3, in the suppression of macrophage activation. Previous studies have demonstrated that Smad3 is critical for the suppression of LPS-mediated inducible nitric oxide (NO) synthase (iNOS) induction, although the role of Smad2 remains to be investigated. In this study, we found that iNOS induction was enhanced in Smad2-deficient bone marrow-derived macrophages (BMDMs) and peritoneal macrophages in vitro and tumor-associated macrophages in vivo, compared with wild-type (WT) macrophages. However, TGF-β1 still suppressed iNOS induction in Smad2-deficient macrophages. In Smad2/3 double knockout (KO) (Smad2/3 DKO) BMDMs, LPS-mediated NO/iNOS induction was more strongly elevated than in Smad2 or Smad3 single KO BMDMs, and its suppression by exogenous TGF-β1 was severely impaired. These data suggest that Smad2 and Smad3 redundantly regulate iNOS induction. Similarly, the production of IL-6 and TNFα, but not IL-10 was augmented in Smad2/3 DKO BMDMs, suggesting that Smad2 and Smad3 also redundantly suppressed some cytokines production. In Smad2/3 DKO macrophages, TLR3- as well as TLR4-mediated IRF3 activation and IFN-β production were strongly augmented, which resulted in hyper STAT1 phosphorylation. Furthermore, IFN-β- and IFN-γ-induced iNOS induction in the absence of TLR signaling and STAT1 transcriptional activity were augmented in Smad2/3 DKO BMDMs. These results suggest that Smad2 and Smad3 negatively regulate iNOS induction in macrophages by suppressing multiple steps in the IRF3-IFN-β-STAT1 pathway.

Keywords: immunosuppression, signal transduction, TGF-β, TLR

Introduction

Immunological suppression systems are important for preventing excess immunity. Dysregulation of these systems often results in immunological disorders such as autoimmune diseases and allergies. Transforming growth factor (TGF)-β is a pleiotropic cytokine that regulates cell growth and differentiation, deposition of the extracellular matrix, fibrosis and immunomodulation (1, 2). Among the three isoforms of TGF-β, TGF-β1 is considered a major anti-inflammatory cytokine that mostly suppresses T-cell proliferation and activation. However, in TGF-β knockout (KO) mice, high levels of inducible nitric oxide (NO) synthase (iNOS), which catalyzes the production of NO from L-arginine, were observed (3, 4). TGF-β1 suppresses NO production from macrophages stimulated with LPS or IFN-γ. TGF-β1 functions as a negative feedback regulator to prevent tissue injury caused by excessive NO from macrophages (5, 6). Although previous reports have suggested that TGF-β1 reduces iNOS mRNA and protein levels (5, 7, 8), the detailed molecular mechanisms of the suppression of iNOS expression by TGF-β1 remain to be clarified.

The major signaling pathway of the TGF-β receptors (TGF-βR) is relatively simple. TGF-β dimers bind to a type II receptor that recruits and phosphorylates a type I receptor. The type I receptor then recruits and phosphorylates a receptor regulated Smad (R-Smad), Smad2 and Smad3. Smad2 and Smad3 then bind to the common Smad, Smad4,
and form a hetero-oligomeric complex. This complex then enters the cell nucleus and, in a cooperative manner with other nuclear cofactors, regulates the transcription of target genes. Apparently, however, there exist Smad-independent pathways such as MAP kinases, RhoA and mTOR pathways.

Although we have shown that Smad2 and Smad3 are redundantly essential for the suppression of T-cell activation and regulatory T-cell (Treg) induction (9), little has been uncovered regarding the roles of Smad2 and Smad3 in macrophage activation. It has been reported that TGF-β inhibits macrophage activation, such as the induction of iNOS and matrix metalloproteinase-12 mostly via Smad3, not via Smad2, by using over-expression studies (10). The over-expression of Smad3 has also been demonstrated to inhibit MyD88-dependent TLR signaling pathways (10). However, it has not been clarified whether Smad3 has a suppressive function at physiological levels, and whether Smad2 and Smad3 have distinctive functions in macrophages.

To address these questions, we generated macrophage-specific Smad2 conditional KO (Smad2 cKO) mice and Smad2/3 double KO mice (Smad2/3 DKO). We found that not only Smad3 but also Smad2 inhibited iNOS mRNA expression induced by LPS stimulation and that Smad2 and Smad3 are redundant for the suppression of iNOS production. Furthermore, we also found that Smad2 and Smad3 are essential for the suppression of IRF3 and STAT1 transcriptional activities because IFN-β production induced by LPS as well as poly (I:C) was strongly enhanced in Smad2/3 DKO bone marrow-derived macrophages (BMDMs) and IFN-γ-mediated iNOS production was also increased in Smad2/3 DKO BMDMs compared with WT BMDMs. This is the first report showing a functional redundancy between Smad2 and Smad3 in TGF-β-mediated macrophage suppression.

Methods

Mice

Tissue-specific Smad2-deficient mice were generated using the cre/loxP system by breeding Smad2floxp mice, which carried a Smad2 allele flanked by loxP sites. Mice expressing cre under the endogenous lysozyme M promoter (LysM-cre) were used to delete Smad2lox in the myeloid compartment (11, 12). Smad3 KO mice with a C57BL/6 background were provided by Dr Saika (Wakayama Medical School) (13). All mice were housed in clean animal rooms under specific pathogen-free conditions. All experiments using these mice were approved by and performed according to the guidelines of the animal ethics committee of Keio University, Tokyo, Japan.

Preparation of macrophages

To isolate peritoneal macrophages (PMs), we injected mice intra-peritoneally with 2 ml of 3% thioglycolate (Sigma, St Louis, MO, USA). Peritoneal exudate cells were isolated from the peritoneal cavity 4 days after injection. Cells were incubated in complete media containing RPMI 1640 supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin, 2 mM glutamine (Nakalai, Kyoto, Japan), 100 nM nonessential amino acids, and 0.05 mM 2-ME (Invitrogen). Three hours after incubation, cells were washed three times with PBS and we used the adherent cells as thioglycolate-elicited PMs for the experiments. BMDMs were prepared from bone marrow cells from femurs and tibias in mice. Bone marrow cells were cultured in RPMI 1640 complete media with 30% L929 conditioned media as a source of macrophage colony-stimulating factor and the 7-day-cultured BMDMs were used for the experiment.

NO assay

NO production was determined indirectly by measuring the accumulation of the stable end product, NO₂⁻, in the culture supernatant using Griess reagent (1% sulfanilamide, 0.1%-N-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5% phosphoric acid). After incubation at room temperature, the absorbance at 570 nm was measured and compared with standard NaNO₂.

Enzyme-linked immunosorbent assay

Cytokine levels in the tissue culture supernatants were determined by ELISA kits according to the manufacturer’s instructions [TNF-α, IL-6 and IL-10 (eBioscience, San Diego, CA, USA) and IFN-β (PBL Biomedical Laboratories, Piscataway, NJ, USA)].

Flow cytometry

Cells were stained with different fluorochrome-coupled antibodies. Anti-CD11b, Anti-F4/80, Anti-class II MHC [I-A/I-E], Anti-CD40 and Anti-CD86 were purchased from eBioscience. Samples were analyzed on FACS Canto (Becton Dickinson-San, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo software (Treestar, San Carlos, CA, USA).

Mice tumor model and tumor-associated macrophages isolation

B16 melanoma cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin and 100 nM nonessential amino acids. 2 × 10⁶ B16 melanoma cells were subcutaneously transplanted to the right lateral region. After 11 days, animals were perfused with 4% paraformaldehyde, the tumors were removed, post-fixed and examined histologically. For immunohistochemistry, paraaffin-embedded sections were dehydrated and then microwaved in 10 mM citrate buffer (pH 6.0) for 15 min. Then, the sections were incubated with the following antibodies: anti-F4/80 (AbD Serotec, Oxford, UK) and iNOS (Santa Cruz Biotechnology, Santa cruz, CA, USA). HISTOFINE MAX-PO kits (Nichirei, Tokyo, Japan) were used for detection. Tumor-associated macrophages (TAMs) were isolated 10 days after transplantation as described (14). Briefly, the subcutaneous tumor was passed through a plastic mesh after being treated with 0.25 mg ml⁻¹ collagenaseD (Roche, Indianapolis, IN, USA) and 0.025 mg ml⁻¹ DNase1 (Roche) for 30 min, the resulting dissociated cells were suspended in PBS, washed once, resuspended in an isotonic 40% Percoll solution (Amersham Biosciences, Uppsala, Sweden) and then centrifuged at 800 × g for 20 min at room temperature. The cell pellet was resuspended in a 0.83% NH₄Cl solution to dissolve red blood cells and washed twice in MACS buffer.
PBS supplemented with 0.5% BSA and 2 mM EDTA). After Fc receptor blocking for 30 min, cells were stained with anti-CD45, anti-CD11b and anti-F4/80 (eBiosciences). Cell sorting was performed using a FACS Aria II cell sorter (BD Biosciences).

Cell culture and reagents
RAW 264.7 cells were maintained in RPMI containing 10% FBS, l-glutamine, penicillin/streptomycin and nonessential amino acids. HEK293T cells were maintained in DMEM supplemented with 10% FBS, l-glutamine, penicillin/streptomycin and nonessential amino acids. LPS (Escherichia coli O111:B4) and poly (I:C) were purchased from Sigma. Recombinant human TGF-β1 and mouse IFN-γ were purchased from Peprotech (Rocky Hill, NJ, USA). Recombinant mouse IFN-γ was purchased from PBL Biomedical Laboratories.

Construction of expression vectors
The cDNAs-encoding mouse Smad2 and Smad3 were cloned from the total RNA of BMDMs by reverse transcription (RT)–PCR and subcloned into the pCMV vector (15). The expression vectors for T7-IPS-1 and IFN-β luciferase reporter plasmid have been described (16). FLAG-IRF3 was subcloned into the pCMV vector. The GAS luciferase reporter plasmid has been described (17). The STAT1c plasmid was kindly provided by Dr T. Ouchi (Northwestern University, USA) (18). The iNOS promoter region (−1505/+1409) was amplified by PCR from mouse genomic DNA and subcloned into pG3V-2 vector (Toyoinki, Tokyo, Japan) using Kpn1/Xho1 sites. All constructs were confirmed by sequencing.

Luciferase assay
RAW cells were transiently co-transfected with the construct reporter plasmid with pRL-TK control use as an internal

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**Fig. 1.** Enhanced production of NO, iNOS and IL-6 in LPS stimulated Smad2-deficient PMs. Thioglycolate-elicited PMs from littermate Smad2<sup>flox/flox</sup> WT and LysM-cre Smad2<sup>flox/flox</sup> cKO (Smad2 cKO) mice were stimulated for 24 h with LPS (10 ng ml<sup>−1</sup>) in the absence or presence of TGF-β1 (10 ng ml<sup>−1</sup>) or anti-TGF-β antibody, 1D11 (10 µg ml<sup>−1</sup>). NO in the culture supernatants were measured using Griess reagent (A), and iNOS and Smad2 levels PMs were examined by Western blotting (B). Cytokines in the culture supernatants were measured by ELISA (C). Results are expressed as mean ± SEM (n = 3). Statistical analysis was performed using the unpaired t-test (*P < 0.05). LPS-stimulated PMs were analyzed by FACS. CD11b and F4/80 double-positive cells were gated (D). Data (B and D) are representative of three independent experiments.
control reporter with expression plasmids using FuGENE 6 (Roche), as described (19). Cells were stimulated and luciferase activities were determined with the commercial dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

Immunoprecipitation and Western blot analysis

For immunoprecipitation, HEK293T cells were co-transfected with expression plasmids using PEI-MAX (Polysciences, Warrington, PA, USA) according to the manufacturer’s instructions. Twenty-four hours after transfection, HEK293T cells were washed once with ice-cold PBS and lysed in the NP-40-lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and protease inhibitor cocktail (Nacalai)] on ice for 10 min and then centrifuged at 15 000 × g for 10 min at 4°C. Proteins from cell lysates were precipitated with anti-Flag (M2) affinity gel (Sigma) or mouse IgG (Santa Cruz) and protein G-sepharose (GE Healthcare, Uppsala, Sweden) overnight at 4°C. The immunoprecipitates were collected by centrifugation and washed four times in the lysis buffer and then Flag fusion proteins were eluted by competition with Flag peptide (Sigma) and eluted samples were subjected to SDS–PAGE. Whole-cell lysates were prepared according to the lysis buffer. Western blot analysis to detect phosphorylated signal transducers and iNOS were described previously (17, 20). Briefly, the immunoprecipitates or whole-cell lysates were resolved through SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were blotted with the indicated antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using Chemi-Lumi One L Western-blotting detection reagents (Nacalai).

Fig. 2. Smad2 cKO mice exhibited mild resistance to B16 melanoma transplantation and increase of iNOS+ TAMs. (A) WT and Smad2 cKO littermate mice were subcutaneously challenged with 2 × 10⁶ B16 melanoma cells (n = 12, each). Lethality was observed for 30 days after B16 challenge. Kaplan–Meier survival curves are depicted as time after tumor challenge. The results were assessed by the log-rank test. P = 0.24. (B) Tumor volume was assessed by measuring three perpendicular diameters. Results are expressed as mean ± SEM (n = 11, each). (C and D) Immunohistochemical staining of infiltrated macrophages. Eleven days after tumor challenge, subcutaneous tumor samples were isolated and used for HE staining and immunostaining for F4/80 and iNOS. Bars, 50 μm. (C) The stained cell numbers in the setting square (D). Results are expressed as mean ± SEM (n = 7). (E) The mRNA expression of iNOS in the TAMs. Left, CD11b-positive gated tumor-infiltrating cells 10 days after tumor transplantation. CD11b+ F4/80+ cells were gated and isolated as TAMs by FACS. Right, total RNA of TAMs was extracted and mRNA expression of indicated genes was assessed by real-time RT–PCR normalized with GAPDH (mean ± SEM, n = 6). Statistical analysis was performed using the unpaired t-test (*P < 0.05).
Fig. 3. Smad2/3 DKO BMDMs exhibited enhanced iNOS and cytokine production. (A and B) Bone BMDMs from littermate WT and Smad KO mice were stimulated for 24 h with LPS (10 ng ml⁻¹) with or without TGF-β1 (10 ng ml⁻¹) or αTGF-β1 antibody (10 μg ml⁻¹). NO in the culture
Antibodies for pSMAD2, pIRF3, IRF3, pSTAT1, pIkB-α, pERK, pp38, p38 and JNK antibodies were purchased from Cell Signaling (Danvers, MA, USA), those for STAT1, IkB-α, ERK2 and iNOS antibodies were from Santa Cruz and those for Smad2/3 and pJNK were from BD Transduction Laboratories (San Jose, CA, USA). Anti-HA antibody was from Covance (Princeton, NJ, USA) and anti-Actin antibody from Sigma.

**Real-time PCR**

Total RNA was isolated from cells using RNA iso (Takara Bio, Shiga, Japan) and RT was performed using the one-step cDNA RT kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the primers in combination with SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) by a CFX384 real-time PCR detection system (Bio-Rad). The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was evaluated as an internal control. The following oligonucleotides were used for mouse iNOS: 5′-CACCTTGGAGTTCAACCATGT-3′ and 5′-ACCACCTGACTTGGGATGC-3′; GAPDH: 5′-TCCACCACCTGTGCTGTA-3′ and 5′-ACCA-CAGTCCATTGCATAC-3′; SPSB1 5′-CGGGGACTCAAGGG-TAAA-3′ and 5′-AGGGCTCAGGATCAAGTGTT-3′.

**Results**

**Smad2-deficient macrophages exhibited enhanced NO/INOS production in response to LPS stimulation**

To investigate the role of Smad2 in macrophages, we generated macrophage-specific Smad2 cKO mice. Smad2lox/lox mice (9) were crossed with a mouse line in which the Cre cDNA was knocked-in to the lysozyme M gene (LysM-Cre mice). Mice homozygous for the loxP-flanked (Smad2lox/lox) gene (WT control) and carrying the LysM-Cre (LysM-Cre Smad2lox/lox) gene (Smad2 cKO) were born at expected Mendelian ratios and exhibited no obvious abnormalities. The Smad2 protein was undetectable in the PMs isolated from Smad2 cKO mice (see Fig. 1B).

First, we examined the inflammatory responses induced by LPS and the inhibitory effect of TGF-β1 in thioglycolate-elicited PMs from Smad2 cKO mice. To eliminate the effect of serum and endogenous TGF-β, we examined the effect of anti-TGF-β antibody in the culture. The levels of NO as well as iNOS protein induced by LPS stimulation were higher in Smad2 cKO PMs than in WT PMs (Fig. 1A and B). Exogenous TGF-β1-mediated NO/INOS suppression was observed in both WT and Smad2 cKO PMs, although levels of NO and iNOS protein in the presence of TGF-β1 in Smad2 cKO PMs were still higher than those in WT PMs. These results suggested that the inhibitory effect of TGF-β1 was partly impaired by Smad2 deficiency; however, other Smads- or Smad-independent pathways compensated for the loss of Smad2. Anti-TGF-β antibody exhibited little effect on NO and iNOS production in both WT and Smad2 cKO PMs. However, low levels of Smad2 phosphorylation were induced by LPS, which disappeared following anti-TGF-β antibody treatment (Fig. 1B). This suggests that endogenous TGF-β1 may play a role in the Smad2-mediated suppression of NO/iNOS induction.

Next, we examined the effect of Smad2 deficiency on LPS-mediated cytokine production and activation marker expression (Fig. 1C and D). The induction of TNFα and IL-10 and the expression of class II MHC, CD40, CD80 and CD86 were almost identical between WT and Smad2 cKO PMs. However, interestingly, LPS-induced IL-6 production was higher in Smad2 cKO PMs than in WT PMs (Fig. 1C). In WT PMs, TNFα and IL-10 induction was not affected by exogenous TGF-β1, although IL-6 was significantly suppressed by TGF-β1. The suppressive effect of TGF-β1 on IL-6 was impaired in Smad2 cKO PMs (Fig. 1C).

Taken together, these results suggested that LPS-induced inflammatory factors were selectively suppressed by TGF-β1/Smad2 in activated macrophages and that NO/iNOS and IL-6 were especially strongly affected by Smad2 deficiency.

Smad2 deficiency in macrophages caused enhanced iNOS production in an in vivo B16 tumor transplantation model

To investigate whether Smad2-deficient macrophages express higher NO/iNOS in vivo, we created a subcutaneous tumor transplantation model with B16 melanoma cells that have been demonstrated to be killed by macrophages in NO-dependent mechanisms (21, 22). We noticed a slight enhancement of survival in Smad2 cKO mice (Fig. 2A). Tumor volume in Smad2 cKO mice was significantly smaller than that in WT mice (Fig. 2B). Immunohistological staining revealed that F4/80-positive TAMs infiltrated the tumor region in both WT and Smad2 cKO mice. The number of F4/80-positive TAMs was not significantly different between WT and Smad2 cKO mice; however, the number of iNOS-positive cells in Smad2 cKO mice was significantly higher than in WT (Fig. 2C and D).

To further characterize Smad2-deficient TAMs, we have isolated TAMs and examined iNOS and cytokine expression by real-time PCR. As shown in Fig. 2E, Smad2-deficient TAMs expressed higher amount of iNOS and TNFα mRNAs than WT TAMs. However, Smad2-KO TAMs also expressed higher levels of IL-10 and arginase-1, suggesting that Smad2-deficient TAMs did not represent simple M1-type macrophages. Nevertheless, these data confirmed that Smad2-deficient TAMs produced more NO than WT TAMs, which may partly account for a suppression of tumor progression in Smad2 cKO mice. These data confirmed that iNOS synthesis is down-regulated by Smad2 both in vivo and in vitro.
Fig. 4. Smad2 and Smad3 inhibited LPS-mediated iNOS mRNA induction and STAT1 phosphorylation. (A–C) BMDMs from littermate WT and Smad KO mice were stimulated for 6 h (B) or 24 h (A and C) with LPS (10 ng ml$^{-1}$) with or without TGF-$\beta_1$ (10 ng ml$^{-1}$) or $\alpha$TGF-$\beta_1$ antibody (10 $\mu$g ml$^{-1}$). Total RNA was extracted and arginase 1 (A), spsb1 (B) and iNOS (C) mRNA levels were analyzed by real-time PCR. The indicated mRNA levels were quantified by real-time PCR and normalized to the expression of ACTIN. (D) BMDMs from littermate WT and Smad KO mice were stimulated with LPS (10 ng ml$^{-1}$) or LPS+TGF-$\beta_1$ for 24 h. Relative luciferase activity was measured using the iNOS-luc construct. The relative luciferase activity was normalized to the activity of the MOCK control. (E) BMDMs from littermate WT and Smad KO mice were stimulated with LPS (10 ng ml$^{-1}$) for 0, 1, 3, and 6 h. Cell lysates were analyzed by Western blotting for pJNK, JNK, pERK, ERK2, p38, and ACTIN. (F) BMDMs from littermate WT and Smad KO mice were stimulated with LPS (10 ng ml$^{-1}$) for 0, 1, 3, 6, and 24 h. Cell lysates were analyzed by Western blotting for pJNK, JNK, pERK, ERK2, p38, and ACTIN. The indicated mRNA levels were quantified by real-time PCR and normalized to the expression of ACTIN.
Smad2 and Smad3 are redundantly essential for iNOS suppression

A previous over-expression study using RAW cells suggests that Smad3 is involved in the suppression of LPS-mediated iNOS induction (10). Thus, we examined whether Smad2 and Smad3 have redundant roles in macrophage activation. To address this, we generated Smad2/3 double KO (Smad2/3 DKO; LysM-Cre Smad2fllox/flox Smad3fllox/flox) mice by crossing Smad2 cKO mice and Smad3 KO mice and compared the iNOS and cytokine production in BMDMs. In Smad2/3 DKO BMDMs, the NO levels induced by LPS were approximately 3-fold higher than those in WT BMDMs (Fig. 3A). Both Smad2 and Smad3 single KO BMDMs also exhibited more significantly enhanced NO production than WT BMDMs. However, the enhancement of NO levels by single Smad deficiency was lower than those observed in Smad2/3 DKO BMDMs. Similar results were observed in iNOS induction in Smad2 single KO BMDMs and Smad2/3 DKO BMDMs (Fig. 3B). The TGF-β1-mediated suppression of NO/iNOS production was severely impaired in Smad2/3 DKO BMDMs (Fig. 3A and B). These results indicated that both Smad2 and Smad3 redundantly and additively suppressed NO/iNOS production. Anti-TGF-β antibody elevated NO/iNOS production in BMDMs, suggesting that endogenous or serum TGF-β had some role in the levels of NO/iNOS in this condition.

Next, we examined cytokines. LPS-induced TNFα levels were higher in Smad2/3 DKO BMDMs than in WT BMDMs, whereas they were comparable between Smad-single KO BMDMs and WT BMDMs (Fig. 3C), suggesting that compensation between Smad2 and Smad3 is very strong for the suppression of TNFα production. Similar to Smad2-deficient PMs, IL-6 levels were higher in Smad2 cKO BMDMs than in WT BMDMs; however, they were much higher in Smad2/3 DKO BMDM than in WT and Smad2 cKO BMDMs (Fig. 3D). IL-6 levels were not affected by Smad3 deficiency (Fig. 3D). These data suggested that both Smad2 and Smad3 were redundantly involved in the suppression of IL-6; however, the contribution of Smad2 was more profound than Smad3. IL-10 was not affected by Smad2/3 DKO BMDMs (Fig. 3E).

These results are consistent with our notion that the suppressive effect of the TGF-β1/Smad pathway had some specificity among LPS-inducible genes and that it has a particularly strong impact on NO/iNOS suppression. Therefore, we focused on the mechanisms of how Smad2 and Smad3 inhibited LPS-mediated NO/iNOS induction.

Smad2 and Smad3 inhibited LPS-mediated iNOS mRNA expression and STAT1 phosphorylation

NO level is determined not only by iNOS but also by arginase 1, which competes with iNOS for their common substrate, L-arginine. Thus, first, we examined the levels of arginase 1 in Smad(s)-deficient macrophages. As shown in Fig. 4(A), TGF-β1 strongly up-regulated arginase 1 expression in WT BMDMs. However, such induction by TGF-β1 was severely impaired in Smad2 cKO BMDMs and partly inhibited in Smad3 KO BMDMs. TGF-β1-mediated arginase-1 induction was almost completely impaired in Smad2/3 DKO BMDMs. These data indicate that arginase 1 is a common target of Smad2 and Smad3, although Smad2 seems to play more important role than Smad3.

It has been shown that TGF-β1 reduces iNOS protein levels by inducing ubiquitin-proteasome-mediated degradation of iNOS (7, 8, 17). Recently, it has been reported that SPSB proteins regulate ubiquitination and iNOS proteasomal degradation (23, 24). Thus, we investigated SPSB1 and SPSB2 expression in Smad(s)-deficient BMDMs. Although SPSB2 expression did not respond to LPS and TGF-β1 stimulation (data not shown), SPSB1 expression levels were up-regulated in WT BMDMs in response to LPS and TGF-β1 stimulation (Fig. 4B). This TGF-β1-mediated SPSB1 mRNA up-regulation was impaired in Smad2/3 DKO BMDMs (Fig. 4B), indicating that SPSB1 was also a target of Smad2/3. This result suggests that TGF-β1-mediated iNOS protein degradation is dependent on Smad2/3 by inducing SPSB1.

Then we examined the role of Smad2/3 on iNOS mRNA levels (Fig. 4C). Similar to protein levels, iNOS mRNA expression 24 h after LPS stimulation was strongly enhanced in Smad(s)-deficient BMDMs and the suppressive effect of TGF-β1 was impaired in Smad2/3 DKO BMDMs. To determine whether Smad2 and Smad3 affected iNOS promoter activity, the iNOS promoter reporter was co-transfection with Smad expression plasmids. As expected, Smad2 and Smad3 inhibited iNOS promoter activities and additional TGF-β1 enhanced the suppressive effect of Smads (Fig. 4D).

Next, we compared the phosphorylation of signaling molecules in response to LPS between WT and Smad2/3 DKO BMDMs. As shown in Fig. 4E, no significant differences were observed in the phosphorylation of ERK, p38 and JNK between these two cells, indicating that MAPK pathways were not affected by Smad2/3. Phosphorylation and degradation of IκBα were slightly enhanced in Smad2/3 DKO, suggesting that Smad2/3 may affect IKK activities. We noticed that STAT1 phosphorylation was enhanced and prolonged in Smad2/3 DKO BMDMs compared with WT BMDMs (Fig. 4F). These data suggested that STAT1 could be an important inhibitory target of Smad2/3.

Smad2/3 inhibited IFN-β production by suppressing IRF3 transcriptional activity

As STAT1 is known to be mostly activated by IFN-β, which is rapidly induced through IRF3 activation by the TRIF pathway activated through LPS/TLR4 or Poly (I:C)/TLR3, we examined the levels of IFN-β induced by LPS and Poly (I:C) in BMDMs.
Role of Smad2/3 in macrophage suppression

**Figure A**

![Graphs showing IFN-β levels in WT, Smad2 KO, and Smad2/3 DKO macrophages under different conditions.](image)

**Figure B**

![Western blots showing pIRF3, IRF3, and ACTIN expression in WT, Smad2 KO, and Smad2/3 DKO macrophages treated with LPS and Poly (I:C).](image)

**Figure C**

![Western blots showing pIRF3, IRF3, and ACTIN expression in WT, Smad2, and Smad3 macrophages treated with LPS.](image)

**Figure D**

![Bar graph showing IFN-β luciferase activity in WT, Smad2 KO, and Smad2/3 DKO macrophages under different conditions.](image)

**Figure E**

![Western blots showing Smad-FLAG, IRF3, and IPS1 interactions in WT, Smad2, and Smad3 macrophages.](image)

**Figure F**

![Western blots showing IRF3-FLAG, Smad2-HA, and IPS1 interactions in WT, Smad2, and Smad3 macrophages.](image)
As expected, Smad2/3 DKO BMDMs produced higher levels of IFN-β in response to both LPS and Poly (I:C) than WT BMDMs (Fig. 5A). IRF3 phosphorylation in response to both LPS and Poly (I:C) stimulation was enhanced in Smad2/3 DKO BMDMs compared with WT BMDMs (Fig. 5B). Consistently, LPS-induced IRF3 phosphorylation was suppressed in Raw cells over-expressed Smad2 or Smad3 (Fig. 5C). These data suggest that Smad2/3 somehow suppresses IRF3 phosphorylation.

Next, we examined the effect of Smad2 and Smad3 on IRF3-mediated IFN-β-promoter activity. As shown in Fig. 5D, both Smad2 and Smad3 inhibited LPS- as well as IPS-1-induced IFN-β-promoter activity. These data confirmed that Smad2 and Smad3 inhibited IRF3 activation. To examine physical interaction between Smad2/3 and IRF3, we performed co-immunoprecipitation experiments. Figure 5(E and F) revealed that both Smad2 and Smad3 bound to IRF3, and interestingly, these interactions were enhanced by IPS-1 co-expression. These data suggested that both Smad2 and Smad3 inhibited IRF3 phosphorylation and transcriptional activity by their direct interaction.

Smad2 and Smad3 inhibited STAT1 transcriptional activity

Although IFN-β production and STAT1 phosphorylation were significantly higher in Smad2/3 DKO BMDMs, we questioned whether the downstream of IFN-β was affected by Smad2/3. To address this question, Smad2/3 DKO BMDMs were stimulated with IFN-β and IFN-γ instead of LPS to directly activate STAT1. As shown in Fig. 6(A–D), IFN-β- or IFN-γ-mediated iNOS mRNA (Fig. 6A and B) and protein (Fig. 6C and D) induction was augmented in Smad2/3 DKO BMDMs, whereas STAT1 phosphorylation was not affected by Smad2/3 deficiencies (Fig. 6C and D). These data indicate that Smad2 and Smad3 have little effect on the signaling of the JAK/STAT pathway. Next, we examined whether Smad2 and Smad3 inhibited STAT1 transcriptional activity. To examine this possibility, we measured STAT1-mediated GAS promoter activation using STAT1 constitutive active-form (STAT1c) (18). As shown in Fig. 6E, Smad2 or Smad3 inhibited STAT1c-mediated GAS promoter activation. Immunoprecipitation studies revealed that both Smad2 and Smad3 bound to endogenous STAT11 constitutively (Fig. 6F). These data suggested that Smad2 and Smad3 inhibited not only IRF3 but also STAT1 by their physical interactions.

Discussion

Negative regulation of inflammatory factors such as pro-inflammatory cytokines, NO and reactive oxygen species is essential to avoid potentially deleterious consequences of excessive immune cell activation. TGF-β1 has been generally considered a major anti-inflammatory cytokine; however, its suppressive mechanisms for inflammatory signals are not fully understood. In this report, we demonstrated for the first time that Smad2 and Smad3 redundantly inhibited LPS-mediated iNOS, IL-6 and TNFα induction in activated macrophages by using gene disruption strategy. Interestingly, compensatory effects of Smad2 and Smad3 were different among these inflammatory factors. Both Smad2 and Smad3 were equally, additively, and redundantly required for suppression of iNOS production. Similar equal contribution of Smad2 and Smad3 were observed in TGF-β1-mediated suppression of the IFN-γ and IL-2 in T cells (9, 25). We have recently demonstrated that Smad2/3 recruited histone methyltransferase Suv39h1 to the IL-2 promoter, thereby inducing suppressive histone methylation and inhibiting TCR-mediated IL-2 transcription. However, unlike IL-2 mRNA, TGF-β1 strongly suppressed iNOS mRNA 24 h after LPS stimulation in macrophages, and the effect of Smad2/3 deficiency was most prominent on relatively late stage. Thus, we suspected that the TGF-β1/Smad pathway modulated secondary steps for the iNOS induction.

Several suppressive mechanisms of TGF-β1 for iNOS expression have been proposed, such as iNOS mRNA degradation, iNOS protein degradation and Smad3 mediated inhibition of NF-κB activity (7, 8, 10, 17, 23, 24). In the present study, we found that SPSB1, which function as part of the ECS E3 ubiquitin ligase complex and regulate iNOS proteasomal degradation, was a target of TGF-β1/Smad2/3 pathway. Thus, Smad2/3 regulates iNOS levels not only by suppressing transcription but also by inducing protein degradation.

Furthermore, by using KO macrophages and over-expression in cultured cells, we demonstrate that Smad2 and Smad3 down-regulate iNOS mRNA levels by suppressing the IRF3-IFN-β-STAT1 pathway. We propose that Smad2/3 inhibit IRF3 phosphorylation and transcriptional activity by a direct interaction, which reduced IFN-β induction. IRF3 has critical roles in the regulation of innate immunity and is a key regulator of IFN-β production (26–29). Although a binding between IRF3 and Smads in activated macrophages has not been reported, crystal structure analysis revealed that IRF3 exhibited structural similarity to the MH2 domain of Smad2.

![Fig. 5. Enhanced IFN-β production in Smad2/3 DKO BMDM.](image-url)
Fig. 6. Smad2 and Smad3 suppressed STAT1 transcriptional activity. (A and B) BMDMs were stimulated for 6 h with IFN-β (200 U ml⁻¹) (A) or IFN-γ (100 ng ml⁻¹) (B). Total RNA was extracted and analyzed by real-time PCR. iNOS mRNA level was normalized with GAPDH. Results are expressed as mean ± SEM (n = 4–5). Statistical analysis was performed using the unpaired t-test. (C and D) BMDMs were stimulated with IFN-β (C) or IFN-γ (D) for indicated periods. Whole-cell lysates were prepared and subjected to Western blotting with indicated antibodies. Data are representative of three independent experiments. (E) RAW cells were transiently transfected with the construct of the GAS reporter plasmid, pRL-TK control plasmid, STAT1 constitutive active-form (STAT1c) plasmid and Smad expression plasmid. After incubation for 24 h, luciferase and pRL-TK activities were measured. Luciferase activity was normalized with pRL-TK activity. Results are expressed as mean ± SEM. Data are representative of three independent experiments. (F) HEK293T cells were transiently co-transfected with either empty or Smad expression plasmid. Twenty-four h later, cell lysates were subjected to immunoprecipitation with indicated antibodies and then protein complexes were subjected to Western blotting with indicated antibodies.
Smad2/3 also suppressed STAT1 transcriptional activity by suppressing STAT1 transcriptional activity by an interaction with Smad3. We have shown by SHAP and immunoprecipitation that Smad3 interacts with NF-κB and NF-κB-dependent promoters in vitro. Smad2/3 also suppressed STAT1 transcriptional activity by suppressing STAT1 transcriptional activity by an interaction with Smad3. Smad2/3 deficiency reduced STAT1 promoter activity in vitro, which was not affected by Smad2/3 deficiency. Although this study did not show a direct interaction between Smad1 and Smad3, co-immunoprecipitation of Smad2/3 and STAT1 with p300 was reported. Thus, Smad2/3 may modulate STAT1 DNA-binding activity through forming a complex with other transcription cofactors.

Other mechanisms remain to be investigated. In astrocytes, TGF-β1 inhibits the induction of class II MHC mRNA not by inhibiting receptor-proximal signaling events but by suppressing the transcriptional induction of CIITA, which is essential for class II MHC expression. Smads activated by TGF-β1 may function as a transcriptional repressor for CIITA. Another report suggests that TGF-β1 inhibits T-bet induction through the induction of protein tyrosine phosphatase Src homology region 2 containing phosphatase-1 (Shp-1) in murine CD4+ T cells. Thus, there seem to be cell- and gene-specific mechanisms for the TGF-β1/Smad-mediated suppression of cytokine signaling. Small reduction of iNOS and IL-6 by TGF-β1 was still observed in Smad2/3 DKO BMDMs (Figs. 3 and 4), suggesting the presence of the Smad2/3 independent mechanism for iNOS and IL-6 suppression. Such multiple, cell-type specific mechanisms may exist for TGF-β1- and Smad-mediated suppression of LPS signaling in macrophages.

It is not clear why LPS-mediated iNOS, IL-6 and TNFα induction was strongly enhanced in Smad2/3 DKO BMDMs in the absence of exogenous TGF-β1. Apparently, endogenous and/or serum TGF-β1 may be involved in this phenomenon because anti-TGF-β antibody partly enhanced LPS-mediated induction of these genes and basal Smad2 phosphorylation disappeared as a result of TGF-β antibody treatment. However, this mechanism cannot explain all of the exogenous TGF-β1-independent effects of Smad2/3 deficiency. Smad2/3 may regulate IRF3 and STAT1 TGF-β1-independently. This idea supports the idea that the over-expression of Smad2 or Smad3 reduced iNOS and IFN-β promoter activities in the absence of TGF-β1 (Figs. 5B and 6E). Alternatively, the lack of Smad2 or Smad3 may affect the development of macrophages. It has also been reported that TGF-β1-signaling deficient hematopoietic stem cells exhibited increased proliferative capacity in vitro, although it has normal self-renewal ability and lineage choice in vivo (37, 38). We also found that more BMDMs were obtained from Smad2/3-deficient BMs than WT BMs (data not shown). We did not observe any differences in the expression of surface markers and receptors including TLR4 between WT and Smad2 cKO macrophages; however, we could not rule out the possibility that the expression of uncharacterized signaling molecules of TLR4 were affected by Smad2 or Smad3 deficiencies in vivo or culture in vitro. Further studies are necessary to clarify this point.

Macrophages are plastic cells; their phenotype depends on their anatomical location and the physiological or pathological context. Classical macrophages (also called M1) and ‘alternative’ activated macrophages (M2) represent two extremes in the spectrum of the macrophage phenotypes. M2 macrophages produce high amounts of IL-10 but not IL-12, express scavenger receptors and exhibit anti-inflammatory and tissue repair functions. In contrast, M1 macrophages, activated by microbial products or IFN-γ, produce large amounts of pro-inflammatory cytokines, express high levels of MHC molecules and are potent killers of pathogens and tumor cells. NO is a mechanism of killing of tumor cells. Since Smad2-deficient TAMs expressed higher levels of iNOS and TNFα than WT TAMs, they seem to be M1 macrophage phenotype; however, arginase-1 and IL-10 were also slightly higher in Smad2-deficient TAMs. Arginase 1 is highly expressed in M2 macrophages. Although we found that arginase 1 expression was strongly reduced in Smad2-deficient BMDMs in vitro, we could not conclude that Smad2-deficient TAMs are typical M1 macrophages. Smad2/3-deficient macrophages resemble M2 macrophages. Both suppressed tumor growth in the transplantation model and enhanced iNOS synthesis; however, Smad2/3 deficiencies did not affect SOCS-1 induction (data not shown). These findings indicated that the M1/M2 phase conversion is not simple, and further study is necessary to verify the hypothesis that the TGF-β1/Smad pathway determines M1/M2 polarization of macrophages.

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