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Smad3-Deficient CD11b<sup>+</b>Gr1<sup>+</b> Myeloid-Derived Suppressor Cells Prevent Allograft Rejection via the Nitric Oxide Pathway

Tingting Wu,*, Chenming Sun,*, Zhigang Chen,† Yu Zhen,*, Jianxia Peng,*, Zhongquan Qi,† Xiao Yang,‡ and Yong Zhao*

Immunosuppressive CD11b<sup>+</b>Gr1<sup>+</b> myeloid-derived suppressor cells and TGF-β have been shown to negatively regulate host immunity against allografts. Our results demonstrated that Smad3-deficient mice or mice reconstituted with Smad3-deficient hematopoietic cells rejected allogeneic skin or heart grafts in a significantly slower manner compared with littermates or wild-type (WT) control mice. Transplanted Smad3<sup>−/−</sup> recipients produced markedly less anti-donor IgG Abs, especially IgG1 and IgG2b subclasses. T cells in alloskin-grafted Smad3-deficient mice were more likely to participate in a Th2-type immune response, as evidenced by more Th2-specific transcription factor, GATA3 expression, and increased IL-4 and IL-10 production, as well as less Th1-specific transcription factor, T-bet expression, and decreased IL-2 and IFN-γ production. More CD11b<sup>+</b>Gr1<sup>+</b> neutrophil infiltration and less monocyte/macrophage and T cell infiltration in allografts were observed in Smad3<sup>−/−</sup> recipients compared with WT recipients. Increased CXCL1 and CXCL2 as well as decreased CCL3, MCP-1, and RANTES chemokines in allografts of Smad3<sup>−/−</sup> recipients were consistently detected by real-time PCR. Further studies indicated that the increased CD11b<sup>+</b>Gr1<sup>+</b> myeloid cells in Smad3-deficient mice were immunosuppressive and responsible for the delayed allograft rejection mainly via an NO-dependent pathway. Thus, this study identifies Smad3 as an intrinsic negative regulator that critically inhibits the differentiation and function of immunosuppressive CD11b<sup>+</b>Gr1<sup>+</b> myeloid-derived suppressor cells. The Journal of Immunology, 2012, 189: 4989–5000.

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Abbreviations used in this article: B6, C57BL/6; BMC, bone marrow cell; CBA, cytometric bead array; cRBC, chicken RBC; DLN, draining lymph node; FCM, flow cytometer; HPRT, hypoxanthine phosphoribosyltransferase; iNOS, inducible NO synthase; i-cNOS, N<sub>O</sub>α-monomethyl-L-arginine; m, mouse; MDSC, myeloid-derived suppressor cell; MST, median graft survival time; PEM, peritoneal exudate macrophage; TG, thioglycollate; Treg, regulatory T cell; WT, wild-type.
strating that Smad3 is an intrinsic factor that inhibits the differentiation and immunosuppressive function of CD11b+Gr1+ MDSCs in mouse transplant models.

Materials and Methods

Mice

BALB/c, C57BL/6 (B6), SCID, C57BL/6-GFP, and Rag1−/− mice were purchased from the Beijing Laboratory Animal Research Center (Beijing, China). The Smad3−/− mice with 129/sv × C57BL/6 background were provided by Dr. X. Yang (Beijing Institute of Biotechnology, Beijing, China) (19). In this mouse line, exon 8 of the Smad3 gene is deleted. Smad3−/− mice were backcrossed for at least six generations to B6 mice in our laboratory and were intercrossed to produce homozygous offspring. The resulting progeny were screened by PCR to identify Smad3−/− mice and Smad3+/− littersmates, which were used as WT control mice. DO11.10 mice, which express a transgenic TCR that recognizes OVA 323–339 peptide, (20), were provided by the Model Animal Research Center of Nanjing University (Nanjing, China). DO11.10 × Smad3−/− mice were produced in our laboratory. All mice were bred and housed in pathogen-free conditions, and all experimental procedures were in accordance with the Institution Guidelines for the Care and Use of Laboratory Animals.

Skin and heart transplantation and histologic examination

Skin and heart grafts from BALB/c mice were transplanted into recipients as described previously (21–24). For skin transplantation, erythema, edema, and hair loss were considered early signs of rejection, whereas ulceration, progressive shrinkage, and desquamation were considered to be the end point of rejection (25). Photographs were taken daily with a digital camera (Canon Powershot A640; Canon, Tokyo, Japan) until the graft was rejected completely. For heart transplantation, undetectable heart impulses for two consecutive days were considered rejection (21). The skin grafts were removed at the indicated time points and rinsed in cold PBS, placed in OCT compound, and immediately frozen in liquid nitrogen for histopathologic examination. Sections (4–6 μm) were fixed in 4% paraformaldehyde and stained with H&E for assessment of cellular infiltration.

Detection of anti-donor Abs in serum

Before transplantation (day 0) and after transplantation at indicated time points, sera from recipients were collected and frozen at −80°C. The IgG isotype of anti-donor alloreactive Abs in sera were measured by flow cytometry using a previously described technique (26, 27). Briefly, 5 × 105 BALB/c splenocytes were used as target cells and incubated for 30 min at 4°C with either negative control serum (diluted 1:10) or with the recipiend mouse serum and FITC-labeled goat-anti-mouse IgG, IgG1, IgG2b, and IgG2c Abs (Santa Cruz Biotechnology) for 30 min at 4°C in the dark. Samples were assayed using a Beckman Coulter Epics XL benchtop flow cytometer (FCM; Beckman Coulter) and were analyzed using FlowJo (Tree Star, Ashland, OR) software. Results were expressed as the mean fluorescence intensity of stained cells subtracted by the mean fluorescence intensity of cells incubated with negative control serum and FITC-labeled secondary Abs.

Bone marrow reconstitution

A total of 2 × 106 bone marrow cells (BMCs) from WT or Smad3−/− mice were injected into C57BL/6-GFP mice or SCID mice lethally irradiated 4 h previously with 8 Gy radiation ([60Co source]. Recipients were tested for chimerism 6 wk later by FCM and after verification were used in bone marrow transplantation experiments.

TCRγ and CD4+ T cell purification and adoptive transfer

TCRγ and CD4+ T cells were sorted from the spleen and lymph nodes (LNs) of WT and Smad3−/− mice using FACSAria II (BD Biosciences) at a purity of ≥98%, and 2 × 105 sorted cells were adoptively transferred into syngeneic Rag1−/− mice via the tail vein. One day after adoptive transfer, the host mice were used as recipients for skin transplantation.

Cytokine bead assay

The quantification of IL-6, IL-17, IFN-γ, TNF-α, IL-4, and IL-10 in the serum from recipients was determined by the mouse cytokometric bead array kit (CBA; BD Biosciences). These experiments were performed according to the manufacturer’s instructions. The intensity of the fluorescence signal was determined with a FACS FCM (BD Biosciences) and analyzed using CBA software.

In vivo T cell migration assay

To detect in vivo T cell migration, 3 × 106 CD4+KJ1-26+ cells from DO11.10 or DO11.10 × Smad3−/− mice were labeled with CFSE (Molecular Probes, Eugene, OR) and injected i.v. into syngeneic BALB/c mice. After adoptive transfer, 50 μg OVA323–339 peptide (inactivated) in 50 μl 1:1 CFA (Sigma-Aldrich) was injected s.c. into the left footpad of recipients, and the right footpad of recipients was used as an unimmunized control. Thirty-six hours later, cells from the left and right popliteal LNs were stained with FITC-KJ1-26 and analyzed by FCM.

T cell proliferation assay

WT and Smad3−/− T cells (2 × 105 cells/well) were cultured in flat-bottom 96-well plates with 2 μg/ml Con A and 5 ng/ml TGF-β for 72 h at 37°C and 5% CO2. The culture medium consisted of RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5 × 10−3 M), and 10% FCS. Cell proliferation was assayed via a BrdU proliferation kit (Filtria Proliferation Kit; Perkin Elmer, Courtaboeuf, France).

In vitro induction of CD4+CD25+ regulatory T cells

FACS-sorted CD4+CD42L−/−CD25+ T cells were stimulated using plate-bound anti-CD3 (6 μg/ml) and anti-CD28 (4 μg/ml) in the presence of 5 ng/ml TGF-β and 10 ng/ml IL-4 for 6 d. The determination of the presence of CD4+CD25+ regulatory T cells (Tregs), cultured cells were stained using PE-Cy5 anti-mCD4 and PE anti-mFoxp3 and analyzed by FCM.

Isolation of skin graft cells

Skin grafts were retrieved and minced into small pieces with a scalpel and then digested for ~1 h at 37°C in RPMI 1640 medium containing 30 U/ml collagenase (type IV; Sigma-Aldrich) and 1 mM CaCl2. Collagenase-pretreated tissues were then ground with the plunger of a 5 ml disposable syringe and passed through a 70-μm nylon cell strainer. Cells were collected after centrifugation at 300 × g for 10 min and resuspended in FACS staining buffer for cell surface marker staining.

Peritoneal cell recruitment

To detect neutrophil and macrophage migration in vivo, 1 ml sterile 3% thiglycollate (TG; warmed to room temperature) was injected i.p. into WT or Smad3−/− mice. Ten hrs after TG injection, peritoneal cells were harvested and analyzed by FCM. To compare WT with Smad3−/− neutrophil migration in vivo, sorted WT CD11b+Gr1+ cells were labeled with PKH26 (Sigma-Aldrich) and Smad3−/− cells were labeled with CFSE (Molecular Probes). A total of 5 × 105 PKH-26-labeled WT CD11b+Gr1+ cells mixed with 5 × 106 CFSE-labeled Smad3−/−CD11b+Gr1+ cells were injected i.v. into WT mice. The recipients then received 1 ml 3% TG treatment over 1 mo to ensure formation of advanced glycation end products (28).

TNF-α, IL-6, and iNOS production by peritoneal exudate macrophages

WT and Smad3−/− peritoneal exudate macrophages (PEMs) were isolated as described previously (29, 30). A total of 5 × 106 PEMs were stimulated with 50 ng/ml IFN-γ (PeproTech) and 1 μg/ml LPS (Escherichia coli III B4; Sigma-Aldrich) in 24-well plates for 24 h at 37°C and 5% CO2. To detect intracellular TNF-α and IL-6 by FCM, 1 μl/ml brefeldin A (BD GolgiPlug; BD Biosciences) was added during the last 8 h of culture (29). TNF-α, IL-6, and iNOS mRNA was measured by real-time PCR.

Phagocytosis of cRBCs by PEMs in vitro

Phagocytosis of chicken RBCs (cRBCs) by CD11b+ (CBA; BD Biosciences) was detected in vitro as described previously (31). Single-cell suspension of cRBCs was freshly obtained. After two washes with PBS, 1 × 106 cRBCs/ml were labeled with 5.0 μM CFSE (Molecular Probes) for 15 min at 37°C. These cells were then washed thoroughly and resuspended at a concentration of 1 × 107/ml. Cell viability was determined by trypan blue exclusion. Cell viability was usually >95%. A total of 1 × 106 PEMs were cocultured with CFSE-labeled cRBCs at a ratio of 1:10 for 2–5 h at 37°C and 5% CO2. To detect phagocytosis of cRBCs, PEMs were labeled with 2% gelatin (Sigma-Aldrich) (32). After 2–5 h, unengested cRBCs were removed by aspirating supernatants and gently washing with cold PBS. Adherent cells were harvested with 5.0 mM EDTA (Sigma-Aldrich) in ice-cold PBS (pH 7.2) and
readjusted. Cells were then stained with PE–anti-F4/80, and the percentage of phagocytosed F4/80+ cells was determined using FC.

**FCM analysis**

Draining lymph node (DLN) cells or spleen cells were prepared as described previously (33). Cell preparations were stained with optimized AB dilutions. For surface marker staining, the following Ab–fluorochrome combinations were used: FITC, PE, or PC5–anti–mouse (mCD4 (RM4-5), PE or FITC–anti–mCD8a (53–67), FITC–anti–mTcRB (H5–57), PE-KJ1–26, FITC–anti–mCD19 (eBioID3), PE or FITC–Cy5–anti–mCD25 (M1/70), PE or PC5–anti–mGr1 (RB6–8C5), FITC or PE–anti–mLy6G (1A8), FITC–anti–mLy6C (AL–21), FITC or PE–anti–F4/80 (BM8), PE–anti–mC1d1 (HL, 1), FITC–anti–mCxCR2 (242216), FITC–anti–mCD20L (MEL–14), FITC–anti–mCD44 (IM7), and Alexa Fluor 488–mCD11b (AFS98). For intracellular staining, FITC–anti–mNiOs (6/iNOS/NOS TypeII) and anti–mGr1 (RB6–8C5) were used. iNOS protein staining was previously described (34), and Foxp3 protein staining was conducted according to the manufacturer’s instructions (eBioscience). For intracellular cytokine staining, splenocytes were collected and left unstimulated or were stimulated for 6 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Calbiochem) in the presence of GolgiStop at the recommended concentrations (BD Pharmingen). Cells were first stained extracellularly with FITC–anti–mCD4 or FITC–anti–mGr1 and were then fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen). They were then stained intracellularly with PE–anti–mIFN–γ (XMG1.2) or PE–anti–mIL–10 (10532G), or FITC–anti–mIL–4 (H57–597), PE–anti–mIL–12, PE–anti–mArg1 (XMG1.2), or PE–anti–mIL–17 (H129.168) using a microplate reader (Bio-Rad). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite.

**RNA preparation and real-time PCR**

Total RNA from skin grafts and spleens of recipients was isolated using RNeasy columns, and contaminating DNA was removed by on-column treatment with RNase-free DNase (Qiagen). For cDNA synthesis, 1 μg total RNA was reverse transcribed using oligo(dT) primers and reverse transcriptase enzyme (Promega), as described previously (35). Real-time PCR was performed with a CFX96 real-time PCR detection system (Bio-Rad), and amplification was carried out in a total volume of 20 μl containing 10 μl SYBR Premix Ex Taq (Takara) to quantitate the PCR product, 0.5 μM of each primer, and 1 μl cDNA prepared as described above. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal control. PCRs were cycled 40 times after initial denaturation (95°C, 5 min) with the following parameters: denaturation 94°C for 15 s, annealing 60°C for 20 s, and extension 72°C for 15 s. After PCR amplification, melting temperature curve analyses were performed, which allowed for the detection of potentially contaminated PCRs. The melt temperature of each PCR product was calculated by determining the temperature at which 50% of the product has melted. Samples were analyzed on a Beckman Coulter Epics XL benchtop FCM (Beckman Coulter) with FlowJo (Tree Star, San Carlos, CA) software.

**In vitro assays measuring the effect of TGF–β1 on NO production**

To determine the effect of TGF–β1 on NO production in WT CD11b+Gr1+ cells, 2 × 10⁵ sorted CD11b+Gr1+ cells isolated from spleens of WT mice 7 d after alloskin transplantation were cultured with LPS (100 ng/ml), TGF–β1 (5 ng/ml; R&D Systems), or TGF–β1 neutralizing Abs (2 μg/ml, clone 1D11; R&D Systems) (36) in 96-well plates for 6 h. Then, 100 μl culture supernatant was used to detect NO. Intracellular iNOS expression was analyzed by FCM (36).

**CD11b+Gr1+ cell apoptosis assay**

One milliliter of 3% TG (warmed to room temperature) was injected i.p. into WT or Smad3+/− mice. Four hours later, 2 × 10⁵ peritoneal cells were isolated and stimulated with LPS (100 ng/ml; Sigma-Aldrich) in 96-well plates for 6 h. Apoptosis was assayed by FCM using Annexin V/PI staining with gated CD11b+Gr1+ cells.

**Induction of CD11b+Gr1+ cells from bone marrow progenitors**

Bone marrow flushed from the tibiae and femora of WT and Smad3+/− mice was subjected to magnetic activated cell sorting against a panel of Abs directed against lineage-committed Ags (Miltenyi Biotec). Hematopoietic stem and progenitor (Lin−) cells were collected and plated at 2 × 10⁵ cells/ml and incubated with recombinant murine GM-CSF (20 ng/ml; PeproTech) for 5 d in DMEM with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 10% FCS. To detect iNOS expression in induced CD11b+Gr1+ cells by FCM, the 5-d cultured cells were stimulated with LPS (100 ng/ml; Sigma-Aldrich) for 4 h.

**OVA immunization of DO11.10 × Smad3−/− mice**

Fifty micrograms of OVA23,33 peptide (Invitrogen) in 50 μl 1:1 CFA (Sigma-Aldrich) was injected s.c. into the left footpad of DO11.10 × Smad3−/− and DO11.10 × Smad3+/− mice, and the right footpad of recipients was used as an unimmunized control. Seven days later, the cells from the left and right popliteal LNs were isolated and analyzed by FCM.

**Statistics**

All graft survival curves were calculated by the Kaplan–Meier method, using the GraphPad Prism software (GraphPad Software, San Diego, CA). The log-rank test was used for graft survival comparison. The median graft survival time (MST) is the time at which half of the subjects reached the respective score. Other data were presented as mean ± SD. Student unpaired t tests for comparison of means were used to compare groups. A p value < 0.05 was considered to be statistically significant.
Results

Delayed allograft rejection in mice with Smad3-deficient hematopoietic cells

To understand the role of Smad3-mediated pathways in the immune response to allograft, we first observed skin allograft survival in Smad3-deficient mice. On the basis of the anti-inflammatory effects of TGF-β, we expected Smad3-deficient mice to show enhanced immunity against allografts. Surprisingly, the MST of BALB/c (H-2k) skin allograft was significantly longer in Smad3−/− mice (B6 background, H-2b; MST = 17 d; n = 15) compared with WT littermates (MST = 11 d; n = 15; p < 0.001; Fig. 1A). As shown in Fig. 1B, from day 7 to 11, alloskin grafts on WT recipients showed progressive loss of hair, dermal necrosis and scab formation. However, the graft on Smad3−/− recipients showed normal gross appearance and hair growth. Histologic examination of skin allograft revealed mild immune cell infiltration in the derma of skin grafts in Smad3−/− recipients at days 2 and 7 after grafting compared with skin grafts in WT recipients, which showed moderate infiltration and necrosis at day 7 (Fig. 1C).

To identify whether Smad3 intrinsically regulates immune cells derived from the hematopoietic compartment or indirectly impacts the rejection response by regulating peripheral tissues and microenvironments, we transplanted BMCs from either WT or Smad3−/− mice into lethally irradiated syngeneic GFP-B6 mice or SCID mice to establish full chimeras. Eight weeks after bone marrow transplant, we grafted allogeneic BALB/c skin on these reconstituted mice. We found that lethally irradiated GFP-B6 and SCID mice reconstituted with Smad3−/− BMCs rejected the allografts with a MST of 15 d, which was significantly longer than the MST of 11 d in mice reconstituted with WT BMCs (p < 0.001; n = 6; Fig. 1D, 1E). Consistent with these results, allogeneic BALB/c heart grafts survived significantly longer in Smad3−/− BMC→B6 chimeric recipients than WT BMC→B6 chimeric recipients (p < 0.01; n = 5; Fig. 1F). Thus, Smad3 deficiency in hematopoietic cells decreases the immune response against allografts in an intrinsic manner.

The Th2 immune system is preferentially activated in response to allografts in Smad3-deficient mice

To determine the intensity of humoral and Th1/Th2 immune responses to allografts in Smad3-deficient recipients of alloskin grafts, we measured the levels of anti-donor IgG subclasses in the serum with FCM assays, as previously described (37), and cytokines, including IL-6, IL-17, IFN-γ, TNF-α, IL-4, and IL-10, with CBA. The level of total anti-donor IgG in Smad3−/− recipients was significantly lower than WT recipients 2 wk posttransplantation (p < 0.001; Fig. 2A). Similarly, anti-donor IgG1, IgG2c, and IgG2b levels were lower in Smad3−/− recipients. In parallel with this observation, significantly less activated/memory CD62LlowCD4+ cells were detected in skin-grafted Smad3−/− mice compared with WT recipients (p < 0.05; Supplemental Fig. 1). Smad3−/− recipients produced less IFN-γ and a remarkably higher quantity of IL-4 and IL-10 compared with WT recipients (p < 0.001; n = 5; Fig. 2B). IL-6, IL-17, and TNF-α levels were not different in Smad3-deficient recipients and WT recipients, except for the enhanced IL-6 level in WT recipients at day 7 (Fig. 2B). In addition, enhanced IL-4 and IL-10 mRNA expression were detected in splenocytes of Smad3−/− recipients by real-time PCR assays 7 d after grafting (data not shown). TGF-β1 mRNA expression in splenocytes of Smad3−/− recipients showed a slight but significant decrease compared with WT mice (data not shown). Notably, expression of the Th1-specific transcription factor, T-bet, was significantly decreased, and expression of the Th2-specific transcription factor, GATA3, was markedly increased in splenocytes of Smad3−/− recipients (p < 0.001 versus WT mice; Fig. 2C). Th17-related retinoic acid-related orphan receptor γT expression did not show detectable alteration, and CD4+CD25+ Treg cells detected in splenocytes of Smad3−/− recipients (p < 0.05; Fig. 2C).

The initiation of antiallograft immunity is known to occur in secondary lymphoid tissues where recipient T cells are activated, undergo clonal expansion, and differentiate into effector cells. In addition, CD4+ T cells activate B cell production of anti-donor
Abs in transplant models. Thus, we analyzed the cells from the DLNs and spleen. We found that the percentage of CD4+Foxp3+ Tregs were slightly increased, whereas the percentage of CD8+ T cells and CD19+ B cells were decreased in spleens and DLN of Smad3 \(^2/2\) mice at day 14 after transplantation (data not shown). Importantly, the percentage of IL-2+CD4+ cells and IFN-\(\gamma\)+CD4+ cells in CD4+ T cells as well as their cell numbers were significantly decreased in the spleens of Smad3 \(^2/2\) recipients 7 d after transplantation (\(p\), 0.001; Fig. 2D). The cytotoxic CD8+IFN-\(\gamma\)+T cells were also decreased in Smad3 \(^2/2\) recipients compared with those in WT recipients (\(p\), 0.001; Fig. 2E). In contrast, IL-4+CD4+ cells and IL-10+CD4+ cells in Smad3 \(^2/2\) recipients were increased in percentage and cell number (\(p\), 0.001; Fig. 2F). Therefore, these data collectively indicate that host immunity of Smad3 \(^2/2\) recipients promotes a skewed Th2-type immune response after alloskin grafting.

Smad3-deficient T cells respond efficiently to alloskin grafts

T cells play a central role in alloskin graft rejection. To see whether intrinsic impairment of T cell function by Smad3 can explain delayed alloskin graft rejection in Smad3-deficient recipients, we first confirmed the potential direct effects of Smad3 on T cell function in vitro and in vivo. T cells showed normal development in Smad3-deficient mice, as determined by the normal thymocyte subset distribution and V\(\beta\) family expression (Ref. 19; data not shown). Smad3-deficient T cells proliferated with Con A stimulation in a similar manner to WT control cells (Fig. 3A). However, TGF-\(\beta\) inhibition on Con A-induced T cell proliferation was less efficient in Smad3-deficient T cells compared with WT control cells (Fig. 3A), consistent with a previous report (19). The percentages of Foxp3+ cells in the freshly isolated naive CD4+ cells (CD4+CD62L\(^{high}\)CD25\(^2\)) from either WT or Smad3 \(^2/2\) mice were \(0.1\%\) as determined by FCM (Fig. 3B). However, after CD4+CD25+ Treg induction from naive CD4+ T cells with TGF-\(\beta\) in vitro, significantly fewer CD4+CD25+Tregs were induced from Smad3-deficient naive CD4+ T cells compared with WT naive T cells (Fig. 3B). IFN-\(\gamma\) production in isolated Smad3-deficient CD4+ T cells and WT cells was similar after stimulation with ionomycin/PMA (Fig. 3C). We also investigated the ability of Smad3-deficient T cells to migrate by using Smad3-deficient CD4+ T cells with a transgenic TCR (referred to as Smad3 \(^2/2\)KJ1-26+CD4+ T cells), which is recognized by KJ1-26 mAb. We adoptively transferred either CFSE-labeled WT CD4+ T cells or Smad3 \(^2/2\)KJ1-26CD4+ T cells into syngeneic BALB/c...
mice. After adoptive transfer, mice were immunized with OVA in CFA. Percentages and cell numbers in DLN were detected 36 h after immunization. The percentage and cell number of CD4+KJ1-26+, CD4+KJ1-26+CD25+, and CD4+KJ1-26+CD69+ cells in DLN of KJ1-26+Smad3−/− T cells was identical to WT KJ1-26+ T cells 36 h after OV A immunization. (I and J) Smad3−/− macrophages demonstrated normal phagocytosis of cRBCs in vitro. (K) Increased NO production by Smad3−/− macrophages after stimulation with LPS + IFN-γ, compared with WT macrophages. (L) Increased TNF-α but not IL-6 protein expression by Smad3−/− macrophages after stimulation with LPS + IFN-γ, compared with WT macrophages. (M) Increased TNF-α and iNOS mRNA and unchanged IL-6 mRNA expression in Smad3−/− macrophages after stimulation with LPS + IFN-γ, compared with WT macrophages. Data are presented as mean ± SD (n = 5), which represent one of two independent experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control WT mice.

FIGURE 3. The direct effects of Smad3 on T cells and macrophages in vitro and in vivo. (A) Sorted Smad3−/− T cells were resistant to TGF-β–mediated immunosuppression in vitro, as determined by cell proliferation stimulated by Con A. (B) Sorted naive Smad3−/− T cells were resistant to TGF-β–induced CD4+CD25+Foxp3+ Treg formation in vitro. (C) IFN-γ in Smad3−/− CD4+ T cells was similar to WT cells after stimulation with ionomycin/PMA. (D–F) Cell migration of adoptively transferred Smad3−/− KJ1-26+ T cells was identical to WT KJ1-26+ T cells 36 h after OVA immunization. (G) Adoptively transferred Smad3−/− T cells rejected alloskin grafts as efficiently as WT T cells in Rag1−/− recipients. (H) Adoptively transferred Smad3−/− CD4+ T cells rejected alloskin grafts as efficiently as WT CD4+ T cells in Rag1−/− recipients. (I and J) Smad3−/− macrophages demonstrated normal phagocytosis of cRBCs in vitro. (K) Increased NO production by Smad3−/− macrophages after stimulation with LPS + IFN-γ, compared with WT macrophages. (L) Increased TNF-α but not IL-6 protein expression by Smad3−/− macrophages after stimulation with LPS + IFN-γ, compared with WT macrophages. (M) Increased TNF-α and iNOS mRNA and unchanged IL-6 mRNA expression in Smad3−/− macrophages after stimulation with LPS + IFN-γ, compared with WT macrophages. Data are presented as mean ± SD (n = 5), which represent one of two independent experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control WT mice.
strated a somewhat higher inflammatory response than WT macrophages in vitro.

**Impaired immune cell infiltration in allografts of Smad3-deficient recipients**

To gain insight into the changes occurring in local graft immune cell subsets and determine the reasons for delayed graft rejection in Smad3-deficient mice, we characterized the cells infiltrating the alloskin graft by FCM analysis at certain time points. We found that the infiltrating leukocyte population consisted mainly of neutrophils (CD11b+Ly6G⁺), inflammatory monocytes (F4/80+Ly6C⁺), CD4⁺ T cells and CD8⁺ T cells. NK cells were also present but in smaller proportions (data not shown). However, Smad3⁻/⁻ recipients exhibited a significantly higher CD11b⁺Ly6G⁺ granulocyte infiltration and a clear reduction in macrophage and T cell populations in skin grafts compared with WT recipients (p < 0.001; Fig. 4A, 4B). Furthermore, real-time PCR assays 4 d after grafting showed that the expression of CXCL1 and CXCL2, chemokines important for neutrophil recruitment, were significantly increased, whereas the expression of chemokines central to T cell recruitment, CCL3 (MIP-1α), MCP-1, and RANTES, were markedly lower in skin grafts in Smad3⁻/⁻ mouse recipients compared with WT mice (p < 0.001; Fig. 4C). Allogeneic Smad3⁻/⁻ skin grafts in BALB/c recipients were rejected as efficiently as WT skin grafts and showed identical innate immune cell infiltration 3 and 7 d after skin grafting (data not shown). These results indicate that Smad3 deficiency in the donor organ does not affect graft survival and immune cell infiltration in allografts. To further determine whether immune cell infiltration is impaired in Smad3-deficient mouse recipients, we performed immune cell recruitment assays in vivo. After inducing peritonitis with 3% TG, the major infiltrating cells were neutrophils during the early time period (4–6 h) and other cells, including macrophages and T cells, during the later time period (>10 h) (Ref. 39; data not shown). Significantly more neutrophils were recruited into the peritoneal cavities of Smad3⁻/⁻ mice in the early stage (4 h; data not shown) and later phase (10 h) (p < 0.001; Fig. 4D, 4E). However, 10 h after induction of peritonitis, low levels of CD11b⁺F4/80⁺ macrophages, CD4⁺ T cells, CD8⁺ T cells, and B cells were detected in the peritoneal cavity of Smad3-deficient mice but still lower than levels found in WT mice (p < 0.001; Fig. 4F–I).

To determine whether Smad3⁻/⁻CD11b⁺Gr1⁺ cells are more sensitive to chemokines, we compared cell migration of cotransferred PKH26-labeled Smad3⁻/⁻CD11b⁺Gr1⁺ cells and CFSE-labeled WT CD11b⁺Gr1⁺ cells in the TG-induced peritonitis model, as described in Materials and Methods. Nearly twice as many Smad3⁻/⁻ cells migrated into the peritoneal cavity compared with WT cells (p < 0.001; Fig. 4J). To determine whether...
differences in sensitivity to cell death accounts for this finding, we measured cell death in freshly isolated and cultured neutrophils. Cell death was similar in Smad3−/− and WT CD11b+Gr1+ cells freshly isolated from the TG-induced peritonitis model, as determined by Annexin V and PI staining (Supplemental Fig. 3A). However, Smad3−/− CD11b+Gr1+ cells cultured with 100 ng/ml LPS for 6 h showed less cell death (p < 0.01; Supplemental Fig. 3B). In addition, after isolated CD11b+Gr1+ cells were stimulated with LPS, significantly more CXCL1 and CXCL2, which are important for neutrophil migration, were expressed by Smad3−/− CD11b+Gr1+ cells compared with WT CD11b+Gr1+ cells (Supplemental Fig. 4). Thus, Smad3-deficient neutrophils are hypersensitive to chemokine attraction and somewhat resistant to cell death but are deficient in subsequent recruitment of other immune cells, including macrophages and T cells.

Smad3-deficient CD11b+Gr1+ myeloid cells prevent allograft rejection

After observing increased CD11b+Ly6G+ myeloid cells in skin grafts, we monitored the presence of CD11b+Gr1+ myeloid cells in the spleen, DLN, and bone marrow in mice at certain time points post skin transplantation. We found that the levels of CD11b+Gr1+ myeloid cells were slightly higher in naive Smad3−/− mice compared with WT mice (day 0; Fig. 5A). More importantly, the percentage of CD11b+Gr1+ cells increased dramatically, up to 2-fold or more, at day 7 in the spleen and DLNs of Smad3−/− mice. This increase was significantly higher than WT mice (p < 0.001; Fig. 5A, 5B). Surprisingly, splenic CD11b+Gr1+ cells of skin-grafted Smad3−/− mice expressed high levels of CXCR2, CD62L, and CD115 molecules (Fig. 5C), which are correlated with the phenotype of MDSCs (reviewed in Refs. 40 and 6). CD11b+ Gr1+ MDSCs are known to consist of two major subsets: granulocytic CD11b+Ly6Cmed and monocytic CD11b+Ly6Chigh cells (40). We therefore assayed these two subsets in graft-bearing WT and Smad3-deficient mice using anti-Ly6C and anti-CD11b mAbs. The proportion of CD11b+Ly6Cmed and CD11b+Ly6Chigh cells was substantially higher in spleens of Smad3-deficient recipients compared with WT recipients (p < 0.001; Fig. 5D), indicating that granulocytic and monocytes increase equally in Smad3-deficient recipients. We then examined whether these induced CD11b+Gr1+ cells in skin-transplanted Smad3−/− mice act as immune suppressors, as described for MDSCs (reviewed in Ref. 6). CD11b+Gr1+ cells isolated from spleens of skin-grafted Smad3−/− mice significantly inhibited CD4+ T cell proliferation in MLR assays (p < 0.001; Fig. 5E). However, CD11b+Gr1+ cells from bone marrow of Smad3−/− mice and from bone marrow or spleens of WT mice did not show any detectable immunosuppressive activity (Fig. 5E). To determine whether the CD11b+Gr1+ cells play a protective role in allo-skin graft rejection in Smad3−/− recipients, we depleted these cells by injecting anti-Gr1 mAb RB6-8C5 twice before and after skin grafting, as described previously (41). Efficient depletion of CD11b+Gr1+ cells was achieved in these mice as evidenced by FCM (data not shown). Depletion of Gr1+ cells in WT recipients did not significantly change alloskin graft survival time (Fig. 5F). However, depleting Gr1+ cells in Smad3−/− recipients clearly reversed the prolonged allograft survival time (MST in Smad3−/− recipients: 17 d versus MST in Gr1-depleted Smad3−/− recipients: 13 d, p < 0.001, n = 5; Fig. 5F). Importantly, adoptive transfer of the sorted CD11b+Gr1+ cells from skin-grafted Smad3−/− mice into syngeneic WT recipients significantly pro-

**FIGURE 5.** CD11b+Gr1+ cells in skin-grafted Smad3−/− mice are capable of immunosuppression in vitro and in vivo. (A) Significantly more CD11b+Gr1+ cells were found in spleens of skin-grafted Smad3−/− mice. (B) Significantly more CD11b+Gr1+ cells were found in DLNs of skin-grafted Smad3−/− mice. (C) CXCR2, CD62L, CD44, and CD115 expression in WT and Smad3−/− CD11b+Gr1+ cells after skin grafting. (D) Significantly higher percentage of CD11b+Ly6Cmed cells and CD11b+Ly6Chigh cells in spleens of skin-grafted Smad3−/− mice. (E) Isolated CD11b+Gr1+ cells from skin-grafted Smad3−/− mice significantly inhibited T effector cell response to allogeneic stimulator cells in vitro compared with WT cells. Assays were performed as described in Materials and Methods. The white and black bars refer to CD11b+Gr1+ cells isolated from WT and Smad3−/− mice, respectively. Data are presented as mean ± SD (n = 5). (F) Depleting Gr1+ cells in Smad3−/− mice with mAb (RB6-8C5) significantly accelerated alloskin graft rejection. Five mice were assayed in each group. (G) Adoptive transfer of CD11b+Gr1+ cells from Smad3−/− mice significantly delayed alloskin graft rejection in syngeneic mice. A total of 1 × 106 CD11b+ Gr1+ cells isolated either from Smad3−/− mice or from WT mice were transferred into syngeneic B6 mice via i.v. injection on day −1. Alloskin grafts were transplanted on day 0, and graft survival was followed daily. Five mice were studied in each group. *p < 0.05, **p < 0.01, ***p < 0.001 for comparisons between indicated groups.
longed the time to alloskin graft rejection (p < 0.01; Fig. 5G), whereas CD11b<sup>+</sup>Gr1<sup>+</sup> cells from WT mice failed to do so. Thus, these data support the conclusion that induced CD11b<sup>+</sup>Gr1<sup>+</sup> cells in transplanted Smad3<sup>−/−</sup> mice have an immunosuppressive ability to prevent aloagraft rejection.

The immunosuppressive ability of Smad3-deficient CD11b<sup>+</sup>Gr1<sup>+</sup> myeloid cells is mediated mainly by NO

A previous report indicated that under immune stress CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs accumulate in lymphoid organs where they inhibit T and B cell function via upregulation of NO production (42). We thus measured the NO levels in sera 7 d after skin transplantation and found that the average NO level was greatly higher in Smad3<sup>−/−</sup> recipients compared with WT mice (12.7 ± 1.1 μM in Smad3<sup>−/−</sup> mice versus 3.4 ± 0.5 μM in WT mice, p < 0.001; Fig. 6A). Consistently, the iNOS protein expression was also significantly higher in Smad3-deficient CD11b<sup>+</sup>Gr1<sup>+</sup> cells compared with WT recipient cells, as detected by intracellular FCM assays (p < 0.001; Fig. 6B). Furthermore, the iNOS mRNA level in the spleens of Smad3<sup>−/−</sup> recipients was significantly higher to levels almost four times greater than those in WT recipients, whereas arginase mRNA expression was lower, as detected by real-time PCR (p < 0.001; Fig. 6C). To determine whether NO production is essential for Smad3-deficient CD11b<sup>+</sup>Gr1<sup>+</sup> immunosuppressive function, we blocked NO production by adding a specific iNOS inhibitor, l-NMMA (5 mM was used, consistent with a previous report; Ref. 43), into the in vitro function assay system, as described in Materials and Methods. l-NMMA significantly reduced NO production almost to the baseline (Fig. 6D) and efficiently blocked the immunosuppressive effects of Smad3-deficient CD11b<sup>+</sup>Gr1<sup>+</sup> granulocytes on T cell response to alloantigens in MLR assays (p < 0.001; Fig. 6E).

NO is produced in a reaction catalyzed by iNOS in which arginine is converted into citrulline and NO. To determine whether arginine depletion caused by increased iNOS contributes to the immunosuppressive effect of CD11b<sup>+</sup>Gr1<sup>+</sup> cells on T cell response, we added l-arginine (2 or 5 mM) to the culture system. Arginine supplementation did not significantly reverse the inhibitory effects of Smad3<sup>−/−</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> cells on T cell proliferation (Fig. 6F), indicating that arginine depletion caused by increased iNOS activity in Smad3<sup>−/−</sup>CD11b<sup>+</sup>Gr1<sup>+</sup> cells is unlikely to be the key reason for immunosuppression. Thus, NO is the major mediator by which Smad3-deficient CD11b<sup>+</sup>Gr1<sup>+</sup> MDSCs suppress the immune response.

CD11b<sup>+</sup>Gr1<sup>+</sup> cells are also known to produce TGF-β (44), and TGF-β has the ability to inhibit production of iNOS in many cells (45–47). To determine whether TGF-β inhibits the immunosuppressive function of CD11b<sup>+</sup>Gr1<sup>+</sup> cells by decreasing iNOS production, we isolated CD11b<sup>+</sup>Gr1<sup>+</sup> cells from WT hosts and examined whether they acquired suppressive function after TGF-β...
function was blocked with neutralizing anti–TGF-β mAb in the culture system. TGF-β neutralization did increase NO production in isolated WT CD11b+Gr1+ cells in the culture system (p < 0.05; Fig. 6G), whereas adding exogenous TGF-β (5 ng/ml) inhibited NO production in WT CD11b+Gr1+ cells (p < 0.01; Fig. 6G; data not shown). Interestingly, after blocking TGF-β signaling in a coculture system, WT CD11b+Gr1+ cells displayed significant suppression of T cell proliferation (p < 0.001; Fig. 6H). These data indicate that TGF-β modulates CD11b+Gr1+ cell function.

Seven days after allografting, more CD11b+Gr1+ cells were detected in the bone marrow of Smad3−/− recipients than WT recipients (p < 0.01; Fig. 6I). To access whether Smad3-deficient bone marrow myeloid progenitors are more sensitive to GM-CSF with respect to MDSC differentiation, we cultured the sorted WT and Smad3−/− bone marrow progenitor cells in the presence of GM-CSF. More of the Smad3−/− myeloid progenitor cells differentiated into CD11b+Gr1+ cells and iNOS+CD11b+Gr1+ cells (p < 0.01; Fig. 6J, 6K). These data suggest that Smad3−/− myeloid progenitor cells may be susceptible to MDSC differentiation in the presence of GM-CSF.

Discussion

Our study demonstrates that Smad3 deficiency in hematopoietic cells causes a significantly delayed allograft rejection in mice. The decreased immunity against allografts is due to increased production of immunosuppressive CD11b+Gr1+ MDSCs, which is induced by Smad3 deficiency in transplanted mouse recipients. The Smad3-deficient CD11b+Gr1+ MDSCs significantly inhibit T cell response to alloantigens, mainly through the NO pathway. Thus, we conclude that Smad3 is an intrinsic inhibitor of immunosuppressive CD11b+Gr1+ MDSC differentiation and function in mice transplanted with allografts.

The immunosuppressive cytokine TGF-β controls immune responses and maintains immune homeostasis through its impact on the proliferation, differentiation, and survival of multiple immune cell lineages. For instance, TGF-β markedly and directly suppresses both the clonal expansion of CD8+ T cells and CD8+ T cell cytotoxicity in vivo (48). TGF-β also inhibits Th1 gene expression in CD4+ T cells in a Smad3-dependent manner (49). As another example, TGF-β, coordinating with IL-21, induces Foxp3 expression and generates Tregs (50). TGF-β, together with IL-6, also induces the lineage-specific differentiation of Th17 cells, a newly defined proinflammatory Th cell population (51, 52). Smad3−/− T cells produce abundant Th2-type cytokines (53).

These effects have also been demonstrated clearly by genetic deletion or attenuation of TGF-β signaling (11). Consistent with these results, our studies and others have shown that deletion of Smad3-dependent TGF-β signaling in T cells promotes severe graft-versus-host disease (our unpublished data and Ref. 38). In our investigation, Smad3-deficient CD4+CD25−T cells were resistant to TGF-β–induced differentiation into CD4+CD25+ Tregs in vitro, as expected. We also anticipated that Smad3 deficiency in T cells would intrinsically increase Th1 cell production and decrease CD4+CD25−Treg production, which together should accelerate allograft rejection and not delay it. Contrary to this expectation, we found increased severity of graft-versus-host disease associated with Smad3-deficient T cells and alloskin graft rejection unchanged by adoptive transfer of Smad3-deficient T cells and CD4+ T cells. These findings suggest that the delayed allograft rejection in Smad3-deficient recipients is unlikely due to the direct effects of Smad3 on T cells.

In addition to suppressing T cell function, TGF-β also has a profound impact on myeloid cell function. TGF-β can downregulate macrophage cell function and phenotype in vitro (54–56).

Our data showed that Smad3-deficient macrophages produced more of the inflammatory cytokine TNF-α. Although it is less well studied, TGF-β has also been noted to inhibit neutrophil activity, such as degranulation (57). Recent studies have suggested that blocking the TGF-β pathway increases the recruitment of neutrophils and inhibits other immune cell responses in guinea pigs with experimental tuberculous pleurisy (58). Interestingly, it is reported that blockade of TGF-β by administration of type I TGF-β receptor kinase inhibitor leads to a marked influx of CD11b+Gr1+ neutrophils with hypersegmented nuclei to tumor sites where they exert cytotoxic activity on cancer cells (59). Our present results in transplant models are somewhat consistent with these reported observations in chronic infection and cancer models. We detected significantly increased CD11b+Gr1+ granulocytes and decreased monocytes/macrophages and T cells in allografts in Smad3-deficient recipients. The inhibitory effect of Smad3 on neutrophil migration into the “alarm” location is likely cell intrinsic, as supported by the following evidence: 1) alloskin grafts were rejected slowly in WT mice constituted with Smad3-deficient hematopoietic cells; 2) TGF-induced peritonitis recruited more CD11b+Gr1+ cells to the peritoneal cavity in WT mice with Smad3-deficient hematopoietic cells than WT mice with WT hematopoietic cells; and 3) Smad3-deficient alloskin grafts were rejected as efficiently as WT alloskin grafts.

It is also noteworthy that the increased presence of CD11b+Gr1+ granulocytes in allografts and spleens in Smad3-deficient mice was associated with partial defects, such as reduced chemokine production. This result is consistent with a study using an infection model (58). In contrast, another study demonstrated that TGF-β receptor blockade resulted in an increased presence of CD11b+Gr1+ granulocytes in tumors and a hypermature phenotype (59). Our results also correspond with this study. Smad3-deficient CD11b+Gr1+ granulocytes demonstrated MDSC phenotypes, such as a high level of CD62L and CD115 expression. In addition, Smad3-deficient CD11b+Gr1+ granulocytes produced a profound quantity of NO and inhibited the T cell response to alloantigens. Depleting this cell population by administration of mAb accelerated allograft rejection in Smad3-deficient mouse recipients. Thus, our study suggests that the increased presence of Smad3-deficient CD11b+Gr1+ MDSCs is at least partially responsible for the delayed graft rejection in Smad3-deficient mice. Our data also offer evidence that the Smad3 pathway, activated by TGF-β and other factors, is a negative regulator for the differentiation and immunosuppressive function of CD11b+Gr1+ MDSCs in mice.

MDSCs represent a heterogeneous population, including monocytic MDSCs expressing CD11b+Ly6Chigh and granulocytic MDSCs with CD11b+Ly6Clow phenotypes (40). The involvement of MDSCs in transplantation has been demonstrated in various models (60–62). Our results in Smad3-deficient mice, which demonstrated inhibitory effects of the increased CD11b+Gr1+ MDSCs on the immunity against allografts, is consistent with these reports (60–62). These data collectively support the critical role of CD11b+Gr1+ MDSCs in preventing allograft rejection. We also suggest that the NO pathway is responsible for this effect. Smad3-deficient CD11b+Gr1+ MDSCs expressed high levels of iNOS and low levels of arginase-1. Blocking NO pathways markedly reversed the immunosuppressive effect of CD11b+Gr1+ MDSCs on the T cell response, indicating that NO is one of the major pathways by which Smad3-deficient CD11b+Gr1+ MDSCs achieve immune suppression.

To summarize, to our knowledge, we identify for the first time Smad3 as an important intrinsic factor that negatively regulates the maturation and function of the immunosuppressive CD11b+Gr1+ MDSCs. Smad3 functions in parallel with other molecules,
including STAT1, STAT3, STAT5, STAT6, and NF-kB, which positively promote the differentiation of MDSCs (reviewed in Ref. 10). Additional insight into the control of MDSCs by Smad3 will not only illuminate the fundamental molecular principle of MDSCs regulation but also facilitate the clinical application of MDSCs in treating immune disorders.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Decreased activated/memory T cells in spleens of allo-skin-grafted Smad3-/- mice compared to WT recipients.

The percentages and cell numbers of CD62L<sub>low</sub>CD4<sup>+</sup> cells and CD62L<sub>low</sub>CD8<sup>+</sup> cells were detected by FCM 7 days after allo-skin grafting. (A) One representation of cell staining detected by FCM. (B) Cell numbers of CD62L<sub>low</sub>CD4<sup>+</sup> cells and CD62L<sub>low</sub>CD8<sup>+</sup> cells are summarized. Data are shown as mean±SD (N=3). *P<0.05, **P<0.01 compared to the control group.
Supplementary Figure 2. Decreased cell number and activation of T cells and increased percentage and cell number of CD11b*Gr1* cells in dLNs of DO11.10<sup>x</sup>Smad3<sup>±/±</sup> mice after immunization with OVA.

The percentage and cell number of CD4<sup>+</sup>KJ1-26<sup>+</sup>, CD4<sup>+</sup>KJ1-26<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>KJ1-26<sup>+</sup>CD69<sup>+</sup> and CD11b<sup>+</sup>Gr1<sup>+</sup> cells in dLNs were measured 7 days after immunization with OVA. (A) Total cell number of CD4<sup>+</sup>KJ1-26<sup>+</sup> cells and CD62L<sup>low/</sup>CD8<sup>+</sup> cells in dLNs are summarized. (B) One representation of CD25 and CD69 staining of CD4<sup>+</sup>KJ1-26<sup>+</sup> gated cells, as detected by FCM. (C) Total cell number of CD4<sup>+</sup>KJ1-26<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>KJ1-26<sup>+</sup>CD69<sup>+</sup> cells in dLNs 7 days after immunization with OVA. Data are shown as mean±SD (N=3). (D) One representation of CD11b and Gr1 staining of dLN immune cells, as detected by FCM. (E) Percentage of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in dLNs 7 days after immunization with OVA. (F) Total cell number of CD11b<sup>+</sup>Gr1<sup>+</sup> cells in dLNs 7 days after immunization with OVA. Data are shown as mean±SD (N=3). *P<0.05, **P<0.01 compared to the control group.
Supplementary Figure 3. In vitro survival of CD11b^Gr1^ cells in Smad3^-/- mice. (A) Percentage of PI^+Annexin V^+ cells among CD11b^Gr1^ cells isolated from the peritoneal cavity of 3% TG-treated mice. (B) Percentage of PI^+Annexin V^+ cells among CD11b^Gr1^ cells after they were cultured with LPS in vitro. Data are shown as mean±SD (N=3). **P<0.01 compared to the control group.
Supplementary Figure 4. CXCL1 and CXCL2 mRNA expression in CD11b+Gr1+ cells isolated from WT and Smad3–/– mice after in vitro LPS stimulation.

(A) CXCL1 mRNA expression in isolated CD11b*Gr1* cells cultured with LPS in vitro. (B) CXCL2 mRNA expression in isolated CD11b*Gr1* cells cultured with LPS in vitro. Data are shown as mean±SD (N=3). **P<0.01 compared to the WT mice.