Essential Role for Smad3 in Regulating MCP-1 Expression and Vascular Inflammation


Abstract—Transforming growth factor (TGF)-β1 is a pleiotropic growth factor with known inhibitory effects on immune cell activation. However, the specific mechanism(s) and in vivo significance of the effectors of TGF-β1 modulation in the context of vascular inflammation are not well characterized. The chemokine monocyte chemoattractant protein (MCP)-1 is critical for the recruitment of macrophages in inflammatory disease states. In this study, we provide definitive evidence that the ability of TGF-β1 to inhibit MCP-1 expression is mediated via its effector Smad3. Adenoviral overexpression of Smad3 potently repressed inducible expression of endogenous MCP-1. Conversely, TGF-β1 inhibition of cytokine-mediated induction of MCP-1 expression was completely blocked in Smad3-deficient macrophages. Consistent with this impaired response, cardiac allografts in Smad3-deficient mice developed accelerated intimal hyperplasia with increased infiltration of adventitial macrophages expressing MCP-1. Previous studies show that MCP-1 inducibility is regulated by an AP-1 complex composed of c-Jun/c-Fos heterodimers. We demonstrate that the inhibitory effect of Smad3 occurs via a novel antagonistic effect of Smad3 on AP-1 DNA-protein binding and activity. Thus, Smad3 plays an essential role in modulating vascular inflammation characteristic of transplant-associated arteriopathy, is important in regulating MCP-1 expression, and plays a critical role in the ability of TGF-β1 to repress stimuli from a major inflammatory signaling pathway. (Circ Res. 2004;94:601-608.)

Key Words: transforming growth factor-β ■ Smads ■ vascular inflammation ■ AP-1 ■ atherosclerosis

Accumulating clinical and experimental studies suggest that chronic inflammation contributes to the development of atherosclerosis, transplant arteriopathy, and restenosis after mechanical injury. A pathophysiological event common to these vascular pathologies is the recruitment and infiltration of monocytes/macrophages into the blood vessel wall.1–4

MCP-1, a member of the C-C chemokine β subfamily, was originally identified for its potent chemotactic activity toward monocytes.5 Several lines of evidence suggest a critical role for MCP-1 in vascular disease states.6 Recent experimental studies demonstrated a reduction in atherosclerotic lesion formation in mice deficient in MCP-1 or its receptor CCR2 in atherosclerotic-prone mice.7,8 Conversely, macrophage-specific overexpression of MCP-1 resulted in the acceleration of vascular lesion size and infiltration of macrophages in atherosclerosis-prone mice.9 MCP-1 also contributes to the development of restenosis after mechanical balloon injury. In several animal models, blockade of MCP-1 or deficiency of CCR2 decreased neointimal hyperplasia after arterial injury.10–12 Although MCP-1 is an important mediator for the development of native atherosclerosis and restenosis, its role in transplant-associated arteriopathy (TxAA) has not yet been defined. The observation that elevated serum levels of MCP-1 in heart transplant patients are associated with the presence of TxAA suggests that MCP-1 may be an important mediator in this process.13 Furthermore, induction of MCP-1 expression correlated with the development of TxAA in rat cardiac allografts and its expression colocalized with macrophages.14 Thus, identification of mechanisms or signaling pathways to inhibit MCP-1 expression may offer novel strategies to diminish the inflammatory response for a broad range of vascular disease states.

Members of the TGF-β superfamily consist of a large group of secreted polypeptides that play essential roles in diverse cellular processes including development, cell growth and differentiation, extracellular matrix deposition, and immune modulation.15–17 With respect to immune modulation, the prototypic ligand TGF-β1 is a particularly potent regulator of inflammatory responses in a number of cell types within the vascular system.18 The role of TGF-β1 in inflammation is highlighted in mice bearing a targeted deletion for this gene. These mice die perinatally and exhibit a systemic inflammatory reaction characterized by multiorgan infiltration of leukocytes.19–22 Thus, Smad3 plays a critical role in regulating vascular inflammation and its mechanism of action is via repression of MCP-1 expression.
kocytes, increased circulating monocytes, and tissue necrosis.\textsuperscript{19,20} Whereas previous studies support a role for TGF-\(\beta\)-1 in inhibiting the expression of MCP-1 and other macrophage activation markers,\textsuperscript{21–23} the specific mechanism(s) and in vivo significance of the effectors of TGF-\(\beta\)-1 modulation are not well understood.

Cellular signaling through the TGF-\(\beta\) superfamily occurs via intracellular mediators, termed Smads, which translocate to the nucleus where they direct transcriptional responses. Three classes of Smads—pathway-restricted, common, and inhibitory—are responsible for coordinating the downstream signaling effects.\textsuperscript{15,17,24} TGF-\(\beta\)/activin receptors phosphorylate the pathway-restricted Smads, Smad2, and Smad3, whereas bone morphogenic protein receptors activate Smads 1, 5, and 8. On phosphorylation, these pathway-restricted Smads may hetero-oligomerize with Smad4, the only common Smad, and translocate to the nucleus where they may participate in regulating transcriptional events. Smad6 and Smad7, known as inhibitory Smads, are structurally divergent from other Smads and function to block TGF-\(\beta\) signaling by preventing activation of pathway-restricted Smads.\textsuperscript{15,17,24}

The importance of Smad proteins in regulating inflammatory events by TGF-\(\beta\)-1 is highlighted by the phenotype of Smad3-deficient mice.\textsuperscript{25–27} These mice exhibit spontaneously activated T cells, impaired mucosal immunity, and abnormal wound healing; however, the contribution of Smad3 in regulating macrophage activation in these mice and its role in vascular inflammation in vivo remains unknown. In the work reported in this study, we demonstrate by both gain and loss of function experiments that the ability of TGF-\(\beta\)-1 to inhibit LPS-mediated induction of MCP-1 in macrophages is Smad3 dependent and involves inhibitory effects on AP-1 activity and DNA-protein binding. Moreover, hearts transplanted into Smad3\textsuperscript{−/−} mice develop accelerated intimal hyperplasia with increased infiltration of adventitial macrophages that express MCP-1. These data indicate a novel mechanism by which TGF-\(\beta\)-1 via Smad3 can suppress inflammatory responses in the vascular system.

Materials and Methods
An expanded Materials and Methods section can be found in the online data supplement at \textit{http://circres.ahajournals.org}.

Results

TGF-\(\beta\)-1 and Smad3 Can Inhibit Cytokine-Induced Expression of MCP-1

In macrophages, stimuli such as LPS can activate MCP-1 expression by stimulating members of the AP-1 family.\textsuperscript{21,28} To examine whether TGF-\(\beta\)-1 may inhibit inducible expression of MCP-1 mRNA and protein in a macrophage cell line, J774a cells were stimulated with the phorbol ester PMA (100 nmol/L) or LPS (10 ng/mL) in the presence or absence of pretreatment with active TGF-\(\beta\)-1 (10 ng/mL) for 30 minutes. As demonstrated in Figure 1, MCP-1 mRNA and protein are expressed at low levels in unstimulated cells and induced markedly by both LPS and PMA. However, pretreatment with TGF-\(\beta\)-1 potently attenuated this induction (Figures 1A and 1B). In contrast, TGF-\(\beta\)-1 increased PAI-1 expression, a well-established target for TGF-\(\beta\)-1.\textsuperscript{17} Previous work has demonstrated that cytokine activation of MCP-1 occurs primarily at the level of transcription.\textsuperscript{29} Induction of MCP-1 expression is mediated in part through two tandem AP-1 sites and an NF-kB site in the proximal promoter of this gene depending on the cell type and stimulus used.\textsuperscript{29–31} Consistent with these results, transient transfection assays using an MCP-1 promoter construct showed an increase in transcriptional activity induced by LPS that could be attenuated by pretreatment with TGF-\(\beta\)-1, in RAW264.7 cells (Figure 1C, left panel). We also examined whether pre-, co-, or postincubation with TGF-\(\beta\)-1 would have similar effects. We found that TGF-\(\beta\)-1 inhibition of the MCP-1 promoter can occur, but is attenuated, if LPS stimulation is allowed to proceed first for 1 to 6 hours in comparison to co- or preincubation with TGF-\(\beta\)-1 (online Figure 1A, in the online data supplement at \textit{http://circres.ahajournals.org}).

Smad proteins constitute a family of intracellular effectors that mediate TGF-\(\beta\) signal transduction and gene expression.\textsuperscript{17} To assess whether the TGF-\(\beta\)-1 inhibition of MCP-1 gene expression was Smad dependent, we performed cotransfection assays of the MCP-1 promoter construct with several Smad expression constructs followed by stimulation with LPS. As shown in Figure 1C, only Smad3 was able to repress the MCP-1 promoter in a manner similar to TGF-\(\beta\)-1, an effect enhanced by treatment with exogenous TGF-\(\beta\)-1. In contrast, the inhibitory Smads, Smad6, and Smad7, transactivated the MCP-1 promoter to levels even higher than that achieved by LPS stimulation. The authenticity of the Smad3 expression construct was verified by its ability to induce the PAI-1 promoter (Figure 1C). Taken together, these data indicate that TGF-\(\beta\)-1 inhibits inducible expression of MCP-1, an effect that may be mediated via Smad3.

Adenoviral Overexpression of Smad3 Inhibits MCP-1 Expression and AP-1 DNA-Protein Binding

To address whether constitutive overexpression of Smad3 can inhibit inducible expression of endogenous MCP-1, we adenovirally overexpressed Ad-Smad3 or Ad-CMV-\(\beta\)-galactosidase (\(\beta\)-gal) in J774a cells followed by stimulation with PMA or LPS. As demonstrated in Figure 2A, whereas TGF-\(\beta\)-1 alone can inhibit PMA- or LPS-induced MCP-1 mRNA, constitutive overexpression of Smad3 markedly augmented this inhibitory effect. As a positive control of TGF-\(\beta\)/Smad responsiveness, we assessed PAI-1 expression under the same conditions. In contrast to the inhibitory effect on MCP-1, overexpression of Smad3 in the presence of TGF-\(\beta\)-1 induced PAI-1 mRNA (Figure 2A).

In theory, the inhibitory effect of Smad3 may occur by direct DNA binding or through interaction with coactivators (eg, p300/CBP), corepressors (eg, TGIF, SnoN, and c-Ski), or sequence-specific transcription factors (eg, AP-1, TFE3, Sp1, Evi-1, and GATA-3).\textsuperscript{17,32} Examination of the \(-486\) bp MCP-1 promoter failed to reveal the presence of a consensus Smad3 binding site. Because transcriptional activation of the MCP-1 promoter is critically regulated by the proximal AP-1 sites,\textsuperscript{29–31} we assessed whether Smad3 can affect AP-1 DNA-protein binding in J774a cells. As demonstrated in Figure 2B, a dominant gel-shift complex is induced after
treatment with PMA and competed away by a wild-type, but not a mutated, competitor oligonucleotide. In addition, supershift studies demonstrate that c-Jun, JunB, and c-Fos are the major AP-1 proteins present. To address whether constitutive overexpression of Smad3 can alter AP-1 DNA–protein binding, we infected J774a cells with adenoviral constructs assayed with the indicated unlabeled wild-type (WT) or mutated (Mut)-competitor probes at 50-molar excess (left) or the indicated AP-1 antibodies (right). The complex representing AP-1 DNA–protein binding is inhibited in the presence of TGF-β1. Ad-Smad3 (middle). For supershift studies (right), the AP-1 radiolabeled probe has been run off the gel.

Figure 1. TGF-β1 can inhibit inducible expression and transcription of MCP-1. A, TGF-β1 inhibits LPS- or PMA-induced MCP-1 mRNA expression in J774a cells. J774a cells were treated with LPS (10 ng/mL) or PMA (100 nmol/L) for 20 hours in the presence or absence of TGF-β1 (10 ng/mL) pretreatment for 30 minutes. Northern blot analysis was performed with 10 μg of total RNA per lane. Blots were hybridized with MCP-1, PAI-1, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading reference. B, TGF-β1 blocks secreted MCP-1 protein. Equal aliquots of conditioned medium were obtained from J774a cells grown under the same conditions as in A and subjected to ELISA. C, TGF-β1 and Smad3 can synergistically inhibit transcription of the MCP-1 promoter. Reporter luciferase constructs of the MCP-1 promoter (−486/+6) and the PAI-1 promoter (−884/+71) were transiently transfected into RAW264.7 cells. After transfection, cells were treated with LPS (10 ng/mL) for 20 hours with or without pretreatment with TGF-β1 (10 ng/mL, 30 minutes). *P < 0.001 vs control; **P < 0.001 vs LPS or PMA; #P < 0.01 vs LPS.

Figure 2. Ectopic expression of Smad3 suppresses LPS- or PMA-induced MCP-1 expression. A, Adenoviral overexpression of Smad3 blocks LPS- or PMA-induced MCP-1 expression while increasing PAI-1 expression. J774a cells were transduced with adenovirus (Ad-βgal or Ad-Smad3) at 100 MOI for 16 hours and treated with PMA (100 nmol/L), LPS (10 ng/mL), and/or 30 minutes of pretreatment with TGF-β1 (10 ng/mL) as indicated. Total RNA was isolated after 20 hours and subjected to Northern analysis. Exogenous (exo) Smad3 mRNA is also shown. B, Ectopic expression of Smad3 blocks PMA-induced AP-1 DNA–protein binding. J774a cells were transduced with adenovirus and treated with PMA (100 nmol/L) or TGF-β1 (10 ng/mL) as described in A. Nuclear extracts were incubated with the 32P-labeled oligonucleotide probe containing the AP-1 site (−122 to −116) of the MCP-1 promoter for mobility gel shift assay. Binding specificities of the DNA complexes were assayed with the indicated unlabeled wild-type (WT) or mutated (Mut)-competitor probes at 50-molar excess (left) or the indicated AP-1 antibodies (right). The complex representing AP-1 DNA–protein binding is inhibited in the presence of TGF-β1. Ad-Smad3 (middle). For supershift studies (right), the AP-1 radiolabeled probe has been run off the gel.
NF-κB binding in J774a cells and raises the possibility that TGF-β mediates gene repression via AP-1 are not well characterized. Although this report suggested a synergistic cooperativity of AP-1 and Smad3 in gene activation, the mechanisms for Smad3-mediated gene repression via AP-1 are not well characterized. Because the regulation of AP-1 transcription factors by TGF-β may vary with the specific family member, we first examined the effect of TGF-β pretreatment on the protein expression levels of c-Jun and c-Fos after stimulating with PMA. As demonstrated in Figure 3A, TGF-β inhibited PMA-induced expression of c-Fos; however, there was no inhibitory effect on c-Jun expression after 6 hours of stimulation (Figure 3A). Although decreased c-Fos expression will prevent heterodimerization, c-Jun homodimerization and AP-1 DNA-binding should still occur. Indeed, reporter gene transfection experiments using c-Jun alone demonstrated transactivation of both the −486 bp MCP-1 promoter construct and an AP-1 concatamer (data not shown). Thus, our observation of decreased AP-1 DNA-binding in the presence of TGF-β, and Smad3 overexpression (Figure 2B) suggests that in response to TGF-β, Smad3 may also interact with c-Jun to prevent its DNA-binding.

To verify that Smad3 functionally interacts with c-Jun, we performed coimmunoprecipitation studies in 293T cells. An aFlag antibody immunoprecipitated c-Jun protein from lysates of cells transfected with Flag-Smad3, c-Jun, and a constitutively active TGF-β type I receptor (TGFβRI), but not with Flag-Smad3+empty vector or c-Jun+empty vector (Figure 3B, left panel). Recent characterization of the Smad3/c-Jun interaction using GST adsorption assays also showed that the N-terminal domain of Smad3 and the basic DNA binding domain and leucine zipper (bZIP) of c-Jun mediate this physical interaction.34 Furthermore, substitution of four critical lysines residues with alanines (at positions 40, 41, 43, and 44) within the N-terminal domain of Smad3 significantly blocked this interaction.34 To assess the degree to which the inhibitory effect observed of Ad-Smad3 plus TGF-β on NF-κB DNA-protein binding (online Figure 1E). Taken together, these data indicate that Smad3 may act as a transcriptional repressor by preventing AP-1 DNA-protein binding in J774a cells and raises the possibility that TGF-β mediates gene repression via AP-1 are not well characterized.

**Figure 3.** TGF-β inhibition of MCP-1 is mediated through a Smad3/c-Jun interaction. A, TGF-β inhibits PMA-induced expression of c-Fos but not that of c-Jun. J774a cells were treated with either vehicle control or PMA (100 nmol/L) in the presence or absence of TGF-β, pretreatment (30 minutes, 10 ng/mL). Whole-cell extracts were harvested at 6 hours after stimulation for immunoblots for c-Jun and c-Fos protein levels. Blots were hybridized with an α-tubulin antibody as a loading reference. B, Smad3 associates with c-Jun, 293T cells were transfected with the indicated expression plasmids. Whole-cell lysates were immunoprecipitated (IP) with anti-Flag antibody, followed by immunoblotting (IB) with either anti-HA antibody to detect Smad3-bound c-Jun or with an anti-HA antibody to verify equal Smad3 expression. C, Smad3(4A) weakly associates with c-Jun, 293T cells were transfected with the indicated expression plasmids. **Ad-Smad3 or Ad-CMV-β-gal (ctrl).** As shown in Figure 2B, although TGF-β alone can inhibit PMA-induced AP-1 DNA-protein binding, constitutive overexpression of Smad3 markedly enhanced this inhibitory effect. In contrast, there was no inhibitory effect observed of Ad-Smad3 plus TGF-β on NF-κB DNA-protein binding (online Figure 1E). Taken together, these data indicate that Smad3 may act as a transcriptional repressor by preventing AP-1 DNA-protein binding in J774a cells and raises the possibility that TGF-β, via Smad3 may regulate the macrophage response to inflammatory stimuli.

**TGF-β Inhibition of MCP-1 Expression Is Mediated by a Smad3-c-Jun Interaction**

The activation of AP-1 by inflammatory stimuli involves the participation of various members of the AP-1 family of bZIP transcription factors and results in their binding to specific DNA sequences.33 These proteins may dimerize with other family members to form transcriptionally active AP-1 complexes. Whereas the Jun family members (c-Jun, JunB, and JunD) can both homo- and heterodimerize with Fos members (c-Fos, FosB, Fra-1, and Fra-2), the Fos family members can only heterodimerize with Jun members.33 AP-1 proteins may also interact with other transcriptional regulators to modulate different signaling pathways. Indeed, a recent investigation verified that AP-1 proteins, c-Jun and c-Fos, can interact with Smad3 to control transcriptional responses to TGF-β.34 Although this report suggested a synergistic cooperativity of AP-1 and Smad3 in gene activation, the mechanisms for Smad3-mediated gene repression via AP-1 are not well characterized. Because the regulation of AP-1 transcription factors by TGF-β may vary with the specific family member, we first examined the effect of TGF-β pretreatment on the
immunoprecipitated c-Jun from lysates transfected with the wild-type Flag-Smad3 construct. In contrast, this immunoprecipitated band is markedly attenuated from lysates transfected with the Flag-Smad3(4A) construct. These findings are consistent with the hypothesis that Smad3 may inhibit the transactivation of AP-1–dependent promoters through a direct interaction of Smad3 with c-Jun. To examine this, we first performed transient transfection experiments with an AP-1-luciferase construct containing seven concatemerized consensus AP-1 sites and verified that TGF-β1 could inhibit induction of AP-1 by LPS or PMA (online Figure 1B). To assess this effect directly, we cotransfected Smad3, c-Jun, and c-Fos. Increasing molar amounts of Smad3 in the presence or absence of TGF-β1, effectively inhibited c-Jun + c-Fos induction of the AP-1-luciferase construct (Figure 3D). However, when the Smad3(4A) construct is cotransfected in a similar manner, no inhibition was detected (Figure 3E). Similar results were observed using the MCP-1 promoter (Figure 3F). Taken together, these data indicate that in response to TGF-β1, Smad3 mediates its inhibitory effect by interacting with c-Jun and interfering with the ability of c-Jun to transactivate AP-1–bearing promoters.

TGF-β1 Inhibition of MCP-1 Expression and AP-1 DNA-Protein Binding Are Blocked in Smad3+/− Macrophages

The studies above support an inhibitory role for Smad3 on MCP-1 expression. To determine whether Smad3 is requisite for TGF-β1-mediated inhibition, we performed loss-of-function studies. Smad3−/− and Smad3+/− bone marrow–derived macrophages were harvested and examined for their responsiveness to LPS in the presence or absence of TGF-β1. As shown in Figure 4A, although TGF-β1 can inhibit LPS-mediated induction of MCP-1 protein in Smad3+/− cells, the inhibitory effect of TGF-β1 is completely blocked in Smad3−/− macrophages. Similarly, although TGF-β1 can inhibit PMA-induced AP-1 DNA-protein binding in Smad3+/− macrophages, there is no inhibitory effect observed in response to TGF-β1 in Smad3−/− macrophages (Figure 4B). These data indicate that Smad3 is required to mediate the inhibitory effect of TGF-β1 on MCP-1 expression and suggest that Smad3 may be critical for regulating inflammatory responses in vivo.

Cardiac Allografts of Smad3−/− Recipient Mice Develop an Accelerated Inflammatory Arteriopathy

Recombinant activated macrophages is critical to the development of transplant arteriopathy, a process characterized by a diffuse and concentric intimal narrowing in blood vessels of the transplanted organ.2 To determine the role of Smad3 in an in vivo model of vascular inflammation, we examined the development of TxAA in hearts transplanted into Smad3−/− or Smad3+/− mice. In light of our observation that Smad3 inhibits MCP-1, we hypothesized that Smad3 deficiency would accelerate TxAA. We transplanted bm12 (major histocompatibility complex (MHC) class II–mismatched) donor hearts heterotopically into C57BL/6 (B6, H-2b) Smad3−/− or Smad3+/− recipients without immunosuppression. Grafts were harvested at 6 weeks after transplantation as we typically detect well-developed neointimal expansion of the coronary arteries after about 8 weeks.35 As shown in Figure 4C, coronary arteries of allografts from Smad3−/− recipient mice exhibit minimal neointima formation at this 6-week time point; however, there is accelerated intimal hyperplasia within allografts from the Smad3−/− mice. Furthermore, there is marked induction of MCP-1 expression in the Smad3−/− recipient allografts in comparison to the Smad3+/− recipient allografts. Indeed, MCP-1 expression colocalized to CD11b-positive macrophages, which accumulated in the adventitia of the coronary arteries of Smad3−/− allografts (Figure 4C). In contrast, there was no difference of low-level immunostaining for CD3+ cells (T lymphocytes) within allografts from either Smad3−/− or Smad3+/− mice (data not shown). In addition, although low-level immunostaining for smooth muscle α-actin (vascular smooth muscle cells) was observed within the neointima of allografts from Smad3−/− mice, there was no immunostaining detected within the minimal neointima of Smad3+/− mice (data not shown). Finally, to rule out the possibility that the accelerated arteriopathy and enhanced MCP-1 expression observed in Smad3−/− mice was not due to a decrease in TGF-β1 expression, we immunostained for TGF-β1. We found that by comparison to Smad3+/−, TGF-β1 expression of Smad3−/− donor hearts is not decreased and is present in both the perivascular and neointimal areas (online Figure 2). Taken together, these findings suggest that Smad3 is critical in modulating the transplant-associated inflammatory process and in regulating the expression of MCP-1 in vivo.

Discussion

The chemokine MCP-1 is critical for the recruitment of macrophages in a variety of inflammatory vascular disease states. As such, identification of mechanisms limiting MCP-1 expression may offer novel strategies to diminish the inflammatory response. One of the most important properties of TGF-β1 is its ability to modulate inflammatory stimuli. Our previous observations demonstrate that TGF-β1 inhibits cytokine-mediated induction of proinflammatory gene expression in macrophages.22,23 However, the mechanism by which TGF-β1 is able to mediate this function and in vivo significance of such effects are not well understood. In this report, we describe a definitive role for the TGF-β downstream effector Smad3 in suppressing inflammatory events within the vascular system in vivo and in macrophages ex vivo in response to TGF-β1. In support, we found that of all the Smads examined, only Smad3 could inhibit induction of MCP-1 transcriptional activity in an analogous manner as TGF-β1 (Figure 1C). In addition, the inhibitory effect of Smad3 was enhanced with the addition of exogenous TGF-β1. Furthermore, adenoviral overexpression of Smad3 significantly repressed inducible expression of endogenous MCP-1 (Figure 2A). Conversely, TGF-β1 inhibition of LPS-mediated induction of MCP-1 expression was completely blocked in Smad3-deficient macrophages (Figure 4A). Consistent with this impaired response, we found abundantly more perivascular CD11b-positive macrophages within allografts from Smad3−/− mice than Smad3+/− mice (Figure 4C). Moreover,
nearly all the MCP-1–positive cells colocalized with these adventitial macrophages (Figure 4C). Perivascular inflammation is an important hallmark in the development of transplant arteriopathy by contributing to adventitial scarring and arterial lumen narrowing. Collectively, these findings are consistent with lesions found early in the development of transplant arteriopathy, typified by a preponderance of inflammatory cells rather than smooth muscle cells. Thus, expression of chemokines such as MCP-1 by adventitial macrophages may allow for potentiation of macrophage infiltration resulting in an accelerated inflammatory arteriopathy as found in the allografts from Smad3−/− mice.

Previous studies highlight the critical role of AP-1 proteins in the induction of MCP-1. Our studies demonstrate by both gain- and loss-of-function strategies that the TGF-β1 inhibition of cytokine-induced AP-1 DNA binding is Smad3 dependent (Figures 2 and 3). Our results also suggest that in response to TGF-β1, the inhibitory effect of Smad3 on AP-1 is mediated through at least two potential mechanisms: by decreasing c-Fos expression and by a direct interaction with c-Jun (Figure 4). The net result of these effects is a reduction in AP-1 DNA binding and transcriptional activity. Although we have demonstrated an inhibitory effect of TGF-β1 and Smad3 on the induction of MCP-1, two recent studies suggest opposite effects. We believe the discrepancy of their observed effects is due to either the failure to coincubate cells with TGF-β1 for the total time that the stimulus was used or differences in cell-type examined (e.g., astrocytic cells).
Our data do not exclude the possibility of additional mechanisms by which Smad3 may inhibit AP-1 function. In light of our observations that JunB is also present in the AP-1 complex (Figure 2B), we cannot exclude the possibility that JunB may also participate in the inhibitory effect of Smad3 on AP-1. Indeed, Smad3 has also been found to interact with JunB in vitro.39 Another possible inhibitory mechanism is coactivator competition. For example, Smad3 can interact with p300, a coactivator that is required for optimal AP-1 mediated transactivation.40 However, because we have previously demonstrated that overexpression of p300 could only partially rescue the inhibitory effects of TGF-β1, alternative inhibitory mechanisms are likely to be involved.23 Thus, our current observations and previously published data support dual inhibitory effects of Smad3 on AP-1 dependent promoters through the direct effect on AP-1 DNA binding and transcriptional activity as well as an indirect effect that may be conferred by coactivator competition. These inhibitory effects may underlie the more potent repression that may be conferred by coactivator competition. These inhibitory effects of Smad3 on AP-1 dependent promoters (eg, MCP-1 and MMP-12) in comparison to non–AP-1–dependent promoters (eg, iNOS) (Figure 1 and Feinberg et al22 and Werner et al23).

Several experimental studies implicate a salutary effect of TGF-β1 on vascular inflammation. For example, blockade of TGF-β1 ligand or the TGF-β type II receptor accelerates the development of atherosclerotic lesion formation in ApoE−/−-atherosclerotic-prone mice.31,41 In patients, the serum concentration of active TGF-β1 is inversely correlated with the severity of atherosclerotic disease.42 In the context of TxAA, cardiac allografts from recipient mice heterozygous for TGF-β1 displayed a marked increase in TxA4 in comparison to wild-type controls.44 The observations in this study that robust macrophage infiltration and accelerated vascular arteriopathy occur in allografts of Smad3-deficient mice coupled with impairment of TGF-β1 inhibition in Smad3-deficient macrophages indicate that Smad3 is the downstream effector mediating the immunosuppressive effects of TGF-β1.

Taken together, these observations indicate that Smad3 plays an essential role in modulating vascular inflammation by regulating MCP-1 expression. Furthermore, we provide evidence that the Smad3 inhibition occurs through a novel antagonistic effect of Smad3 on the AP-1 signaling pathway in macrophages. To our knowledge, this is the first report to demonstrate an essential role of this Smad protein in vascular inflammation in vivo. Targeting the Smad3 pathway may provide a novel antiinflammatory strategy for transplant arteriopathy and atherosclerosis.

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Materials and Methods

Cell culture and reagents—J774a and RAW264.7 cells (American Type Culture Collection) were grown as described.1 8-10 week old mouse bone marrow-derived monocytes were isolated from extruded bone marrow plugs by density-gradient centrifugation employing Lymphocyte Separation Medium (ICN Biomedicals) and subsequent adherence to cell culture dishes as described.1 Bone-marrow derived monocytes were cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 ug/ml), and M-CSF (50 ng/ml) for 6 days at which time M-CSF was removed from the culture medium and cells were grown in DMEM containing 3% FBS. We obtained LPS (salmonella typhi) and 12-O-tetradecanoylphorbol-13-acetate (PMA) from Sigma and active TGF-β1 from R&D Systems. The MCP-1 and PAI-1 promoter constructs were obtained from A. Garzino-Demo (Univ. Maryland) and D. Vaughan (Vanderbilt), respectively. The Smad3(4A) and c-Jun expression constructs were kindly provided by R. Derynck (Univ. California, San Francisco) and A. Mauviel (Paris), respectively. The c-Fos expression construct was obtained from M. Karin (Univ. California, San Diego).

RNA Extraction and RNA Blot Analysis—Total RNA was isolated from cultured cells by guanidium isothiocyanate extraction and centrifugation through cesium chloride. RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters. The filters were hybridized with 32P-labeled, random-primed cDNA probes, washed, and exposed as described previously. Mouse cDNA probes for PAI-1 and Smad3
were generated as previously described\(^1\) and a mouse MCP-1 cDNA probe was obtained as a gift from B. Rollins (Harvard Medical School).

**Western Analyses, Immunoprecipitation Studies, and MCP-1 Measurements**—Western blot analyses were performed as described.\(^1\) Immunoblots were incubated with the appropriate primary antibodies for c-Jun (sc-45), c-Fos (sc-52), p-Smad3 (sc-11769) (Santa Cruz Biotechnology), \(\alpha\)-tubulin (Sigma), or the epitope tags (Flag or HA) (Sigma) and were detected using HRP secondary antibodies and chemiluminescence. For immunoprecipitation assays, 293T cells were transfected with the indicated expression plasmids and harvested with RIPA buffer 48 hrs later. Lysates were subjected to immunoprecipitation with either 4 ug of aFlag M2 monoclonal antibody (Sigma) or 4 ug of IgG\(_1\) control antibody (Sigma) at 4°C for 2 hrs followed by incubation with protein A/G sepharose overnight at 4°C. The beads were washed and proteins separated by SDS-PAGE as previously described.\(^2\) For MCP-1 ELISAs, release of MCP-1 was measured by sandwich ELISA using the standards and antibodies according to the protocol of the manufacturer (R&D Systems).

**Transient Transfections and Adenoviral Infection**—RAW264.7 cells were transfected with Fugene\(^{\text{TM}}\) 6 Transfection Reagent (Roche Molecular Biochemicals) as described.\(^1\) The total amount of plasmid DNA was kept constant within each experiment. Luciferase actitvity was normalized to \(\beta\)-galactosidase activity (to correct for differences in transfection efficiency) by cotransfecting the pCMV-\(\beta\)gal plasmid or verified by total protein content. All transfections were performed in triplicate from at least three
independent experiments. The expression plasmids for Smad1, 2, 3, 4, 6 and 7 have been described previously\(^1\) and were expressed at comparable levels as verified by immunoblotting (Supplemental Fig. 1C). The AP-1 concatamer construct was obtained from Strategene. For adenoviral infection of J774a cells, replication-deficient human adenovirus vectors expressing mouse Smad3 or LacZ (encoding for β-galactosidase) under the control of the CMV promoter (Ad-Smad3 or Ad-βgal) were used (a gift from K. Miyazono, Tokyo). Cells seeded at 2 x 10\(^6\)/10 cm\(^2\) dish were infected with the adenoviral vectors at 100 MOI (multiplicity of infection) and incubated for 16 hours prior to treatment with PMA, LPS, or TGF-β1 as indicated. Cell transduction efficiencies were tested by in situ X-Gal staining (near 100\%) and expression of Smad3 was verified by Western analysis. Under these conditions, overexpression of Smad3 resulted in a low-level of phosphorylated Smad3 in comparison to Smad3 + TGF-β1 (Supplemental Fig. 1D).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays**—Nuclear extracts from J774a or bone marrow-derived macrophages were prepared and mobility shift analyses were performed as previously described.\(^1\) DNA probes were generated to the AP-1 (TRE) element at position –122 of the human MCP-1 promoter as double-stranded oligonucleotides corresponding to the sequence: ‘5-GCTTGACTCCGCC-3’. Supershift antibodies for c-Jun (sc-45), c-Fos (sc-52), JunB (sc-46), JunD (sc-11375), FosB (sc-48) (Santa Cruz Biotechnology), or IgG\(_1\) (Sigma) were incubated with nuclear extracts for 2 hrs at 4º C prior to adding the radiolabeled oligonucleotide.
*Heterotopic Heart Transplantation and Immunohistochemistry*—Vascularized, heterotopic abdominal transplantation of donor hearts (8-12 weeks old) were performed, monitored, and harvested after 6 weeks as previously described. Ischemic time was typically less than 30 minutes and the viability of the cardiac allografts were assessed by daily abdominal palpation. For immunohistological assessment of heart allografts, Paraffin sections were stained with hematoxylin and eosin (H & E) and immunostained for MCP-1 and macrophages (CD11b) as described. Quantitation of the relative intimal thickness was determined by dividing the area within the internal elastic lamina by that of the lumen. Frozen sections were stained for TGF-β1 (sc-146). Smad3 mice were provided by X-F Wang (Duke Univ.). These mice have all been back-crossed 11 generations to the C57BL/6 (B6) background and were used as recipients (age 8-12 weeks). Donor hearts from bm12 mice (major histocompatibility complex (MHC) class II-mismatched) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the Harvard Medical School animal facilities and experiments conformed to approved animal care protocols.

*Statistics*—Data are expressed as mean±SE. For comparison between two groups, the unpaired Student’s *t* test was used. For multiple comparisons, ANOVA followed by unpaired Student’s *t* test was used. A value of *P* < 0.05 was considered significant.
References


Supplemental Figure Legends

Supplemental Figure 1. A) Time course of TGF-β1 treatment on the MCP-1 promoter. The MCP-1 promoter (–486/+6) was transiently transfected into RAW264.7 cells as described in the “Materials and Methods.” After transfection, cells were treated with LPS (10 ng/ml) for 20 hrs with or without treatment with TGF-β1 (10 ng/ml) at the indicated time points. B) Effect of TGF-β1 on LPS or PMA-induction of the AP-1 concatamer. The AP-1 concatamer was transiently transfected into RAW264.7 cells and treated with LPS (10 ng/mL) or PMA (100 nM) for 20 hrs with or without treatment with TGF-β1 (10 ng/ml, 30 min pretreatment). C) Expression of Smad proteins. The Smad constructs 1-4, 6, 7 were transiently transfected into 293T cells and harvested 48 hours later along with the respective control vectors for immunoblots for Smad expression levels with the indicated antibodies. Blots were hybridized with an α-tubulin antibody as a loading reference. D) Phosphorylation of Smad3 under growing and serum-free conditions. J774A cells were transduced with Ad-βgal (Ctrl) or Ad-Smad3 at 100 MOI and treated in the presence of regular growing medium (DMEM, 10% FBS), serum-free medium, or TGF-β1 as a positive control. Whole-cell lysates were immunoprecipitated (IP) with anti-Flag antibody for Ctrl-Flag or Smad3-Flag, followed by immunobloting (IB) with a phospho-Smad3 antibody to detect phospho-Smad3. E) Ectopic expression of Smad3 does not prevent LPS-induced NF-kB DNA-binding. J774a cells were transduced with adenovirus and treated with LPS (10 ng/mL) or TGF-β1 (10 ng/ml, 30 min pretreatment). Nuclear extracts were incubated with the 32P-labeled oligonucleotide probe containing the NF-kB sites of the MCP-1 (–90 to –80) or iNOS (–85 to –75) promoters for mobility gel
shift assays as indicated. Data (means ± S.E.) were subjected to ANOVA. *, $P < 0.001$ versus LPS; **, $P < 0.001$ versus LPS or PMA.

Supplemental Figure 2. TGF-β1 expression in allografts from Smad3+/+ or Smad3−/− mice. Vascularized heterotopic cardiac transplantations were performed and harvested after 6 weeks as described in the text for immunohistochemistry analyses. Sections were stained with antibodies for TGF-β1 or IgG isotype control as indicated. TGF-β1 staining is present in both the perivascular and neointimal areas in allografts from Smad3−/− mice and is present in the perivascular areas in allografts from Smad3+/+ mice.
Supplemental Figure 1

A

(Basic Luciferase Values)

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B

(Full Luciferase Values)

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C

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D


E


NF-κB (-90 to -80), MCP-1 pro

NF-κB (-85 to -75), iNOS pro