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TLR agonists regulate PDGF-B production and cell proliferation through TGF-β/type I IFN crosstalk

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Transforming growth factor-β (TGF-β) and type I interferon (IFN) autocrine/paracrine loops are recognized as key mediators of signaling cascades that control a variety of cellular functions. Here, we describe a novel mechanism by which Toll-like receptor (TLR) agonists utilize these two autocrine/paracrine loops to differentially regulate the induction of *PDGF-B*, a growth factor implicated in a number of diseases ranging from tumor metastasis to glomerulonephritis. We demonstrate that CpG-specific induction of *PDGF-B* requires activation of Smads through TGFβ1 autocrine/paracrine signaling. In contrast, polyinosinic:polycytidylic acid strongly represses CpG's as well as its own intrinsic ability to induce *PDGF-B* mRNA through type I IFN-mediated induction of *Smad7*, a negative regulator of Smad3/4. Furthermore, we have shown that this crosstalk mechanism translates into similar regulation of mesangial cell proliferation. Thus, our results demonstrate the importance of crosstalk between TGF-β and type I IFNs in determining the specificity of TLR-mediated gene induction.

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Introduction

Recognition of bacterial DNA is a mechanism that the immune system utilizes to respond to bacterial infections. Hypomethylated CpG oligonucleotide-motif DNA (CpG), found commonly in bacterial genomes, is recognized by Toll-like receptor 9 (TLR9) expressed by a number of different immune cell types (Du *et al*, 2000; Hemmi *et al*, 2000; Takeshita *et al*, 2001). Upon uptake of CpG by the cell into endosomal compartments, TLR9 binds to CpG and becomes activated and signals a potent inflammatory, antibacterial response (Takeshita *et al*, 2001).

TLR9 is one of 11 TLR family members that are the first line of defense for the immune system as reviewed by Modlin and Cheng. Each TLR recognizes a different set of pathogen-associated molecular patterns (PAMPs) (Akira *et al*, 2001), such as LPS, unmethylated CpG DNA and dsRNA, which are conserved aspects of bacteria, viruses and other microbes. While all TLRs contain TIR domains and signal through similar adaptor proteins, they each elicit a variety of highly specific gene programs that are best suited for fighting the many different pathogens recognized by each TLR (Poltorak *et al*, 1998a, b; Rock *et al*, 1998). TLRs 3 and 9 are both endosomal TLRs that recognize nucleic acids (Alexopoulou *et al*, 2001; Ahmad-Nejad *et al*, 2002; Matsushima *et al*, 2004); however, they activate different signaling cascades and induce different genes and functional outcomes.

We have previously reported that the TLR3 and TLR4 agonists, polyinosinic:polycytidylic acid (polyI:C) and LPS, induce an antiviral gene program in macrophages through the combinatorial activation of NF-κB and IRF3 (Doyle *et al*, 2002). Through activation of these transcription factors, polyI:C and LPS induce type I interferons (IFNs) that signal through type I IFN receptor (IFNAR) activation of the JAK/STAT pathway to initiate an antiviral gene program, characterized by apoptosis and growth inhibition. PolyI:C induces type I IFNs through TRIF/TRAF6 activation of NF-κB and TRIF/TBK1 activation of IRF3 (Fitzgerald *et al*, 2003a; Jiang *et al*, 2004; Perry *et al*, 2004). LPS induces type I IFNs through TRIF/TRAM heterodimers that activate IRF3 through TBK1 and NF-κB through MyD88/IRAK4/TRAF6 (Cao *et al*, 1996; Wesche *et al*, 1997; Fitzgerald *et al*, 2003b). Other TLR agonist, such as CpG and PGN, fail to induce this antiviral gene program in macrophages.

While TLR3- and TLR4-specific induction of antiviral gene programming arises from combinatorial control of NF-κB and IRF3, how TLR9 induces inflammatory and proliferative genes remains unknown. Even though TLR9 and TLR4 share the signaling pathway of MyD88/IRAK4/TRAF6 to activate NF-κB and a variety of other transcription factors and protein kinases, TLR9 alone specifically induces strong expression of genes such as *MARCO* (Doyle *et al*, 2004). Microarray data from our laboratory indicate that there are a number of genes that are solely induced by CpG and not polyI:C or LPS in macrophages. How various TLR agonists' regulation of NF-κB and other transcription factors affects CpG-specific induction of these genes and the resulting phenotype is important, as many of these genes are associated with diseases.

Here, we describe a novel crosstalk mechanism by which TLR9 and TLR3 agonists differentially regulate gene induction through combinatorial control of NF-κB and Smads. Analyzing *PDGF-B*, a proliferation/migration-inducing

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growth factor implicated in a number of diseases such as tumor metastasis and glomerulonephritis (Floege *et al*, 1993; Cao *et al*, 2004), we show that, while both TLR9 and TLR3 agonists activate NF-κB, which is necessary for *PDGF-B* induction, only CpG is able to activate Smad3/4 through the TGF-β autocrine/paracrine loop. PolyI:C induction of a type I IFN autocrine/paracrine loop prevents polyI:C's intrinsic ability to induce *PDGF-B* as well as actively blocks CpG induction of *PDGF-B* through type I IFN/STAT1 induction of the TGF-β/Smad3/4 inhibitor *Smad7*. Thus, this work demonstrates how two seemingly unrelated autocrine/paracrine pathways crosstalk to determine a novel CpG-mediated proliferative signal that requires both NF-κB and TGF-β-activated Smads.

Results

CpG activation specifically upregulates PDFG-B in a MyD88/IRAK4-dependent manner

In our efforts to search for genes uniquely induced by individual TLR agonists, we have identified by microarray analysis *PDGF-B* as a gene that was specifically induced by CpG stimulations, but not by polyI:C or lipid A, in bone marrow-derived macrophages (BMMs) (Figure 1A). Since both CpG and *PDGF-B* are major mediators of inflammatory phenotypes but their connection has not yet been established, we decided to focus on the molecular mechanisms and specificity of TLR-mediated upregulation of *PDGF-B*. We first confirmed CpG-induced upregulation of *PDGF-B* mRNA in BMMs by quantitative real-time PCR (Q-PCR) analysis. As shown in Figure 1B, CpG specifically induced *PDGF-B* but not *IFN*-β mRNA, whereas polyI:C upregulated *IFN*-β but repressed basal *PDGF-B* mRNA levels. PDGF-BB protein production was also analyzed by Western blot using whole-cell lysates collected from BMMs stimulated by CpG, which potently induced PDGF-BB protein production (Figure 1C).

To further elucidate how CpG-specific gene induction occurs, *PDGF-B* induction by CpG was analyzed in BMMs deficient in known TLR9 signaling proteins MyD88 and IRAK4. Induction of *PDGF-B* by CpG was compared to polyI:C-specific induction of IFN-β and its dependent genes. As shown in Figure 1D, CpG-induced upregulation of *PDGF-B* was abolished in BMMs derived from either MyD88- or IRAK4-deficient mice, while polyI:C induction of the type I IFN-dependent gene *Mx1* was not affected in these cells. Thus, CpG-specific induction of *PDGF-B* is MyD88–IRAK4 dependent.

Smad4 is recruited to the PDGF-B promoter in response to CpG and not polyI:C

In order to determine the mechanisms responsible for CpG-specific induction of *PDGF-B*, the promoter regions of *PDGF-B* and the TLR3/4-specific gene *IFN*-β were analyzed based on previously reported data (Figure 2A) (Khachigian *et al*, 1994, 1995; Kujoth *et al*, 1998; Maniatis *et al*, 1998; Rafty and Khachigian, 1998; Taylor and Khachigian, 2000). While both genes share NF-κB binding sites, *PDGF-B*'s promoter region contains binding sites not found in *IFN*-β. One such binding site, Smad3/4, is not directly associated with TLR signaling but has been shown to be required for TGFβ1 induction of *PDGF-B* (Taylor and Khachigian, 2000). In order to determine the role of NF-κB and Smads in CpG-induced *PDGF-B* mRNA, we analyzed binding of p65 and Smad4 in the promoter region of *PDGF-B*.

Chromatin immunoprecipitation (ChIP) analysis after stimulation of BMMs by CpG or polyI:C was carried out to analyze Smad4 and p65 recruitment to the *PDGF-B* promoter *in vivo*. As shown in Figure 2B and C, CpG recruits both Smad4 and p65 to the endogenous *PDGF-B* promoter. In contrast, polyI:C only recruits p65 but not Smad4 to the promoter regions of both *IFN*-β and *PDGF-B*. Differential binding of Smad4 and p65 suggests that NF-κB is involved

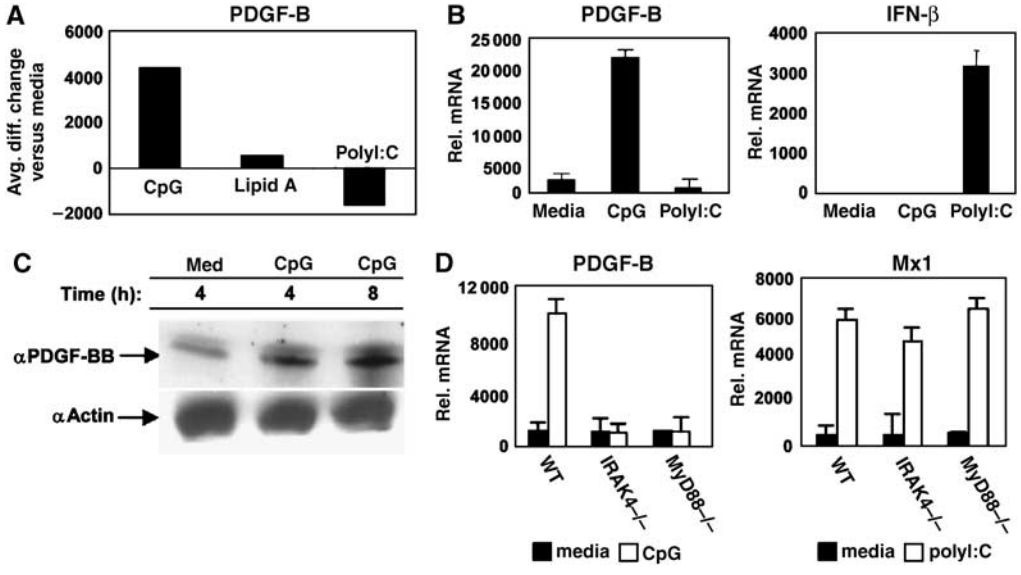

Figure 1 CpG specifically upregulates *PDGF-B* mRNA in a MyD88-IRAK4-dependent manner. (**A**) BMMs were stimulated with CpG (100 nM), polyI:C (1 μg/ml) or lipid A (1 ng/ml) for 4 h. Total RNA was collected and subjected to microarray analysis as described in Materials and methods. Values in graph are average difference change versus media. (**B**) BMMs were stimulated with CpG (100 nM) or polyI:C (1 μg/ml) for 4 h. RNA was collected and analyzed by Q-PCR. (**C**) BMMs were stimulated with CpG (100 nM). Whole-cell lysate was collected and analyzed by Western blot with anti-PDGF-BB or anti-actin. (**D**) BMMs (WT, MyD88−/−, IRAK4−/−) were stimulated with CpG (100 nM) or polyI:C (1 μg/ml) for 4 h. RNA was collected and analyzed by Q-PCR.

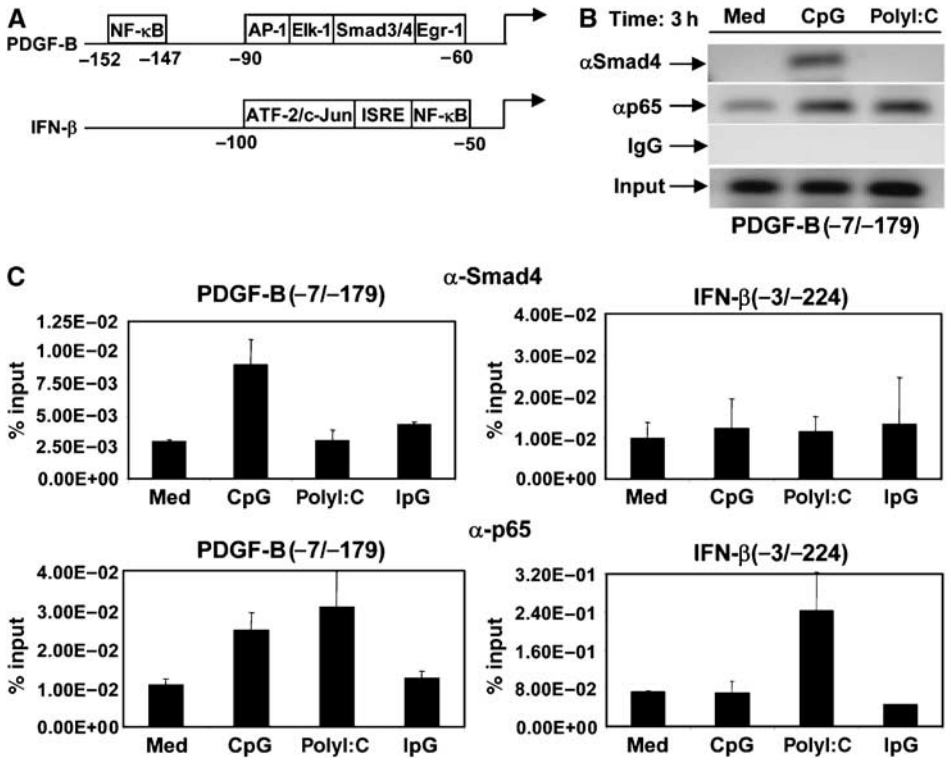

Figure 2 Smad4 is recruited to the *PDGF-B* promoter by CpG but not polyI:C. (**A**) Diagram of *PDGF-B* and *IFN*-β promoters. (**B, C**) BMMs were stimulated with CpG (100 nM) or polyI:C (1 μg/ml) for 3 h. Cells were fixed with formaldehyde. ChIP with anti-Smad4 or anti-p65 was performed on sonicated samples, washed thoroughly and analyzed by PCR/gel electrophoresis and Q-PCR, normalized to input.

but not sufficient for induction of *PDGF-B*, while Smad4 binding stimulated by CpG and not polyI:C suggests a key role for Smads in CpG-specific induction of *PDGF-B*.

CpG-specific induction of PDGF-B is regulated by Smads and NF-κB

In order to further illuminate the function of Smad recruitment to the *PDGF-B* promoter by CpG, wild-type and Smad3-deficient BMMs were stimulated with polyI:C, CpG or TGFβ1. Q-PCR analysis revealed that *PDGF-B* induction by CpG or TGFβ1 was reduced with the loss of Smad3 (Figure 3A, left panel). Furthermore, the loss of Smad3 had no significant effect on polyI:C-specific induction of *IFN*-β in BMMs (Figure 3A, right panel). Thus, maximal induction of *PDGF-B* by CpG or TGFβ1 requires Smad3. The partial defects on *PDGF-B* induction in Smad3-deficient cells might be due to the redundancy of other Smad family members, as Smad2 and Smad4 have also been shown to activate genes through binding at Smad3/4 binding sites (Nakao *et al*, 1997).

In order to determine the role of NF-κB in CpG-mediated induction of *PDGF-B*, a RAW 264.7 macrophage cell line (Raw-IκB-DA) overexpressing a constitutively active form of IκB was used. These Raw-IκB-DA cells were previously shown to be defective in NF-κB signaling (Doyle *et al*, 2002). Raw-IκB-DA cells stimulated with CpG induced only minimal amounts of *PDGF-B* compared to wild-type RAW 264.7 macrophage (Raw-WT) cells (Figure 3B, left panel). Furthermore, comparing polyI:C induction of *IFN*-β in Raw-WT and Raw-IκB-DA cells confirms that polyI:C induction of *IFN*-β shares the requirement of NF-κB for maximal gene induction (Figure 3B, right panel). These data and the data from Figure 2 strongly suggest that binding of NF-κB to the promoter region of *PDGF-B* is required for CpG induction of *PDGF-B*.

Based on these data, CpG induction of *PDGF-B* requires coordinate activation of NF-κB and Smads. Furthermore, while polyI:C-specific and CpG-specific genes in BMMs both require NF-κB for maximal induction, it appears that Smads confer specificity to CpG induction of *PDGF-B*.

CpG upregulates PDGF-B through induction of TGFβ1

Since CpG-specific induction of *PDGF-B* involves Smads, we compared CpG's ability to induce Smad3/4 binding with that of polyI:C and recombinant TGFβ1. As shown in Figure 4A, CpG is a more potent inducer of Smad binding to the Smad3/4 consensus sequence (Santa Cruz) than polyI:C. Upon 3 h of stimulation, only CpG and recombinant TGFβ1 show increased Smad3/4 binding. Furthermore, supershift experiments were performed in RAW 264.7 cells stably expressing Flag-Smad3 to ensure that the shifted complex contained Smads (Figure 4A, right panel). To analyze CpG's and polyI:C's ability to induce Smad3/4 or NF-κB transactivation, we transfected RAW 264.7 cells with a TGF-β-responsive luciferase construct, 3TP-Luc, or an NF-κB luciferase construct, κB-Luc. CpG and TGFβ1 were able to potently activate the 3TP-Luc construct, while polyI:C was unable to do so (Figure 4B, right panel). In contrast, both CpG and polyI:C were able to potently activate κB-Luc (Figure 4B, left panel).

Because current dogma associates Smad activation with TGF-β, we hypothesized that TGFβ1 may be an intermediate factor linking CpG to Smads and *PDGF-B*. We analyzed the possible role of TGFβ1 in CpG induction of *PDGF-B* by using the anti-TGFβ1 neutralizing antibody. As shown in Figure 4C, CpG induction of *PDGF-B* mRNA is inhibited by anti-TGFβ1

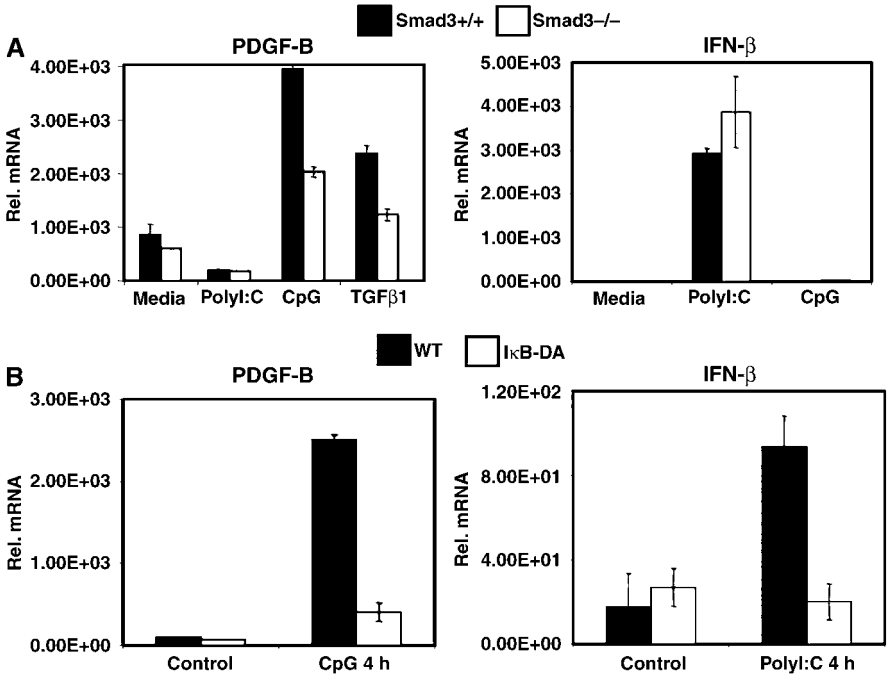

Figure 3 CpG induction of *PDGF-B* mRNA is regulated by Smads and NF-κB. (**A**) BMMs were stimulated with polyI:C (1 μg/ml), CpG (100 nM) or TGFβ1 (2.5 ng/ml) for 4 h. RNA was collected and analyzed by Q-PCR. (**B**) Raw-WT and Raw-IκB-DA cell lines were pretreated with tamoxifen (200 nM) for 2 h and stimulated with CpG (100 nM) or polyI:C (1 μg/ml) for 4 h. RNA was collected and analyzed by Q-PCR.

neutralizing antibody, but not by control IgG. These data show clear evidence for the involvement of TGFβ1 in CpG-mediated induction of *PDGF-B*.

Recent studies indicated that LPS was able to induce TGFβ1 production in macrophages (Sly *et al*, 2004). To compare the ability of CpG and polyI:C to induce TGFβ1, we analyzed the levels of TGFβ1 by ELISA upon stimulation of BMMs with CpG, LPS or polyI:C. While CpG induces more potent Smad activation than polyI:C, all three TLR agonists induce TGFβ1 (Figure 4D). Sequence analysis indicated that there is a strong NF-κB consensus site in the proximal region of the TGFβ1 promoter (data not shown). In order to determine the role that NF-κB may play in CpG-induced TGFβ1 production, we analyzed TGFβ1 production in the Raw-IκB-DA cell line. As can be seen in Figure 4E, CpG induction of TGFβ1 is greatly reduced in the Raw-IκB-DA cell line compared to wild-type RAW 264.7 macrophages. Thus, it appears that TGFβ1 production by CpG is dependent on NF-κB.

PolyI:C inhibits induction of PDGF-B through activation of the type I IFN pathway

The above studies suggest that CpG-specific Smad activation and *PDGF-B* upregulation are not due to differential abilities of CpG and polyI:C to induce TGFβ1 production. To further determine the mechanism responsible for CpG-specific *PDGF-B* upregulation, we costimulated BMMs with CpG and polyI:C and found that polyI:C was not only able to abolish CpG-induced upregulation of *PDGF-B*, but also reduce the basal levels of *PDGF-B* in a dose-dependent manner (Figure 5A). This indicates that polyI:C may trigger an inhibitory signal to actively repress *PDGF-B* mRNA induction.

Since type I IFNs mediate much of TLR3's gene program, we determined the role of type I IFNs on the inhibition of CpG signaling by costimulating BMMs with CpG and recombinant IFN-β. As seen in Figure 5B, similar to polyI:C, IFN-β strongly inhibited CpG-induced *PDGF-B* mRNA upregulation. Furthermore, polyI:C failed to inhibit CpG induction of *PDGF-B* in BMMs derived from IFNAR- or STAT1-deficient mice, suggesting that the JAK/STAT pathway plays an important role in polyI:C-mediated inhibition of *PDGF-B* production (Figure 5C and D). Interestingly, in contrast to wild-type BMMs, polyI:C itself strongly induced *PDGF-B* in IFNAR−/− and STAT1−/− BMMs (Figure 5C and D). These results, together with the data in Figure 4D, suggest that polyI:C has the intrinsic ability to induce *PDGF-B* presumably through a TGFβ1-mediated autocrine/paracrine pathway. However, this ability is repressed by type I IFNs, another autocrine/paracrine pathway induced by polyI:C, but not by CpG. Indeed, treatment of BMMs with TGFβ1 strongly induces the mRNA levels of *PDGF-B* and this TGFβ1-induced *PDGF-B* upregulation is inhibited by polyI:C or IFN-β, indicating the opposing effects of TGFβ1 and IFN-β in *PDGF-B* expression (Figure 5E).

It has previously been reported that CpG can induce type I IFNs in plasmacytoid dendritic cells (pDCs) and bone marrow-derived dendritic cells (BMDCs) (Kadowaki *et al*, 2001; Hemmi *et al*, 2003). Furthermore, it has been shown that compared to conventional CpG used in our experiments (CpG-B), CpG A/D-type (CpG-A) is a more potent inducer of IFN-α production. In BMMs, both forms of CpG did not induce type I IFNs or type I IFN-dependent secondary genes like Mx1 and were unable to induce STAT1 phosphorylation (see Supplementary data). In pDCs, however, both CpGs were unable to induce *PDGF-B* mRNA while various type I IFNs and the type I IFN-dependent secondary gene, Mx1, were induced (see Supplementary data).

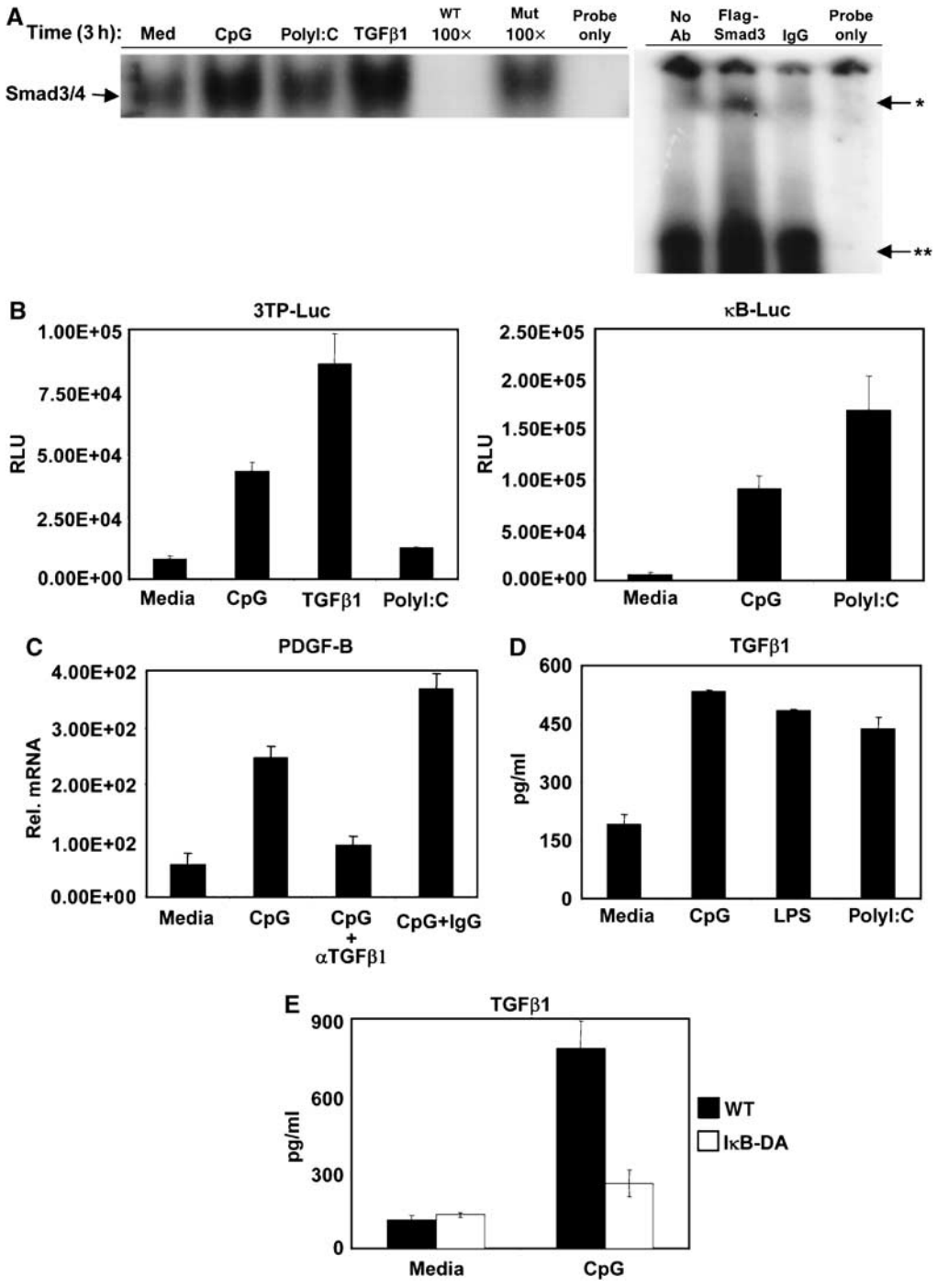

Figure 4 CpG more potently activates Smad3/4 than polyI:C through TGF-β. (**A**) BMMs were stimulated with CpG (100 nM), polyI:C (1 μg/ml) or TGFβ1 (2.5 ng/ml). Nuclear extract was collected and analyzed by EMSA with Smad3/4 consensus probe (Santa Cruz). CpG sample was used for 100 × WT/Mut cold competition. Supershift experiments on samples stimulated with CpG (100 nM) were carried out with anti-Flag (M2) or mouse IgG. (*) Flag-Smad3 supershifted complex; (**) native complex. (**B**) RAW 264.7 cells were transfected with 1 μg 3TP-Luc or κB-Luc and stimulated with CpG (500 nM), TGFβ1 (5 ng/ml) or polyI:C (5 μg/ml) for 24 h. Luciferase activity was measured and normalized to β-galactosidase activity. Graph shown is the average of three independent experiments. (**C**) BMMs were stimulated with CpG and anti-TGF-β neutralizing antibody or control IgG (2 μg/ml) for 4 h. RNA was collected and analyzed by Q-PCR. (**D**) BMMs were serum starved for 6 h and then stimulated with CpG (500 nM), LPS (10 ng/ml) or polyI:C (1 μg/ml) in serum-free media for 24 h. The supernatant was analyzed by ELISA for TGFβ1. (**E**) Raw-WT or Raw-IκB-DA cells were serum starved overnight and stimulated with CpG (100 nM) in serum-free media for 24 h. The supernatant was analyzed by ELISA for TGFβ1.

PolyI:C repression of CpG-mediated Smad3/4 activation and PDGF-B mRNA induction occurs through Smad7

Since both CpG and polyI:C induce TGFβ1 but only CpG specifically induces Smad3/4 activation, we propose that polyI:C repression of *PDGF-B* may be mediated through inhibition of TGF-β signaling and activation of Smads. To further study this hypothesis, we analyzed the effect that polyI:C may have on CpG-mediated Smad3/4 activation. BMMs were stimulated with CpG, polyI:C or both, and nuclear extract was collected. EMSA with the Smad3/4

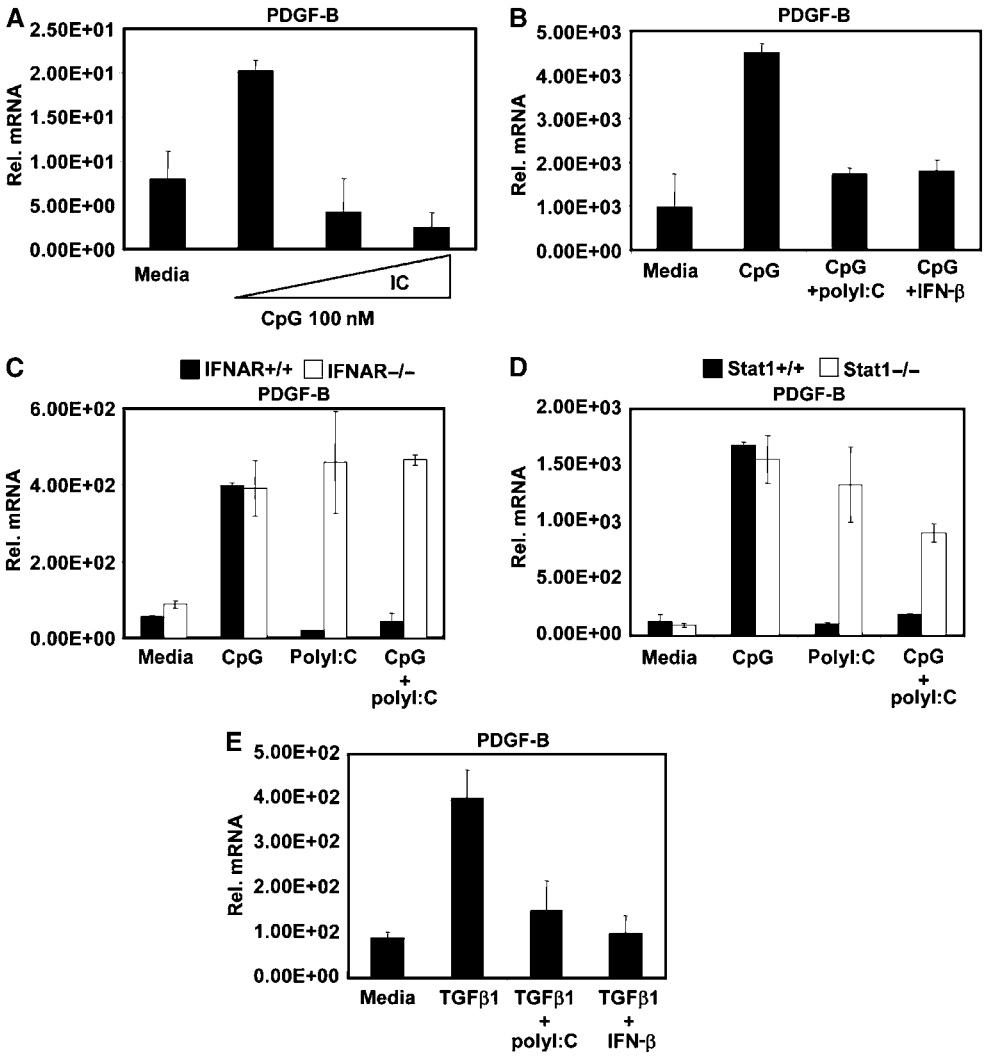

Figure 5 PolyI:C inhibits induction of *PDGF-B* mRNA through activation of the type I IFN pathway. (**A**) BMMs were stimulated with CpG (100 nM) and increasing amounts of polyI:C (0, 1 and 10 μg/ml). RNA was collected and analyzed by Q-PCR. (**B**) BMMs were stimulated with CpG and polyI:C (1 μg/ml) or recombinant IFN-β (200 U/ml). RNA was collected and analyzed by Q-PCR. (**C**) IFNAR+/+ and IFNAR−/− BMMs were stimulated with CpG (100 nM), polyI:C (1 μg/ml) or both for 4 h. RNA was collected and analyzed by Q-PCR. (**D**) Repeated (C) with Stat1+/+ and Stat1−/− BMMs. (**E**) BMMs were stimulated with TGFβ1 (2.5 ng/ml) and polyI:C (1 μg/ml) or recombinant IFN-β (200 U/ml). RNA was collected and analyzed by Q-PCR.

consensus probe revealed that polyI:C inhibited CpG's ability to induce Smad3/4 binding activity (Figure 6A).

It has been previously reported that STAT1 activation can inhibit Smad3/4 signaling through induction of Smad7 protein (Ulloa *et al*, 1999). In order to determine if polyI:C can induce *Smad7* through the type I IFN autocrine/paracrine pathway, wild-type and IFNAR-deficient BMMs were stimulated with cell-free conditioned media (CM) from wild-type BMMs stimulated with CpG or polyI:C. Q-PCR analysis revealed that polyI:C_CM, but not CpG_CM, potently induces *Smad7* mRNA (Figure 6B, left panel). Furthermore, polyI:C_CM induction of *Smad7* requires IFNAR (Figure 6B, right panel). In addition, similar experiments were performed in STAT1-deficient BMMs and showed that polyI:C_CM induction of Smad7 also requires STAT1 (Figure 6B, lower panel).

To further determine the effect of Smad7 on *PDGF-B* mRNA induction by CpG and TGFβ1, we have generated RAW 264.7 macrophage cell lines with stably integrated pcDEF3 (MT vector) or pcDEF3-Flag-Smad7 (Flag-Smad7). As shown in Figure 6C, overexpression of Smad7 leads to a loss of *PDGF-B* mRNA induction by both CpG and TGFβ1. Since polyI:C induction of Smad7 through type I IFNs seems to mediate repression of *PDGF-B* mRNA induction, we analyzed the requirement of Smad7 in polyI:C repression of *PDGF-B* mRNA induction through dominant-negative and siRNA studies. As demonstrated in Figure 6D, expression of Flag-Smad7DN or knockdown of Smad7 by Smad7 siRNA oligos resulted in an inability for polyI:C to repress CpG-induced *PDGF-B*. Thus, Smad7 appears to be a key mediator of polyI:C-type I IFN repression of *PDGF-B*.

CpG and polyI:C regulation of PDGF-BB determines mesangial cell proliferation

PDGF-BB is a major mediator of glomerulonephritis, primarily through the induction of mesangial cell proliferation (Floege *et al*, 1999). Furthermore, macrophage recruitment by CD4+ T cells is central to the development of glomerulonephritis (Ikezumi *et al*, 2004). Presumably, stimulated

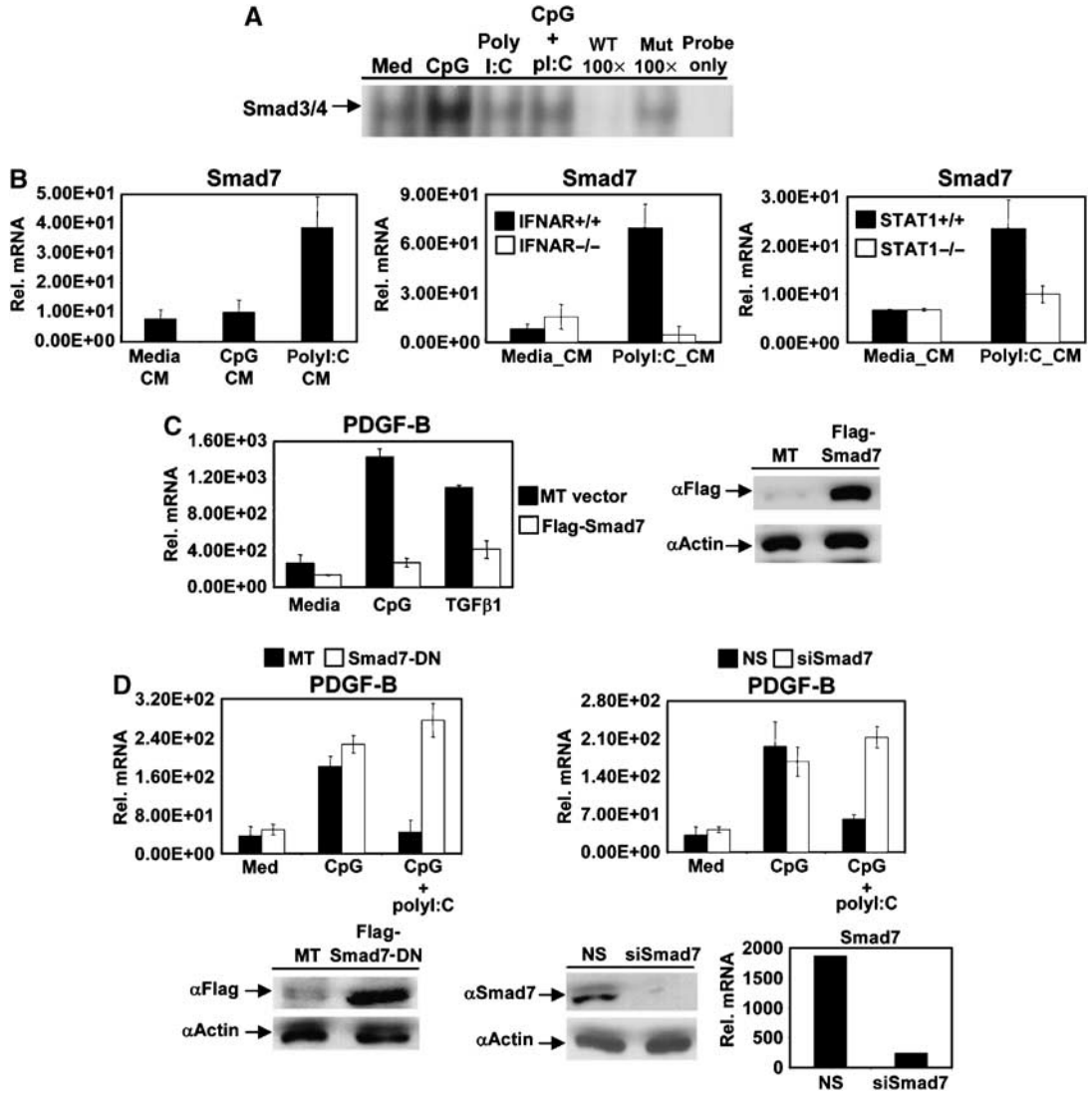

Figure 6 PolyI:C repression of CpG-mediated Smad3/4 activation and *PDGF-B* mRNA induction is mediated through Smad7. (**A**) BMMs were stimulated with CpG (100 nM), polyI:C (1 μg/ml) or both for 3 h. Nuclear extract was collected and analyzed by EMSA with Smad3/4. Media sample was used for 100 × WT/Mut cold competition. (**B**) IFNAR + / + and IFNAR−/− BMMs were treated for 30 min with CM collected from IFNAR + / + BMMs that were stimulated with CpG (100 nM) or polyI:C (1 μg/ml) for 2 h. STAT1 + / + and STAT1−/− BMMs were stimulated with STAT1 + / + BMM CM for 4 h. RNA was collected and analyzed by Q-PCR. (**C**) Raw-MT and Raw-Flag-Smad7 cell lines were stimulated with CpG (100 nM) and TGFβ1 (2.5 ng/ml) for 4 h. RNA was collected and analyzed by Q-PCR. Western blot with anti-Flag (M2) or anti-actin was performed to confirm expression of Flag-Smad7. (**D**) Raw-MT and Raw-Flag-Smad7-DN cell lines were stimulated with CpG (100 nM) with or without polyI:C (1 μg/ml). RNA and protein expressions were carried out as in (C). RAW 264.7 cell lines were transfected with Smad7 or nonspecific oligos (100 nmol). At 36 h after transfection, RNA and protein levels were analyzed.

macrophages release a host of cytokines and factors critical to induction of glomerulonephritis. In order to determine if CpG-specific induction of *PDGF-B* mRNA by macrophages could affect mesangial cell proliferation, we analyzed mesangial cell proliferation by [^{3}H]thymidine incorporation following stimulation with CM from BMMs stimulated by CpG, polyI:C or both. CpG was able to potently induce mesangial cell proliferation, while polyI:C inhibited basal and CpG-induced proliferation (Figure 7A). Furthermore, polyI:C's ability to inhibit mesangial cell proliferation was lost in IFNAR-deficient BMMs (data not shown), confirming the importance of type I IFNs in polyI:C-mediated inhibition of cell proliferation.

Since CpG specifically induces PDGF-BB protein production in BMMs, we analyzed the role of PDGF-BB in CpG-induced mesangial cell proliferation. Anti-PDGF-BB neutralizing antibodies inhibit CpG-induced mesangial cell proliferation in a dose-dependent manner (Figure 7B), suggesting that PDGF-BB plays a major role in CpG-induced mesangial cell proliferation. Thus, our current studies demonstrate that TLR-mediated positive and negative regulation of *PDGF*-B mRNA translates into control of mesangial cell proliferation.

Discussion

The diversity of PAMPs that are recognized by 11 different TLRs provides the immune system with a powerful first line of defense. Each TLR signals a unique set of genes most suited to ward off the type of pathogen that they recognize (Modlin and Cheng, 2004). Even though these TLRs share

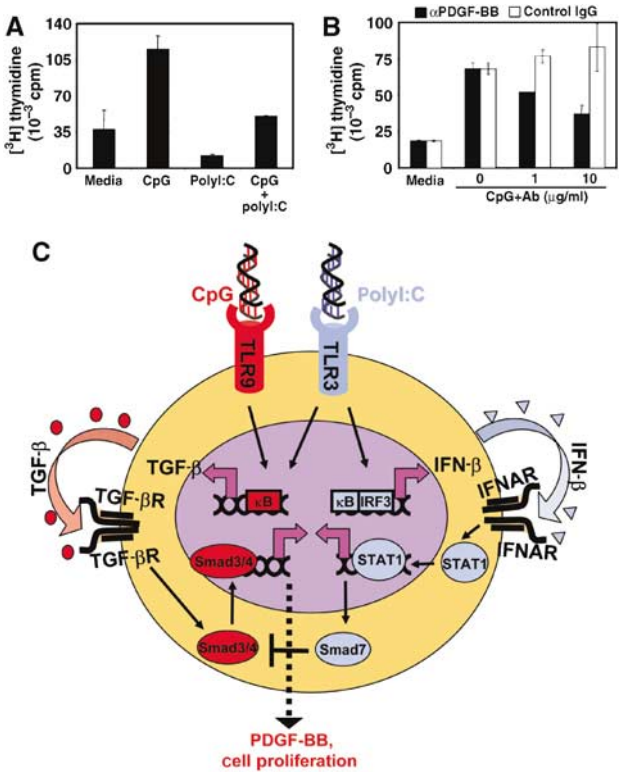

Figure 7 Maximal CpG induction of mesangial cell proliferation involves PDGF-BB. (**A**) Mesangial cells were serum starved for 3 days and then stimulated for 48 h with CM from BMMs stimulated overnight with CpG (100 nM), polyI:C (1 μg/ml) or both. [^{3}H]thymidine was added 6 h prior to collection. Cells were harvested and analyzed by scintillation counter. Graphs are the average of three independent experiments. (**B**) CM from BMMs stimulated overnight with CpG (100 nM) was thoroughly mixed with anti-PDGF-BB neutralizing antibody or control IgG (0, 1 or 10 μg/ml) and used to stimulate mesangial cells as in (A). (**C**) Model of CpG and polyI:C regulation of *PDGF-B*. CpG and polyI:C induce TGF-β production through NF-κB activation. TGFβ1 autocrine/paracrine loop activates Smad3/4, which induces *PDGF-B*. PolyI:C also induces the IFN-β autocrine/paracrine loop, activating Stat1 and inducing Smad7. Smad7 inhibits polyI:C activation of Smad3/4, prohibiting *PDGF-B* induction.

many signaling similarities, specificity through coordinate regulation of transcription factors determines very different phenotypes. This is evident in a novel mechanism that we describe here in BMMs, by which TLR9 and TLR3 specifically enhance or repress proliferative phenotypes through crosstalk between two seemingly unrelated autocrine/paracrine signaling networks, TGF-β and type I IFNs (Figure 7C).

Our results indicate that CpG-specific upregulation of *PDGF-B* proceeds through a TGF-β autocrine/paracrine signaling network. This is evident by the observation that, through NF-κB, CpG can induce TGFβ1 production and anti-TGFβ1 neutralization antibody can block CpG-induced upregulation of *PDGF-B*. TGF-β produced by CpG-stimulated cells can trigger Smad3/4 activation through either an autocrine or paracrine manner. Within 3 h of CpG stimulation of macrophages, both Smad3/4 and NF-κB subunits can be detected by ChIP analysis in the endogenous promoter region of the *PDGF-B* gene. These transcription factors may have synergistic effects on the transcription of *PDGF-B*, as macrophages lacking Smad3 or overexpressing IκB are defective in CpG-induced *PDGF-B* upregulation. While both CpG and polyI:C activate NF-κB, only CpG is able to activate Smads and induce their binding to the *PDGF-B* promoter, which may explain TLR9's unique ability to specifically induce *PDGF-B*. In addition, induction of *PDGF-B* by CpG is also impaired in macrophages derived from MyD88- and IRAK4-deficient mice, suggesting the overall signaling network from CpG to *PDGF-B* initiates through the MyD88-dependent pathway. Interestingly, TLR9-specific induction of gene activity through the coordinate activation of NF-κB and Smads is not limited to *PDGF-B*, but has also been seen in other TLR9-specific genes such as *MARCO* and *lysyl oxidase* (data not shown).

In contrast to CpG-specific activation of Smad3/4 in BMMs, CpG, LPS and polyI:C all seem to be able to induce TGFβ1 production, suggesting potential negative regulation of TGF-β autocrine/paracrine signaling by LPS and polyI:C. Indeed, addition of polyI:C strongly inhibits CpG-induced Smad3/4 activation and *PDGF-B* upregulation. Our results further demonstrate that the inhibition of TGF-β signaling by polyI:C depends upon TLR3-mediated type I IFN autocrine/paracrine signaling network. In fact, our studies show that recombinant IFN-β alone is able to inhibit TGFβ1 induction of *PDGF-B*. Macrophages derived from IFNAR1- and STAT1-deficient mice not only lost the ability of polyI:C to inhibit CpG-induced *PDGF-B* upregulation, but also enable polyI:C itself to strongly upregulate *PDGF-B*. This repression of TGF-β-mediated Smad3/4 activation appears to be through IFN/STAT-dependent induction of *Smad7*. Smad7 protein is one of the inhibitory Smads that can inhibit Smad signaling, through either competing with activating Smads for binding to activated TGF-β type I receptors, recruiting E3-ubiquitin ligases such as Smad ubiquitination regulatory factor 1 (Smurf1) and Smurf2 to promote receptor ubiquitination and degradation (Ebisawa *et al*, 2001; Zhang *et al*, 2001), or recruiting a complex of GADD34 and the catalytic subunit of protein phosphatase 1 to dephosphorylate the activated TGF-β type I receptor (Shi *et al*, 2004). We found that polyI:C can strongly upregulate *Smad7* mRNA in a type I IFN/STAT1-dependent manner and overexpression of Smad7 in macrophages diminished CpG- and TGFβ1-induced upregulation of *PDGF-B*. Furthermore, downregulation of Smad7 results in an inability of polyI:C to repress CpG-induced *PDGF-B*. Interestingly, in cell types where CpG induces type I IFNs, CpG is unable to induce *PDGF-B*, presumably due to the crosstalk between type I IFNs and TGF-β signaling pathways. Thus, our studies have uncovered a TLR-mediated negative regulatory pathway, emphasizing the importance of both positive and negative signaling networks in regulating the specificity of gene expression profiles induced by individual TLRs.

PDGF-BB has been shown to be a critical growth factor in the progression of various diseases where uncontrolled proliferation of certain cell types occurs. A well-documented example is mesangial cell proliferation by PDGF-BB in glomerulonephritis (Floege *et al*, 1993, 1999). We have shown that CpG induces similar mesangial cell proliferation through its induction of PDGF-BB. Our results further suggest that TLR-mediated induction of PDGF-BB uses TGF-β as a critical positive regulator, although TGF-β alone may not be sufficient to promote mesangial cell proliferation (Blom *et al*, 2001). We also provide evidence that type I IFNs are critical to polyI:C negative regulation of TGF-β signaling, PDGF-BB production and mesangial cell proliferation. TGF-β and type

I IFN signaling are both considered to be critical mediators of a number of functions. Until now, however, there has never been an association between the two autocrine/paracrine loops. We have presented a clear system where crosstalk between these two signaling networks regulates *PDGF-B* and a proliferative signal that could potentially affect a variety of functions and diseases. Furthermore, this work provides insights into possible therapeutic approaches to controlling irregular PDGF-BB expression and function.

Materials and methods

Cell culture and reagents

Murine BMMs were differentiated from marrow as described previously (Doyle *et al*, 2002). Mice deficient in Smad3 and STAT1 were previously described (Durbin *et al*, 1996; Datto *et al*, 1999). Mice deficient in MyD88 and littermate controls were obtained from Shizuo Akira (University of Osaka, Osaka, Japan). Mice deficient in IRAK4 were obtained from Wen-Chen Yeh (University of Toronto, Toronto, Canada). IFNAR-deficient mice were obtained as previously described (O'Connell *et al*, 2004). Cells from F5 C57Bl/6 littermate wild-type mice were used as wild-type controls for experiments using cells from F5 C57Bl/6 $IFNAR^{-/-}$ mice. C57/Bl6 mice were used for all experiments not involving the MyD88-deficient mice (Jackson ImmunoResearch Laboratories). RAW 264.7 murine macrophage cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Stable Raw-Flag-Smad7 or Raw-MT vector was made by transfecting Raw 264.7 cells with 5 μg pcDEF3-Flag-Smad7 or 5 μg pcDEF3 and 0.5 μg pBabe-puro with Superfect (Qiagen) and selected with puromycin. Stable Raw-Flag-Smad3 or Raw-MT vector was made by transfecting Raw 264.7 cells with 5 μg pCMV5-Flag-Smad3 or 5 μg pCMV and 0.3 μg pBabe-puro with Superfect (Qiagen) and selected with puromycin. Stable Raw-Flag-Smad7-DN or Raw-MT vector was made by transfecting Raw 264.7 cells with 1 μg pBabe-Flag-Smad7DN or 1 μg pBabe with Superfect (Qiagen) and selected with puromycin. Stable cell lines expressing dominant active forms of IκBα-ER (RAW-IκB-DA) were used as previously described (Doyle *et al*, 2002). Specific TLR activation was achieved using CpG oligonucleotides for TLR9 (Invitrogen), polyI:C for TLR3 (Amersham Biosciences) and *Escherichia coli* LPS for TLR4 (Sigma-Aldrich). Inhibition of TGFβ1 and PDGF-BB was carried out with anti-TGFβ1 neutralizing antibody and anti-PDGF-BB neutralizing antibody (R&D Systems), respectively. Macrophage colony-stimulating factor-containing medium was obtained by growing L929 cells 4 days past confluency and then harvesting the CM.

RNA quantitation

For microarray studies, BMMs were treated with media or 1 ng/ml lipid A (Sigma-Aldrich) for 4 h or 1 μg/ml polyI:C or 100 nM CpG for 4 or 12 h. After stimulation, total RNA was extracted and labeled cRNA synthesized as described previously (Doyle *et al*, 2002). The labeled cRNA was used to hybridize to Affymetrix Mu11K chip sets and data were analyzed using Affymetrix Microarray Suite 4.0 data mining software. To identify PAMP-induced genes, comparisons were performed using the media-only-treated sample as a baseline. Genes were considered induced if they displayed (a) a three-fold change relative to baseline, (b) they had a poststimulation average difference (a value representing absolute expression level) of at least 500 and (c) they had an average difference change relative to baseline of at least 600 (roughly twice chip background). Average difference change values for genes were processed using Cluster. For Q-PCR, total RNA was isolated and cDNA synthesized as described previously (Doyle *et al*, 2002). PCR was then performed using the iCycler thermocycler (Bio-Rad). Q-PCR was conducted in a final volume of 25 μl containing *Taq* polymerase, 1 × *Taq* buffer (Stratagene), 125 μM dNTP, SYBR Green I (Molecular Probes) and Fluoroscein (Bio-Rad), using oligo-dT cDNA or random hexamer cDNA as the PCR template. Amplification conditions were 95°C (3 min), 40 cycles of 95°C (20 s), 55°C (30 s) and 72°C (20 s). The following primers were used to amplify 100–120 bp fragments: *PDGF-B*: *5′CCACTCCATCCGCTCCTTT*, *3′AAGTCCAGCTCAGCCCCAT*; *Smad7*: *5′CCTCCTCCTTACTCCAGATA*, *3′ACGCACCAGTGTGACCGATC*; IFN-β and Mx1 primers were previously described (Doyle *et al*, 2002).

Western, EMSA and ELISA protein analysis

For Western blots, cell lysates were incubated at room temperature for 5 min with EB lysis buffer (10 mM Tris–HCl buffer, pH 7.4, containing 5 mM EDTA, 50 mM NaCl, 0.1% (wt/vol) BSA, 1.0% (vol/vol) Triton X-100, protease inhibitors), size-separated in 10% SDS–PAGE and transferred to nitrocellulose. The anti-PDGF-BB and anti-Smad7 antibodies were obtained from Santa Cruz. Anti-Flag (M2) antibody was obtained from Sigma-Aldrich. For EMSA, BMMs were serum starved with 0.5% FBS complete DMEM for 6 h and stimulated with TLR ligands and nuclear extract was prepared as previously described (Doyle *et al*, 2002). Smad3/4 consensus probe and mutant sequence were obtained from Santa Cruz Biotech. For ELISA, BMMs were serum starved with 0.5% FBS complete DMEM for 6 h and stimulated with TLR ligands in serum-free media for 24 h. TGFβ1 ELISA (R&D systems) was carried out according to the manufacturer's protocol.

Reporter assay

All plasmids were purified using Endo-free Maxiprep (Qiagen). RAW 264.7 cells were transfected with 1–3 μg 3TP-Luc or κB-Luc with 100 ng LacZ by Superfect (Qiagen). Following transfection, cells were stimulated with CpG (500 nM), TGFβ1 (5 ng/ml) or polyI:C (5 μg/ml) for 24 h. Cells were lysed in 200 μl reporter lysis buffer (Promega). Luciferase activity was measured and normalized to β-galactosidase activity as previously described (Kanasaki *et al*, 2003).

Chromatin immunoprecipitation

Inactivated and activated cells were fixed at room temperature for 10 min by adding formaldehyde directly to the culture medium to a final concentration of 1%. The reaction was stopped by adding glycine at a final concentration of 0.125 M for 5 min at room temperature. After three ice-cold PBS washes, the cells were collected and lysed for 10 min on ice in cell lysis buffer (5 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 8.0), 85 mM KCl, 0.5% NP-40, protease inhibitors). The nuclei were resuspended in nuclei lysis buffer (50 mM Tris–HCl (pH 8.1), 10 mM EDTA, 1% SDS, protease inhibitors) and incubated on ice for 10 min. Chromatin was sheared into 500- to 1000-bp fragments by sonication and was then precleared with protein A or protein G-Sepharose beads. The purified chromatin was diluted with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl (pH 8.1), 167 mM NaCl, protease inhibitors) and immunoprecipitated overnight at 4°C using 2–4 μg of anti-Smad4, anti-p65 (Santa Cruz Biotech) or control rabbit IgG. Immune complexes were collected with protein A or protein G-Sepharose beads and were then washed thoroughly and eluted. After protein–DNA crosslinking was reversed and the DNA was purified, the presence of selected DNA sequences was assessed by PCR or Q-PCR. Q-PCR was analyzed by MyIQ (Bio-Rad) and samples normalized to input. PCR products were analyzed on 2% agarose gel. Primers used for ChIP were as follows: *PDGFPro(−197/−7)*: *5′CCAAGAGGCTAGATTCACAGTCAC*, *3′TTCAGCTGTTCCGGCCTTT*; *IfnBPro(−224/−2)*: *5′CCAGCAATTGGTGAAACTGTACA*, *3′CCTTCAT GGCCTGTGCTATTTAT*.

Smad7 siRNA preparation

Smad7 siRNA experiments were performed as described previously (Davoodpour and Landstrom, 2005). Briefly, the targeted sequence for the Smad7 siRNA duplex (5′-*AA GCUCAAUUCGGACAACAAG*-3′) or nonspecific siRNA duplex was synthesized by Invitrogen. Duplex oligonucleotides were transfected using Lipofectamine (Invitrogen) at a ratio of 100 nmol of RNA to 1.5 μl of Lipofectamine in serum-free, antibiotic-free media. Media were changed after 4–6 h and experiments were performed 36 h post-transfection.

Cell proliferation

All work was carried out in 96-well plates. Mouse mesangial cells (ATCC) were serum starved in 1% FBS complete RPMI for 3 days and then stimulated for 48 h with CM from BMMs stimulated overnight with CpG (100 nM), polyI:C (1 mg/ml) or both. [^{3}H]thymidine (0.5 μCi; 1 Ci = 37 GBq) was added 6 h prior to collection and collected on a 96-well filtermat with an automated harvester. Results were analyzed by scintillation counter.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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