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Molecules and Cells

IFN-γ Down-Regulates TGF-β1-Induced IgA Expression through Stat1 and p300 Signaling

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IFN-γ has been shown to either up- or down-regulate the expression of specific TGF-β1-induced target genes. We investigated the effect of IFN-γ on TGF-β1-induced IgA isotype expression. We found that IFN-γ inhibited not only TGF-β1-induced germ-line (GL) α transcription, but also IgA secretion by TGF-β1-stimulated murine B cells. Overexpression of Stat1 diminished TGF-β1-induced, Smad3/4- and Runx3-mediated GLα promoter activity. Overexpression of p300 also increased the promoter activity, while its effect was abrogated by co-transfected Stat1. Stat1 interfered with the Smad3:p300 interaction, likely due to a stronger Stat1:p300 binding affinity. These results indicate that Stat1 can inhibit GLα transcription through binding to p300. Further, overexpression of SOCS1, a JAK inhibitor, diminished the antagonistic effect of IFN-γ on TGF-β1-induced GLα transcription and IgA secretion. These results indicate that JAK/Stat1-mediated IFN-γ signaling antagonizes TGF-β1-induced GLα transcription, mainly through deprivation of p300 from Smad3, resulting in decreased IgA synthesis.

INTRODUCTION

Immunoglobulin (Ig) class switch recombination (CSR) results in juxtaposition of the rearranged $V_HD_HJ_H$ genes upstream of a new C_H gene (Mandler et al., 1993; Shi and Stavnezer, 1998). This switch mechanism endows antibodies with different biological functions for removal of diverse pathogens. On the other hand, transcription of the corresponding unrearranged C_H gene to produce germ-line transcripts (GLTs), precedes transcription of productive Ig mRNA (Lorenz et al., 1995; Shi and Stavnezer, 1998). These GLTs are a prerequisite for subsequent switch recombination.

It is well established that TGF-β1 induces IgA isotype switching (Coffman et al., 1989; Kim and Kagnoff, 1990a; 1990b; Sonoda et al., 1989) and increases GLα transcripts (GLT_α) in murine, human, and rabbit B cells (Islam et al., 1991; Lebman et al., 1990; Shockett and Stavnezer, 1991). TGF-β1 signals from the membrane to the nucleus through TGF-β1 receptors and their downstream effectors, termed Smad proteins (Smad3 and Smad4) (Massague, 1998; Okadome et al., 1994). Upon stimulation by TGF-β1, activated Smad3/4 heterodimeric complexes translocate into the nucleus where they bind specific DNA sequences in target promoters, thereby activating transcription (Nakao et al., 1997). We have previously shown that Smad3/4, Runx3, and p300 mediate TGF-β1-induced GLα transcription and IgA secretion (Park et al., 2001; 2003).

IFN-γ, a pleiotropic cytokine produced by T cells and NK cells, plays fundamental roles in both innate and acquired immune responses. Activated IFN-γ receptors and their associated protein tyrosine kinase, JAK, phosphorylate Stat1, which then forms homodimeric complexes. These Stat1 complexes translocate to the nucleus where they bind specific DNA sequences, such as TTCNNNGAA (IFN-γ activated sites, GAS), in target promoters, thereby activating transcription (Boehm et al., 1997; Song et al., 2002). We have recently shown that TGF-β1 and IFN-γ independently up-regulate the expression of B cell-activating factor belonging to the TNF family (BAFF) through Smad3/4 and PKA/CREB pathways, respectively (Kim et al., 2008). However, there is evidence that IFN-γ can antagonize TGFβ-induced target gene expression by either increasing Smad7 expression (Ulloa et al., 1999) or inhibiting p300 action (Ghosh et al., 2001).

In this study, we examined the effect of IFN-γ on TGF-β1-induced IgA isotype expression in murine B cells. We found that IFN-γ inhibits not only TGF-β1-induced germ-line (GL) α promoter activity, but also IgA secretion. Further, Stat1 signaling was mainly responsible for the inhibitory activity of IFN-γ. p300 interacted with Stat1, thus possibly reinforcing the antagonistic activity of IFN-γ on TGF-β1-induced, Smad3/4-mediated IgA expression.

MATERIALS AND METHODS

Animals

BALB/c mice were purchased from Orient. Co., Ltd. (Gyeonggido, Korea) and maintained on an 8:16 h light:dark cycle in an animal environmental control chamber (Myung Jin Inst. Co., Korea). Animal care was in accordance with the institutional guidelines of Kangwon National University.

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Cell culture and reagents
The murine B cell line, A20.3, was provided by Dr. Janet Stavnezer (University of Massachusetts Medical School, USA). The murine B lymphoma cell line, CH12F3-2A (surface, μ^+) (Nakamura et al., 1996), was provided by Dr. T. Honjo (Kyoto University, Japan). Cells were cultured at 37°C in a humidified CO_2 incubator (Sanyo, Japan) in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (HyClone Labs, USA). Murine spleen B cell suspensions were prepared as previously described (Murray et al., 1987). Spleen B cells (1×10^6 cells/ml) were cultured with LPS (12.5 μg/ml, *E. coli* 0111:B4, Sigma), TGF-β1 (0.2 ng/ml, R&D Systems, Inc., USA) and/or IFN-γ (10 ng/ml, R&D Systems).

Expression and reporter plasmids
The expression plasmids containing the cDNA for Stat1 and dominant negative (DN)-Stat1, which were subcloned into pRc/CMV (Bromberg et al., 1996), were generously provided by Dr. J.E. Darnell, Jr. (Rockefeller University, USA). DN-Stat1 is a single amino acid substituted mutant of Stat1 (Tyr-701 → Phe-701). Phosphorylation of this tyrosine is required for Stat1 homodimerization or heterodimerization with other Stat proteins before DNA binding and the formation of a transcriptionally active complex (Shuai et al., 1993). Genes encoding Smad3 (Zhang et al., 1996b) and Smad4 (Hahn et al., 1996) were subcloned into Flag-pcDNA3 (Imamura et al., 1997), and were provided by Dr. M. Kawabata (JFCR Cancer Institute, Japan). Dr. S. Hiebert (St Jude Children's Research Hospital, USA) provided the expression plasmid for Runx3 (Meyers et al., 1996). The expression plasmid for p300 (Janknecht et al., 1998) was provided by Dr. R. Janknecht (Mayo Graduate School, USA). The expression plasmid for SOCS1 (pEF-BOS/ SOCS1) (Naka et al., 1997) was provided by Dr. Fujimoto (National Institute of Biomedical Innovation, Japan). The reporter plasmid GLα-130/+14 Luc (Shi and Stavnezer, 1998) was provided by Dr. J. Stavnezer.

Transfection and luciferase assay
Transfection was performed by electroporation with a Gene Pusler II (Bio-Rad, USA), as previously described (Park et al., 2001). Reporter plasmids were co-transfected with expression plasmids and pCMVβgal (Stratagene, USA). Luciferase and β-gal assays were performed as previously described (Park et al., 2001).

Reverse transcription-polymerase chain reaction (RT-PCR)
RNA preparation, reverse transcription, and PCR were performed as previously described (Park et al., 2001). Primers for PCR were synthesized by Bioneer Corp. (Korea): GLT_α sense 5′-CTACC ATAGG GAAGA TAGCCT-3′ and antisense 5′-TAATC GTGAA TCAGG CAG-3′ (product size 206 bp); β-actin sense 5′-CATGT TTGAG ACCTT CAACA CCCC-3′ and antisense 5′-GCCAT CTCCT GCTCG AAGTC TAG-3′ (product size 320 bp). All reagents for RT-PCR were purchased from Promega (USA). PCR reactions for GLT_α and β-actin were performed in parallel to normalize cDNA concentrations within each set of samples. PCR products were resolved by electrophoresis on 2% agarose gels.

Isotype-specific ELISA
Antibodies produced in B cell cultures were detected by ELISA as previously described (Park et al., 2001).

Immunoprecipitaton and Western blotting
CH12F3-2A B lymphoma cells were transfected as above with the appropriate expression vectors. After 24 h incubation, cells were collected, lysed, and subjected to immunoprecipitation with an anti-p300 antibody (Santa Cruz Biotechnology, Inc., USA) using protein A-Sepharose (Amersham Pharmacia Biotech, Sweden). For Western blot analysis, total cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to PVDF membranes (Bio-Rad Laboratories, USA). Specific immunodetection was carried out by incubation with an anti-Smad3 mAb (Zymed laboratories Inc., USA) or an anti-FLAG Ab (Sigma, USA), followed by peroxidase-conjugated goat anti-mouse IgG Ab (Pierce, USA), and visualized by chemiluminescence (Supersignal detection kit, Pierce).

Statistical analysis
Statistical differences between experimental groups were determined by analysis of variances. Values with $p < 0.01$ by an unpaired two tailed Student's *t* test were considered significant.

RESULTS

IFN-γ inhibits TGF-β1-induced IgA expression
Several studies have demonstrated that IFN-γ down-regulates TGF-β1 targeted gene expression (Ghosh et al., 2001; Higashi et al., 2003a; Kuga et al., 2003; Ulloa et al., 1999; Wen et al., 2004; 2007). We have previously shown that TGF-β1 increases GLT_α expression through Smad3/4, leading to IgA secretion (Park et al., 2001). Therein, we investigated the effect of IFN-γ on TGF-β1-induced IgA isotype expression. As shown in Fig. 1A, IFN-γ substantially decreased TGF-β1-induced IgA secretion by LPS-activated B lymphoma cells. Further, IFN-γ also decreased IgA production by LPS-activated primary spleen B cells. To assess the inhibitory mechanisms of IFN-γ, we first examined the effect of IFN-γ on TGF-β1-induced GLT_α expression. As shown in Fig. 1B, TGF-β1 increased the expression of endogenous GLT_α. This increase was abrogated by IFN-γ treatment. IFN-γ also inhibited TGF-β1-induced GLα promoter activity (Fig. 1C). These results suggest that IFN-γ, represses TGF-β1-induced GLα transcription, resulting in decreased IgA secretion.

Roles of Stat1 and p300 in the antagonistic activity of IFN-γ on GLα promoter activity
Since Stat1 is a key mediator in IFN-γ signaling (Darnell et al., 1994), we examined whether Stat1 is involved in TGF-β1-induced GLT_α expression. As shown in Fig. 2A, overexpression of Stat1 strengthened the antagonistic effect of IFN-γ on TGF-β1-induced GLT_α expression. Smad3/4 and Runx3 mediate TGF-β1-induced GLα transcription and IgA expression (Park et al., 2001; 2003). Therefore, we analyzed the role of Stat1 in the GLα promoter activity under the influence of overexpressed Smad3/4 or Runx3. Overexpression of Smad3/4 further augmented the TGF-β1-induced GLα promoter activity (Fig. 2B). This increase was significantly diminished by IFN-γ treatment. Moreover, this antagonistic activity of IFN-γ was also strengthened by overexpressed Stat1, and weakened by overexpressed DN-Stat1. A similar pattern of GLT_α regulation was observed when Runx3 was overexpressed (Fig. 2B). These results suggest that IFN-γ repressed TGF-β1-induced GLT_α expression mainly through Stat1.

p300 interacts with phosphorylated Smad3 and coactivates TGF-β1-targeted genes (Ghosh et al., 2000; Janknecht et al., 1998; Kanamaru et al., 2003; Nishihara et al., 1998). Further, we have demonstrated that p300 acts as a transcriptional coactivator for Smad3/4 and Runx3 in TGF-β1-induced GLα transcription (Park et al., 2003). On the other hand, early studies have also demonstrated that p300 directly interacts with Stat1

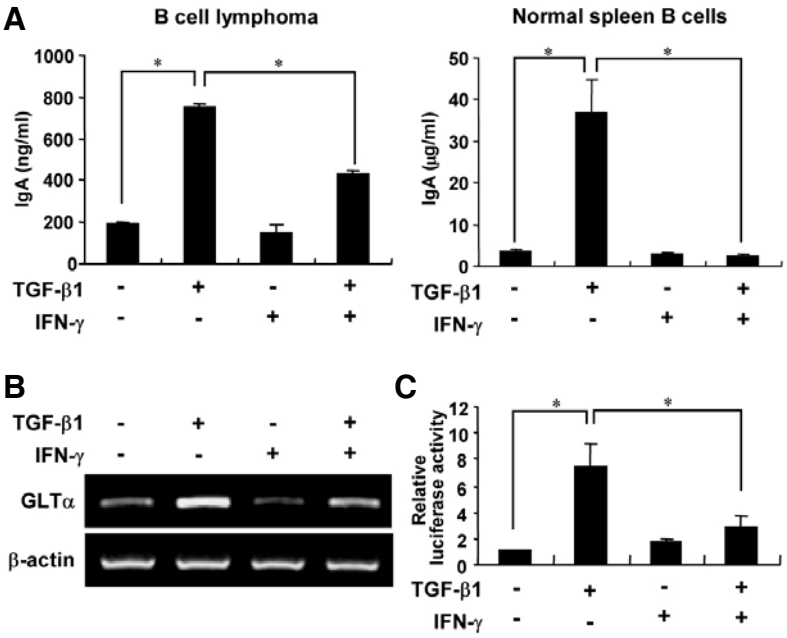

Fig. 1. IFN-γ down-regulated TGF-β1-induced IgA expression and GLα transcription in murine B cells. (A) CH12F3-2A B lymphoma cells and primary spleen B cells were stimulated with LPS (12.5 μg/ml) and combinations of TGF-β1 (0.2 ng/ml) and IFN-γ (10 ng/ml). Secreted IgA levels were determined by ELISA. Data are averages of triplicate cultures with SEM error bars. (B) GLT_{α} levels were measured by RT-PCR 24 h after CH12F3-2A B lymphoma cells were stimulated as above. (C) A20.3 B lymphoma cells were transfected with the GLα promoter reporter (15 μg). Cells were then stimulated with TGF-β1 (1 ng/ml) and IFN-γ (10 ng/ml). Luciferase activity was determined 16 h later. Transfection efficiency was normalized to β-gal activity. Data represents the average luciferase activities from three independent transfections with SEM error bars. $^{*}p < 0.01$.

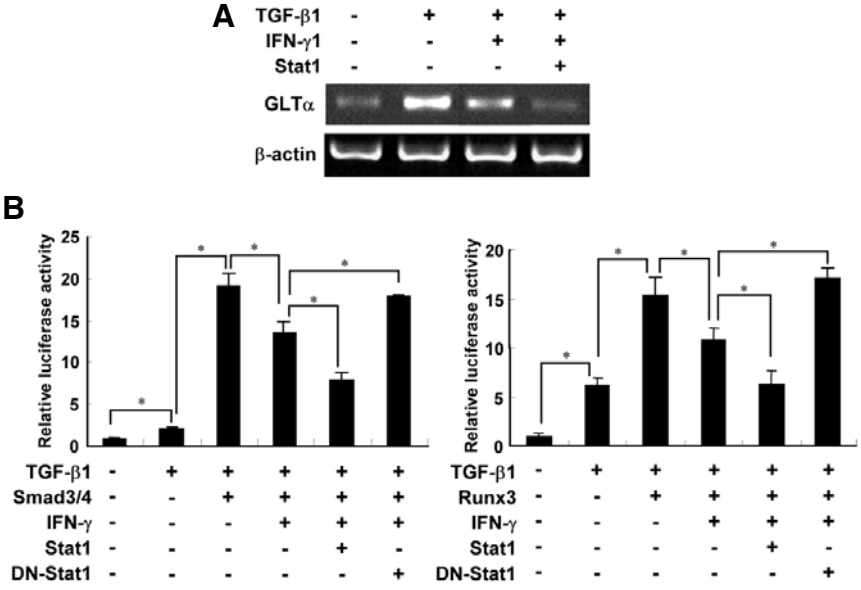

Fig. 2. Effects of IFN-γ and overexpression of Stat1 on TGF-β1-induced GLα transcription. (A) CH12F3-2A B lymphoma cells were transfected with 15 μg of Stat1 or empty vector. Induction and analysis were performed as in Fig. 1B. (B) A20.3 B lymphoma cells were transfected with 15 μg of GLα promoter reporter and expression plasmids (5 μg of Smad3 and Smad4, 10 μg of Stat1, DN-Stat1, and Runx3). Induction and analysis were performed as in Fig. 1C. Data are average luciferase activities of three independent transfections with SEM error bars. $^{*}p < 0.01$.

and enhances IFN-γ-induced, Stat1-mediated transcriptional activity (Horvai et al., 1997; Zhang et al., 1996a). It has been demonstrated that IFN-γ activates Stat1 and enhances the interaction of p300 with phosphorylated Stat1. This interaction down-regulates TGF-β1-induced, Smad3-dependent transcription by directly competing for binding between Smad3 and p300 to regulate transcription of the type I collagen gene (Ghosh et al., 2001). Therein, it was important to distinguish the role of p300 in GLα transcriptional regulation by TGF-β1 and IFN-γ. As shown in Fig. 3A, over-expression of p300 further augmented the TGF-β1-induced, Smad3/4-mediated GLα promoter activity. However, this increase of promoter activity was markedly abolished by overexpressed Stat1 in the presence of IFN-γ. These results suggest that p300 may interact with Stat1 more strongly than Smad3, ultimately leading to a decrease in GLα promoter activity. We examined this possibility by determining the physical interactions among Smad3, Stat1, and p300. TGF-β1 treatment increased the binding of Smad3 to p300 (Fig. 3B, lanes 2 and 3). This binding virtually disappeared during IFN-γ treatment and overexpression of Stat1 (Fig. 3B, lanes 3 and 4). p300 strongly interacted with Stat1, indicating that p300 bound to IFN-γ-activated Stat1, and therefore supported IFN-γ antagonistic activity on TGF-β1-induced, Smad3/4-mediated GLT_{α} expression.

Overexpressed SOCS1 rescues the inhibitory effect of IFN-γ on TGF-β1-induced IgA expression

SOCS1 is a JAK inhibitor and inhibits IFN-γ activation of Stat1 (Endo et al., 1997; Yasukawa et al., 2000). Similarly, it has been recently shown that SOCS1 is necessary for Th17 differentiation by suppressing antagonistic effects of IFN-γ on TGF-

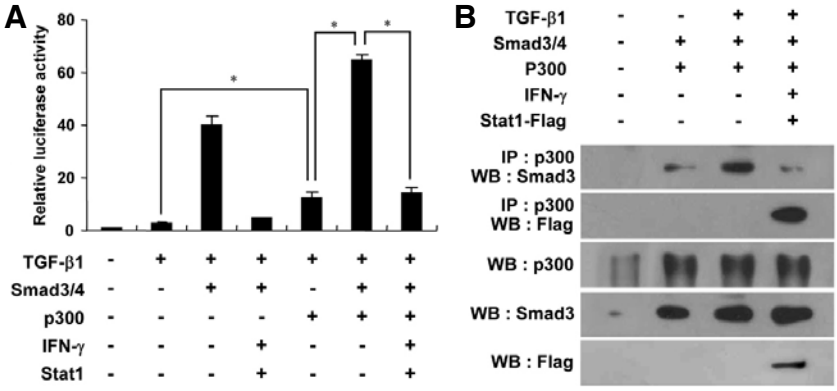

Fig. 3. IFN-γ/Stat1 interferes with the interaction between Smad3 and p300. (A) A20.3 B lymphoma cells were transfected with 15 μg of GLα promoter reporter and expression plasmids (2 μg of Smad3 and Smad4, 20 μg of p300, 10 μg of Stat1, or empty vector). Induction and analysis were performed as in Fig. 1C. Data are average luciferase activities of three independent transfections with SEM error bars. (B) Interactions among Smad3, p300, and Stat1 were examined by immunoprecipitation (IP) followed by Western blot (WB) in CH12F3-2A B lymphoma cells. IPs were performed with anti-p300 Ab, and coimmunoprecipitated Smad3 and Stat1 proteins were detected by WB with anti-Smad3 and anti-Flag Ab, respectively. *$p < 0.01$.

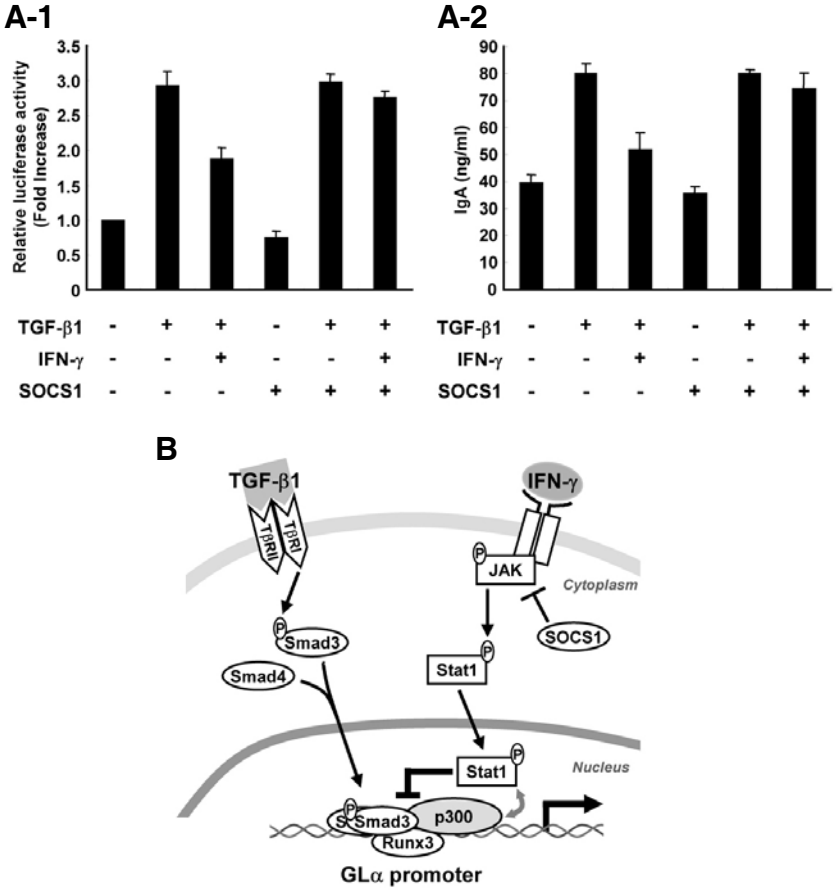

Fig. 4. SOCS1 hinders the antagonistic effect of IFN-γ on TGF-β1-induced IgA expression. (A-1) A20.3 B lymphoma cells were transfected with 15 μg of GLα promoter reporter and SOCS1 expression plasmid (pEF-BOS/SOCS1, 10 μg) or empty vector (pEF-BOS, 10 μg). Induction and analysis were performed as in Fig. 1C. Data are average luciferase activities of three independent transfections with SEM error bars. (A-2) CH12F3-2A B lymphoma cells were transfected with 10 μg of pEF-BOS/ SOCS1 or pEF-BOS, and were stimulated with LPS (2.5 μg/ml) and a combination of TGF-β1 (0.2 ng/ml) and IFN-γ (10 ng/ml). Supernatants were harvested after three days and the levels of IgA were determined by ELISA. Data are averages of triplicate cultures with SEM error bars. (B) Proposed mechanism by which IFN-γ suppresses TGF-β1-induced GLα transcription.

β1-mediated Smad3 activity (Tanaka et al., 2008). Therein, we examined the involvement of SOCS1 in TGF-β1-induced IgA expression. As shown in Fig. 4A, overexpression of SOCS1 counteracted the antagonistic effect of IFN-γ on TGF-β1-induced IgA expression, both at the promoter and the Ig secretion levels. These results provide additional evidence that IFN-γ/Stat1 signals inhibit TGF-β1-induced GLα transcription, leading to IgA class switching.

DISCUSSION

Here, we showed that IFN-γ repressed TGF-β1-induced IgA expression and Stat1 mediated this antagonistic activity. We also found that overexpression of Stat1 substantially abolished the GLα promoter activity enhanced by overexpressed Smad3/4 or Runx3. These results clearly show that Stat1 is the major transducing molecule that mediates the inhibitory signal of IFN-γ. We have previously shown that p300 co-activates Smad3/4- and Runx3-mediated GLα transcription and IgA isotype expression in response to TGF-β1 (Park et al., 2003). Nevertheless, in the present study, a marked increase of GLα promoter activity by overexpression of Smad3/4 and p300 under the influence of TGF-β1 was virtually eliminated by IFN-γ/Stat1 signaling (Fig. 3). Subsequently, we observed that Stat1 bound

p300, which may lead to deprivation of p300 from Smad3. Consistent with these results, it has been proposed that IFN-γ induces binding between Stat1 and p300 and inhibits TGF-β-induced, Smad3/4-mediated type I collagen gene expression (Ghosh et al., 2001). Similarly, YB-1 mediates the inhibitory effect of IFN-γ on TGF-β-induced, Smad3-mediated α2(I) procollagen gene (*COL1A2*) transcription through preferential interaction with p300 (Higashi et al., 2003a; 2003b). Therefore, the coactivator action of p300 in Smad3-mediated target gene expression is likely to be prohibited by p300-binding molecules that transduce IFN-γ signals. In this regard, it would be worthwhile to determine whether YB-1 in response to IFN-γ hinders Smad3/p300-mediated GLα transcription and IgA expression by TGF-β1.

IFN-γ/Stat1 inhibited TGF-β1-induced, Runx3-mediated GLα promoter activity. p300 is known to interact with Runx2 and coactivate Runx2-dependent transcription (Kitabayashi et al., 1998). Stat1 can bind to the runt domain of Runx2 and inhibit the nuclear localization of Runx2 (Kim et al., 2003; Miyazono et al., 2004). Thus, Stat1 may bind to the Runx3 runt domain leading to decreased GLα promoter activity. We are currently examining the dynamics of interactions among Runx3, Stat1, and p300 at the promoter level.

IFN-γ antagonizes the action of TGF-β by inducing inhibitory Smad7 expression in multiple cell types (Soto et al., 2003; Ulloa et al., 1999; Weng et al., 2007). Therefore, we examined Smad7 expression and found that IFN-γ indeed enhances Smad7 transcription induced by TGF-β1 (data not shown). This suggests that Smad7 is, at least in part, involved in the antagonistic activity of IFN-γ on TGF-β1-induced GLα transcription.

The experiments presented here suggest the following mechanism by which IFN-γ suppresses TGF-β1-induced GLα transcription (Fig. 4B). Upon stimulation by TGF-β1, Smad3 becomes phosphorylated by activated TGF-β1 receptors (TβRII, TβRI) and forms complexes with Smad4. These Smad complexes cooperate with Runx3 and p300 on the GLα promoter to activate GLα transcription. On the other hand, IFN-γ activates Stat1 via JAK and phosphorylated Stat1 strongly binds to p300. Consequently, IFN-γ inhibits TGF-β1-induced, Smad3-mediated GLα transcription through preferential binding between Stat1 and p300. SOCS1 interferes with this inhibitory action of IFN-γ through deactivating JAK activity, leading to the reduction of Stat1 phosphorylation and subsequent GLα promoter activity.

These data suggest that IFN-γ can act as an inhibitory cytokine in TGF-β1-stimulated IgA isotype expression in murine B cells. However, we have recently demonstrated that IFN-γ, through the PKA/CREB pathway, can synergize with TGF-β1 in BAFF expression by murine macrophages (Kim et al., 2008). These results suggest that the function of IFN-γ in association with TGF-β1 varies between cellular contexts. Thus, functional outcomes of IFN-γ and TGF-β1 signaling seem to be dependent on availability and quantities of transducing mediators participating in the crosstalk between the two cytokines. Nevertheless, our results reveal that IFN-γ, a typical Th1 cytokine, has an important impact on TGF-β1-induced, Smads/Runx3-mediated IgA isotype switching through Stat1 and p300 signaling.

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