TGF-β Does Not Inhibit IL-12- and IL-2-Induced Activation of Janus Kinases and STATs

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The immune system is an important target for the cytokine TGF-β1, whose actions on lymphocytes are largely inhibitory. TGF-β has been reported to inhibit IL-12- and IL-2-induced cell proliferation and IFN-γ production by T cells and NK cells; however, the mechanisms of inhibition have not been clearly defined. It has been suggested by some studies that TGF-β blocks cytokine-induced Janus kinase (JAK) and STAT activation, as in the case of IL-2. In contrast, other studies with cytokines like IFN-γ have not found such an inhibition. The effect of TGF-β on the IL-12-signaling pathway has not been addressed. We examined this and found that TGF-β1 did not have any effect on IL-12-induced phosphorylation of JAK2, TYK2, and STAT4 although TGF-β1 inhibited IL-2- and IL-12-induced IFN-γ production. Similarly, but in contrast to previous reports, we found that TGF-β1 did not inhibit IL-2-induced phosphorylation of JAK1, JAK3, and STAT5A. Furthermore, gel shift analysis showed that TGF-β1 did not prevent activated STAT4 and STAT5A from binding to DNA. Our results demonstrate that the inhibitory effects of TGF-β on IL-2- and IL-12-induced biological activities are not attributable to inhibition of activation of JAKs and STATs. Rather, our data suggest the existence of alternative mechanisms of inhibition by TGF-β. The Journal of Immunology, 1999, 162: 2974–2981.

Interleukin-12 is a critical cytokine produced by phagocytic cells and APCs including monocytes/macrophages and dendritic cells in response to microbial pathogens. The biologic effects of IL-12 are exerted principally on T cells and NK cells and serve to promote cell-mediated immune response. IL-12 induces proliferation and IFN-γ secretion and enhances the cytolytic activity of NK cells and cytotoxic T cells. Importantly, it also drives the differentiation of naïve CD4+ cells to Th1 cells (1, 2). The role of IL-12 in cell-mediated immunity is exemplified by IL-12 knockout mice, which are impaired in their ability to generate a normal Th1 response and to produce IFN-γ (3).

IL-12 transmits signals by binding to a receptor complex composed of two subunits, β1 and β2 (4). Both subunits are required for the formation of high affinity receptors and subsequent signaling (4, 5). Like other type I cytokine receptors, IL-12 receptor subunits do not possess intrinsic tyrosine kinase activity but rather function by associating with the members of the Janus family of kinases (JAK) (6). IL-12 induces tyrosine phosphorylation of JAK2 and TYK2 (7), which in turn phosphorylate other substrates including the transcription factors STAT4 and STAT3 (8, 9). Activated STATs translocate to the nucleus and regulate gene transcription (6, 10). Whether STATs are directly involved in mediating the biological effects of IL-12, such as IFN-γ production, is presently not clear, and our knowledge of IL-12 target genes is far from complete.

While cytokines like IL-12 and IL-2 promote immune responses, other cytokines like TGF-β suppress immune cell function. TGF-β1 inhibits IL-2- and IL-12-induced effects, including proliferation, IFN-γ production, and the cytotoxic activity of NK cells and T cells (11–17). Deficiency of TGF-β1 results in severe immune dysregulation, as evidenced by TGF-β1 knockout mice, which develop severe inflammatory disease in several organs and die of overt autoimmune reaction (18, 19).

An issue of great importance is how the immunosuppressive effects of TGF-β are mediated. Although there is ample documentation of the inhibitory effects of TGF-β, an understanding of the mechanism by which these effects on immune cell function occur is lacking. Conceivably, one mechanism might be that TGF-β interferes with early signaling events induced by cytokines; specifically, it could be envisioned that TGF-β might inhibit JAK or STAT activation. However, several studies on the effects of TGF-β on activation of JAKs and STATs have provided conflicting results. TGF-β1 was shown to inhibit IL-5-induced activation of JAK2 and STAT1, and this was suggested to underlie the inhibitory effect of TGF-β on eosinophils (20). Similarly, TGF-β was reported to inhibit IL-2-induced activation of JAKs and STATs (21, 22), although the exact findings of these two studies were somewhat contradictory. Han et al. (22) reported inhibition of IL-2-induced phosphorylation of JAK1, JAK3, STAT5, and STAT3 by TGF-β1. However, Bright et al. (21) observed inhibition of only JAK1 and STAT5 and no inhibition of IL-2-induced phosphorylation of JAK3 or STAT3. In contrast, the suppression of IFN-γ-induced class II MHC gene expression by TGF-β2 was found not to be the result of inhibition of phosphorylation of JAKs or STATs (23), but rather due to inhibition of class II trans-activator (CIITA) (24, 25).

The effect of TGF-β on IL-12 signaling has not been previously reported. In light of the conflicting reports in different systems, it was of interest to address this issue. We found that TGF-β1 had no effect on IL-12-induced phosphorylation of JAK2, TYK2, and STAT4. We also found that TGF-β1 did not inhibit activated STAT4 from binding DNA. Thus, in contrast to what has been
reported previously for some cytokines, the inhibitory effect of TGF-β on IL-12-induced activities is not explained by an effect on activation of JAKs and STATs. More surprisingly, in contrast to previous reports, we found that TGF-β1 did not inhibit IL-2-induced activation of JAKs and STATs in human T cells and an NK cell line. These studies suggest that alternative mechanisms, beyond inhibition of proximal signaling elements, are likely to account for the critical immunologic functions of TGF-β.

**Materials and Methods**

Recombinant human TGF-β1 and IL-12, and human IFN-γ immunoassay kits were purchased from R&D Systems (Minneapolis, MN). Human IL-2 was provided by Dr. C. Reynolds (National Cancer Institute, Frederick, MD). Mouse anti-polyclonal Abs (clone 4G10) and polyclonal anti-JAK2 and -TYK2 Abs were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-TYK2 Ab was purchased from Transduction Laboratories (Lexington, KY). Polyclonal rabbit anti-STATA Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-JAK1 Ab was provided by Dr. A. Larner (Cleveland Clinic Foundation, Cleveland, OH). The rabbit polyclonal Abs against human JAK1 and STAT5A were raised in our laboratory (26, 27). RNA extraction kit (RNAgents Total RNA Isolation System, Promega, Madison, WI) and RiboQuant Multiprobe RNase Protection System (PharMingen, San Diego, CA) were purchased.

**Cell culture**

The IL-12-responsive human NK cell line NK3.3 was provided by Dr. J. Kombi (Arkansas Cancer Research Center, Little Rock, AR) and was cultured as described previously (7, 8). PBMC were isolated from peripheral blood of normal healthy donors by Ficoll-Paque gradient centrifugation, activated with PHA (2 μg/ml) for 72 h, and cultured for an additional day in presence of IL-2 (40 IU/ml). Typically, this resulted in >95% CD3+ cells. Before stimulation with cytokine, cells were washed with acidified PBS containing 1 mM EDTA and 2 mM Na3VO4 and incubated at 4°C for 30 min. The complexes were resolved on a 6% denaturing polyacrylamide gel, and transferred to Immobilon (Millipore, Bedford, MA). Immuno precipitation and immunoblotting

Immunoblotting with Abs to the JAKs or STATs was conducted by blocking the membrane in TBS containing 5% nonfat dried milk and 0.1% Tween and incubating sequentially with primary Ab, HRP-conjugated goat anti-rabbit IgG (Boehringer Mannheim) or HRP-conjugated sheep anti-mouse IgG and detected by use of ECL. Before reprobing, the membranes were stripped in 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 0.7% 2-ME at 70°C.

**Results**

**TGF-β1 inhibits the production of IFN-γ induced by IL-12 and IL-2**

The inhibitory effect of TGF-β1 on T cell proliferation and production of IFN-γ by IL-12 and IL-2 has been reported previously (11, 13, 15, 17, 32). To establish that our system responded appropriately to TGF-β1 and to verify the activity of TGF-β1 used, we treated NK3.3 cells with TGF-β1 and stimulated with varying concentrations of IL-12 or IL-2 for 24 h and assayed cell-free supernatants for IFN-γ. Consistent with previous findings, both IL-12 and IL-2 induced IFN-γ production in NK3.3 cells (Fig. 1, A and B). IL-12 stimulation resulted in modest induction of IFN-γ (Fig. 1, A and B) whereas IL-2 was a more potent inducer of IFN-γ production (Fig. 1B). When a combination of both IL-2 and IL-12 was used, a synergistic effect on IFN-γ production was observed (Fig. 1B). Treatment with TGF-β1 resulted in approximately 30–45% inhibition of IFN-γ induction by either IL-12 (Fig. 1, A and B) or IL-2 (Fig. 1B) or the combination of both (Fig. 1B). To confirm that similar results would be obtained with primary human cells, we determined the effect of TGF-β1 on activated T lymphocytes since activated, but not resting, T cells express IL-12 receptors (33). We found that both IL-2 and IL-12 induced IFN-γ production in activated T cells and that the combination of IL-2 and IL-12 was synergistic (Fig. 1C). Treatment with TGF-β1 resulted in partial inhibition of IFN-γ production by IL-2 or IL-12 separately or in combination (Fig. 1C).

We next determined whether TGF-β1 influenced the steady state levels of IFN-γ mRNA. To this end, we conducted RNase protection assay. As shown in Fig. 1D, stimulation with IL-2 or IL-12 (lanes 3 and 5, respectively) resulted in increased amounts of IFN-γ transcript as compared with control (lane 1). In accordance with the data shown in Fig. 1, A-C, IL-2 was a more potent

**Materials**

Total RNA was extracted from NK3.3 cells after preincubation of cells with TGF-β1 (10 ng/ml) for 15 min and stimulated with IL-2 or IL-12 for 4 h. RNase protection assay was conducted as follows. 32P-labeled RNA probe was synthesized by incubating probes from the multiprobe template set with T7 RNA polymerase for 1 h. DNA was digested with 2 units of DNase I (Boehringer Mannheim), and RNA probes were extracted with phenol and chloroform and precipitated with ethanol. Labeled RNA probes were hybridized overnight at 56°C with equal amounts of target RNA (5 μg), following which free probe and single-stranded RNA were digested with RNase (Life Technologies, Gaithersburg, MD). The protected mRNA fragment was extracted with phenol and chloroform, precipitated with ethanol, resolved on 6% denaturing polyacrylamide gel, and subjected to autoradiography. **RNase protection assay**

Cell extracts were prepared from cytokine-stimulated NK3.3 cells, and RNase was provided as described (30, 31), using a 32P-labeled double-stranded oligonucleotide corresponding to the GRRI of the human FcγRI (5′-AGCATGGTTCAGTTGATGATTCCCGAAGAAG-3′) for IL-12 studies and the GAS-like element of the CD23 promoter (5′-AAGACCTATTTAAGAAATCTTAC-3′) for IL-2 studies. Cell extracts were incubated with the labeled oligonucleotide for 20 min at room temperature. In supershift assays, this complex was further incubated with anti-STATA Ab (5 μl) at 4°C for 30 min. The complexes were electrophoresed through a 4.5% nondenaturing acrylamide gel and subjected to autoradiography.

**Preparation of cytoplasmic and nuclear extracts**

Cytoplasmic and nuclear extracts were prepared as previously described (28) with some modifications. Cytokine-stimulated cells were washed in 1× PBS containing 1 mM EDTA and 2 mM Na3VO4 and incubated at 4°C for 5–10 min in 1 ml of hypotonic solution (20 mM HEPES (pH 7.9), 10 mM KCl, 1 mM EDTA, 1.5 mM MgCl2, 10% glycerol, 0.2% Nonidet P-40, 2 mM Na3VO4, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM DTT). Cells were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were transferred to a microfuge tube and centrifuged at 1500 rpm for 5 min. Supernatants were saved and used as cytoplasmic extract. The crude nuclei were washed twice with 200 μl of hypotonic solution and centrifuged at 1500 rpm for 3 min. The nuclei were then resuspended in high salt buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 20% glycerol, 2 mM Na3VO4, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM DTT) and incubated at 4°C for 30 min, followed by incubation at room temperature for 10 min. Two hundred units of DNase I (Boehringer Mannheim, Indianapolis, IN) were added and incubated at room temperature for 10 min. The samples were centrifuged at 14,000 rpm at 4°C for 5 min, and supernatants were collected as nuclear extracts.

**Immunoprecipitation and immunoblotting**

Rested cells (3 × 107 NK3.3 cells or 5 × 107 T cells) were incubated at 37°C with TGF-β1 (10 ng/ml) in 12 ml media for various times, followed by stimulation with IL-2 or IL-12 for 15 min at 37°C. Stimulated cells were washed and lysed with buffer containing 0.5% Triton X-100 (29). Clarified lysates were immunoprecipitated with either anti-STATA, -STAT5A, -JAK1, -JAK2, -TYK2, or -JAK3 antisera conjugated to protein A-coupled Sepharose beads. The immunoprecipitates were resolved on 8% SDS-polyacrylamide gel and transferred to Immobilon (Millipore, Bedford, MA).

Immunoblotting with antiphosphotyrosine was conducted after blocking the membrane in Tris-buffered saline containing 1% fish gelatin, 2% goat serum, 0.1% BSA, and 0.5% Tween by sequential incubation with antiphosphotyrosine Ab, biotinylated goat anti-mouse IgG, and HRP-conjugated streptavidin (Oncogene Science, Cambridge, MA). The phosphoproteins were detected by use of enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL).
inducer of IFN-γ transcripts than IL-12, and the combination of IL-12 and IL-2 showed a synergistic effect. Treatment with TGF-β1 resulted in a decrease in the induction of IFN-γ mRNA by IL-2 and IL-12 separately (lanes 4 and 6, respectively) or in combination (lane 8). Equal amounts of GAPDH and L32 transcripts confirmed that equal amounts of target RNA was used. Thus, TGF-β1 inhibits steady state levels of IFN-γ message. To confirm that the TGF-β1 used in our experiments was active, we also conducted a standard growth inhibition assay of CCL64 mink lung epithelial cells and found that TGF-β1 inhibited maximal growth at concentration below 0.5 ng/ml (data not shown).

TGF-β1 does not inhibit IL-12-induced phosphorylation of JAK2 and TYK2

One mechanism by which TGF-β1 could inhibit the function of IL-12 is by inhibition of the early signaling steps regulated by IL-12. The most proximal event thus far defined in IL-12 signaling is the phosphorylation of JAK2 and TYK2 (7). Therefore, we first looked to see whether the phosphorylation of these kinases was inhibited by TGF-β1. NK3.3 cells were treated with TGF-β1 for various times and stimulated with IL-12. Cell lysates were immunoprecipitated with Abs to JAK2 or TYK2, and the immunoprecipitates were blotted with anti-phosphotyrosine. As seen in Fig. 2, stimulation of cells with IL-12 resulted in phosphorylation of JAK2 (lane 2), while treatment with TGF-β1 alone did not result in phosphorylation of JAK2 (lane 8). Preincubation of cells with TGF-β1 for 10 min to 18 h had no effect on phosphorylation of JAK2 (Fig. 2A, lanes 3-7). Similarly, IL-12-induced phosphorylation of TYK2 (Fig. 2B, lane 2), and treatment of cells with TGF-β1 had no effect on IL-12-induced phosphorylation of TYK2 (Fig. 2B, lanes 3-7). To confirm that equal amounts of JAK2 or TYK2 were loaded, the above blots were stripped and rebotted with Abs to JAK2 or TYK2 (Fig. 2, A and B, lower panels). To be certain that this was also true in primary human cells, we conducted the same experiment in activated T cells and found no inhibition of IL-12-induced phosphorylation of TYK2 (Fig. 2C) or JAK2 (data not shown) by TGF-β1. These data demonstrate that the proximal event in IL-12-induced JAK-STAT pathway, namely, phosphorylation of JAK2 and TYK2, was unaffected by TGF-β1; furthermore, the expression of these proteins was neither inhibited nor enhanced by TGF-β1.

TGF-β1 does not inhibit phosphorylation of STAT4 in response to IL-12

Activated JAKs phosphorylate cytokine receptors, thus providing docking sites for STATs (34, 35). STATs are also phosphorylated by JAKs, allowing them to dimerize, translocate to the nucleus, and activate gene transcription (6, 10). Since the initial events in IL-12 signaling were not inhibited by TGF-β1, we looked to see whether a subsequent downstream signaling event, namely, the phosphorylation of STAT4, was inhibited by TGF-β1. As shown

![FIGURE 1. Inhibition of IL-12- and IL-2-induced IFN-γ production by TGF-β1.](image)

A, NK3.3 cells (1 × 10⁶) were treated with TGF-β1 (10 ng/ml) for 15 min followed by stimulation with varying concentrations of IL-12 for 24 h. Cell-free supernatants were assayed for IFN-γ. One representative experiment of the three conducted at different times is shown here. B, NK3.3 cells or (C) activated T cells (1 × 10⁶) were treated with TGF-β1 (10 ng/ml) for 15 min followed by stimulation with IL-12, IL-2, or IL-12 and IL-2 in combination for 24 h. Cell-free supernatants were assayed for IFN-γ. One representative experiment of the three conducted at different times is shown here. D, NK3.3 cells were preincubated with or without TGF-β1 (10 ng/ml) for 15 min and stimulated with IL-2 (1000 IU/ml), IL-12 (10 ng/ml), or both in combination for 4 h. mRNA was prepared from each of these, and RNase protection assay was conducted as described in Material and Methods.
inhibit IL-12-induced phosphorylation of STAT4 (Fig. 3A, lanes 3-8) and stimulated with IL-12 (10 ng/ml) (lanes 2-7) for 15 min. Cell lysates were immunoprecipitated with anti-JAK2 Ab (A) or anti-TYK2 Ab (B and C) and immunoblotted with anti-phosphotyrosine Ab (A, B, and C, upper panels). The blots were stripped and rebotted with anti-JAK2 antiserum (A, lower panel) or anti-TYK2 antiserum (B and C, lower panels).

FIGURE 3. IL-12-induced phosphorylation of STAT4 is not inhibited by TGF-β1. A, NK3.3 cells or (B) activated T cells were either untreated (lane 1) or treated with TGF-β1 (10 ng/ml) for the indicated times (lanes 3-8) and stimulated with IL-12 (10 ng/ml) (lanes 2-7) for 15 min. C, NK3.3 cells were treated with varying concentrations of TGF-β1 for 4 h (lanes 2-4, 6-8, 10-12, and 14-16) and stimulated with varying concentrations IL-12 (lanes 5-16) for 15 min as indicated. D, NK3.3 cells were treated with TGF-β1 (lanes 2, 4, 6, 8, 10, 12, and 14) for 30 min and stimulated with IL-12 (10 ng/ml) (lanes 3-14) for times as indicated. Cell lysates were immunoprecipitated with antiserum against STAT4, immunoblotted with anti-phosphotyrosine Ab (A, B, C, and D, upper panels) and stripped and rebotted with anti-STAT4 antiserum (A, B, C, and D, lower panels).

in Fig. 3, stimulation of cells with IL-12 resulted in phosphorylation of STAT4 (lane 2), and, as expected, treatment with TGF-β1 alone did not result in phosphorylation of STAT4 (lane 8). Incubation of cells with TGF-β1 for 10 min to 18 h followed by stimulation with IL-12 did not inhibit phosphorylation of STAT4 (Fig. 3A, lanes 3-7) as compared with control cells (Fig. 3A, lane 2). That the levels of STAT4 in these immunoprecipitates were equal was determined by stripping the blot and reblotting with anti-STAT4 Ab (Fig. 3A, lower panel). Thus, treatment with TGF-β1 affected neither the level of expression of STAT4 nor its phosphorylation. As before, we also conducted the same experiment with activated human T cells and found that TGF-β1 did not inhibit IL-12-induced phosphorylation of STAT4 (Fig. 3B, lanes 3-7).

To further confirm the lack of effect of TGF-β1 on IL-12-induced STAT4 phosphorylation, we assessed the effect of varying concentrations of both IL-12 and TGF-β1. As seen in Fig. 3C, IL-12-induced phosphorylation of STAT4 (Fig. 3C, lanes 5, 9, and 13) and treatment with various concentrations of TGF-β1 had no effect on IL-12-induced phosphorylation of STAT4, regardless of the amount of IL-12 (Fig. 3C, lanes 6-8, 10-12, and 14-16). We further tested a potential effect of TGF-β1 on a broad time course of STAT4 activation. NK3.3 cells were treated with TGF-β1 for 30 min followed by stimulation with IL-12 for various times (15 min-22 h). As shown in Fig. 3D, STAT4 is rapidly phosphorylated by IL-12, and this persists up to 22 h (lanes 3, 5, 7, 9, 11, and 13); treatment with TGF-β1 (lanes 4, 6, 8, 10, and 14) did not inhibit IL-12-induced phosphorylation of STAT4. Thus TGF-β1 did not inhibit IL-12-induced STAT4 phosphorylation at any dose that we tested nor did it alter the kinetics of STAT4 activation.

We have previously shown that STAT4 undergoes serine phosphorylation in response to stimulation with IL-12; this is manifested by a retardation in mobility and may be important in regulating transcriptional activation (36). Therefore, we considered the possibility that TGF-β1 might inhibit serine phosphorylation of STAT4, even though tyrosine phosphorylation of STAT4 was not inhibited by TGF-β1. As seen in Fig. 3, A and B (lanes 3-7), and Fig. 3C (lanes 6-8, 10-12, and 14-16) the appearance of the slower
migrating form of STAT4 was not affected by TGF-β1 in NK3.3 cells and primary T cells. Furthermore, TGF-β1 failed to inhibit IL-12-dependent trans-activation of a STAT reporter construct (data not shown), an event that is thought to be dependent upon tyrosine and serine phosphorylation (36). These data argue against TGF-β1 inhibition of serine phosphorylation of STAT4. However, in view of the fact that sites of serine phosphorylation on STAT4 have not been mapped, we cannot exclude with certainty that TGF-β1 might affect this modification.

Activation of STATs by tyrosine phosphorylation is necessary for dimerization and binding to DNA (37, 38). Since IL-12-induced phosphorylation of STAT4 was not inhibited by TGF-β1, we expected that TGF-β1 would have no effect on IL-12-induced DNA binding complex. As further proof of the lack of effect of TGF-β1 on IL-12-mediated STAT activation, we conducted an EMSA. Extracts from cells stimulated with IL-12 showed binding to a 32P-labeled double-stranded oligonucleotide corresponding to the GRR of the FcyRII (Fig. 4A, lane 3) while unstimulated cells did not (Fig. 4A, lane 1). Supershift of the GRR binding complex on addition of antiserum to STAT4 showed that the DNA binding complex contained STAT4 (Fig. 4A, lanes 4, 6, 8, 10, and 12). Treatment with TGF-β1 for varying times before stimulation with IL-12 failed to inhibit the formation of STAT4 GRR complex (Fig. 4A, lanes 5, 7, 9, and 11). We also considered the possibility that TGF-β1 might inhibit nuclear targeting of STAT4. To investigate this, NK3.3 cells were pretreated with TGF-β1 and stimulated with IL-12 (Fig. 4, B and C). STAT4 was immunoprecipitated from cytoplasmic and nuclear extracts, and the immunoprecipitates were blotted with anti-phosphotyrosine. As seen in Fig. 4, B and C, unstimulated cells showed minimal phosphorylation of STAT4 in both cytoplasmic and nuclear extracts. Importantly, most of STAT4 protein was present in the cytoplasmic extract of unstimulated cells (Fig. 4, B and C, lane 1), with negligible amounts in the nuclear extract (Fig. 4, B and C, lane 5) whereas, upon stimulation, STAT4 was readily detected in the nucleus (Fig. 4B, lane 6, and 4C, lane 7). On stimulation with IL-12, phospho-STAT4 was detected in both cytoplasm and nucleus. As seen in Fig. 4, B and C, pretreatment with TGF-β1 did not prevent nuclear translocation or phosphorylation of STAT4 at times ranging from 15 min to 24 h. Taken together, the data presented in Figs. 2, 3, and 4 clearly argue that TGF-β1 does not directly inhibit IL-12-induced activation of JAKs and STATs.

TGF-β1 does not inhibit IL-2-induced phosphorylation of JAK3, JAK1, and STAT5

Since previous reports had suggested that IL-2-induced phosphorylation of JAKs and STATs could be inhibited by TGF-β1 (21, 22), we sought to determine whether IL-12 and IL-2 signaling differed in this respect. We therefore investigated the effect of TGF-β1 on the IL-2-induced phosphorylation of JAK3, JAK1, or STAT5. As shown in Fig. 5, A and B, IL-2-induced phosphorylation of JAK3 and JAK1 (Fig. 5, A and B, respectively, lane 2). Treatment with TGF-β1 for varying times, however, did not result in inhibition of IL-2-induced phosphorylation of either JAK3 or JAK1 (Fig. 5, A and B, respectively, lanes 3-7). Similarly, IL-2-induced phosphorylation of STAT5A (Fig. 5C, lane 2). Treatment with TGF-β1 did not result in inhibition of IL-2-induced phosphorylation of STAT5A (Fig. 5C, lanes 3-6). Since these results in NK cells contradicted previous reports (21, 22), we sought to confirm the result in primary human T cells. As shown in Fig. 5, IL-2 induced phosphorylation of JAK3 and JAK1 (lane 2 in Fig. 5, D and E, respectively). Treatment with TGF-β1 did not result in inhibition of IL-2-induced phosphorylation of either JAK3 or JAK1 (Fig. 5, D and E, respectively, lanes 3-7) in T cells. Further, TGF-β1 did not affect IL-2-induced phosphorylation of STAT5A in primary T cells (Fig. 5F, lanes 3-7).

Since phosphorylation of JAK1, JAK3, and STAT5A was not inhibited by TGF-β1, we conducted EMSA to confirm the lack of effect of TGF-β on the binding of STAT5A to DNA. Extracts from cells stimulated with IL-2 showed binding to 32P-labeled GRR probe. For supershift assays, 5 μl of anti-STAT4 Ab was added after incubation of the probe with cell extracts (lanes 2, 4, 6, 8, 10, and 12). B, NK3.3 cells were untreated (lanes 1 and 5) or treated with TGF-β1 (10 ng/ml) (lanes 3-12) for 15 min. Whole cell lysates were prepared, and EMSA was conducted using 32P-labeled GRR probe. For supershift assays, 5 μl of anti-STAT4 Ab was added after incubation of the probe with cell extracts (lanes 2, 4, 6, 8, 10, and 12). B, NK3.3 cells were untreated (lanes 1 and 5) or treated with TGF-β1 (10 ng/ml) (lanes 3, 4, 7, and 8) and stimulated with IL-12 for 15 min. C, NK3.3 cells were untreated (lanes 1 and 5) or treated with TGF-β1 (10 ng/ml) (lanes 2, 4, 6, and 8) for 30 min and stimulated with IL-12 for 24 h. STAT4 was immunoprecipitated from cytoplasmic and nuclear extracts, immunoblotted with anti-phosphotyrosine (A and B, upper panels) and stripped and rebotted with anti-STAT4 antisera (A and B, lower panels).

![FIGURE 4. IL-12-dependent STAT4 binding to DNA and nuclear translocation of activated STAT4 is not inhibited by TGF-β1.](image)

- **A**: IL-12-induced STAT4 binding to DNA in the absence (lane 1) or presence of TGF-β1 (lane 2) for 15 min. Whole cell lysates were prepared, and EMSA was conducted using 32P-labeled GRR probe. For supershift assays, 5 μl of anti-STAT4 Ab was added after incubation of the probe with cell extracts (lanes 2, 4, 6, 8, 10, and 12). B, NK3.3 cells were untreated (lanes 1 and 5) or treated with TGF-β1 (10 ng/ml) (lanes 3-12) for 15 min. Whole cell lysates were prepared, and EMSA was conducted using 32P-labeled GRR probe. For supershift assays, 5 μl of anti-STAT4 Ab was added after incubation of the probe with cell extracts (lanes 2, 4, 6, 8, 10, and 12).
- **B**: IL-12-induced STAT4 expression in nuclear extracts. Extracts from cells stimulated with IL-12 showed binding to 32P-labeled oligonucleotide corresponding to the GAS (Fig. 6, lane 2), while unstimulated cells did not (Fig. 6, lane 1). Treatment with TGF-β1 for the indicated times before stimulation with IL-2 did not inhibit formation of a GAS binding complex (Fig. 6, lanes 3-8). These complexes were supershifted by addition of antisera to STAT5A (data not shown), confirming that the GAS binding complex contained STAT5A. These data showed that phosphorylated STAT5A, like STAT4, was capable of forming a DNA binding complex following treatment with TGF-β1. We further looked to see whether IL-2-induced STAT-mediated gene transcription was inhibited by TGF-β1, by using a luciferase reporter construct under the control of the STAT binding site and minimal promoter.
We found that TGF-β1 had no effect on IL-2-induced transcription of the reporter gene (data not shown). Thus, the data presented in Figs. 5 and 6 indicate that TGF-β1 does not inhibit IL-2-induced activation of the early events in IL-2 signaling, namely, JAK and STAT phosphorylation and activated STAT binding to DNA.

**Discussion**

Although many studies have documented the inhibition of IL-2- and IL-12-induced effects on T cells and NK cells by TGF-β1, the mechanism(s) of this inhibition is poorly understood. In this study, we investigated the effect of TGF-β1 on early events in IL-12 and IL-2 signaling, specifically, the activation of JAK2, TYK2, and STAT4. Since we have not found STAT3 to be activated by IL-12 in NK3.3 (39) and in human T cells (data not shown), we have not addressed its role in mediating the inhibitory effect of TGF-β1. Our data indicate that TGF-β1 does not inhibit IL-12-induced phosphorylation of JAK2, TYK2, or STAT4 or the binding of STAT4 to DNA. Further, our data also show that IL-2-induced phosphorylation of JAK1, JAK3, or STAT5A is not inhibited by TGF-β1 in NK3.3 cells or primary human T cells; TGF-β1 also does not inhibit the formation of an IL-2-induced DNA binding complex. Thus, the inhibitory effects of TGF-β1 on IL-12- and IL-2-induced biological activities is unlikely to be due to inhibition of JAK or STAT activation.

We thought it important to communicate these findings in light of the contradictory findings pertaining to the effect of TGF-β on JAKs and STATs. For IL-5, TGF-β1 appears to inhibit phosphorylation of JAK2 and STAT1 in eosinophils (20). For IFN-γ, the suppression of IFN-γ-induced MHC class II gene expression by TGF-β2 evidently does not involve the inhibition of phosphorylation of JAKs and STATs but rather involves the inhibition of CIITA (24, 25). Studies on the effect of TGF-β on IL-2-mediated activation of JAKs and STATs have provided even more contradictory information. Bright et al. (21) showed the inhibition of IL-2-induced phosphorylation of JAK1, JAK3, or STAT5A in Con A-activated murine T cells but found no effect on phosphorylation of JAK3 or STAT3. In contrast, Han et al. (22) showed the inhibition of IL-2-induced phosphorylation of JAK1, JAK3, STAT5A, and STAT3 in Con A blasts of nonobese diabetic mouse splenocytes. We, however, found no significant inhibition of phosphorylation of any of these proteins by TGF-β1 in human primary T cells and an NK cell line.

It is difficult to offer a clear explanation for the difference between our data on primary human T cells and the above described
LACK OF INHIBITION OF JAKs AND STATs BY TGF-β1

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