

# The effects of interleukin-6, leukemia inhibitory factor and interferon-gamma on STAT DNA binding and c-fos mRNA levels in cortical astrocytes and C6 glioma cells

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## Abstract

**OBJECTIVES:** The purpose of this study was to compare the effects of neurotrophic cytokines on the JAK/STAT transcription factors and the immediate early gene, *c-fos*, in C6 glioma cells and in primary astrocyte cultures.

**METHODS:** In this study we compared the effects of interleukin 6, leukemia inhibitory factor and interferon gamma on C6 glioma cells and on primary astrocyte cultures using electrophoretic mobility shift assay and quantitative solution hybridization/Northern Blot analysis.

**RESULTS:** We show that interleukin 6, leukemia inhibitory factor and interferon gamma treatment induce the nuclear STAT3 and STAT1 proteins to bind to the *sis* inducible element of *c-fos* in both C6 glioma cells and primary cortical astrocytes. Furthermore, quantitative solution hybridization and Northern blot analysis show the differential regulation of *c-fos* mRNA levels by interleukin-6 (8.1 and 4.0 folds) and leukemia inhibitory factor (5.4 and 3.2 folds) in C6 and astrocyte cultures (respectively). However, interferon gamma increases in *c-fos* mRNA levels (2.9 fold in both C6 and astrocyte cultures) were not significant in a one way ANOVA.

**CONCLUSION:** This study suggests that two initial cytokine signaling pathways are present and functional in C6 glioma cells and in primary astrocyte cultures.

## Introduction

Interleukin 6 (IL-6) and Leukemia inhibitory factor (LIF), are multifunctional members of the gp130 cytokine family, that include oncostatin M (OSM), and ciliary neurotrophic factor (CNTF). These cytokines are synthesized by a variety of cells and have broad range of biological activities, that include stimulation of acute-phase proteins in hepatocytes, hematopoietic cell development, and regulation of neuronal and astrocytic development, differentiation, and inflammation [1–6]. Through the activation of JAK/STAT proteins in neuroblastoma cell lines, in primary hippocampal neurons, in sympathetic ganglia, and following transient ischemia in cortex and striatum astrocytes [7–11], these cytokines regulate the expression of many genes, including *c-fos*, *jun B*, opioid peptides and receptors, neuropeptides, and nitric oxide synthase [12–20]. In this study we compare the effects of IL-6, LIF and interferon gamma (IFN- $\gamma$ ) on activation of a functional JAK/STAT/*c-fos* signaling pathway in C6 glioma cell line and in primary cortical astrocyte cultures.

## Materials and methods

C6 glioma cells were obtained from American Type Tissue Collection (Manassas, VA) and grown in DMED with 10% fetal bovine serum (Life Technologies, NY). Primary astrocyte cultures were established from the cortex of one day old Fischer rats (Charles River, NY), as previously reported [21–23]. Briefly, the brains of rat pups were removed, cortex dissected, and seeded in 6 well tissue culture plates in DME medium containing 10% fetal bovine serum (Life Technologies, NY). At confluence (2–3 weeks), greater than 98% of the cells were astrocytes as determined by immunocytochemistry for glial fibrillary acidic protein (GFAP) [21]. For cytokine treatments, the cells were grown in serum-free media for 3 hrs before the addition of IL-6 (R&D System, Minneapolis, MN), LIF (R&D System, Minneapolis, MN), or IFN- $\gamma$  (Genzyme, Cambridge, MA). Duplicate or triplicate culture dishes were used for each drug treatment and were repeated at least once. Procedures involving the use of animals followed the *Guidelines for the Care and Use of Laboratory Animals* set forth by the NIH.

Nuclear extracts were prepared after 15 min of vehicle (0.1% BSA) or cytokine addition as described previously [12;13]. The m67, an oligonucleotide that is similar to the sis inducible element of *c-fos* gene [24], was incubated with the nuclear extracts (5–10  $\mu$ g) in 20 mM Hepes (7.9), 40 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.5 mM DTT and 4% Ficoll for 20 min at room temperature with the probe. The reaction products were fractionated on a 4% nondenaturing polyacrylamide gel. Antisera to STAT1 and STAT3 proteins (Santa Cruz Biotech., Santa Cruz, CA) were preincubated with the extracts for 10 min before the addition of the probe.

Forty five minutes after the addition of vehicle (0.1% BSA) or cytokines to the cultures, total RNA was extracted using Trizol reagent (Life Technologies, Grand Island, NY). Northern blot analysis was performed using 20  $\mu$ g of total RNA by the glyoxal method [12;13]. *C-fos* mRNA levels were measured by solution

hybridization assay as previously described [12;13]. Briefly, aliquots of total RNA extracts were hybridized to P<sup>32</sup>-riboprobes overnight and the mixture was then subjected to ribonuclease A and T1 treatment for 1 hr. Comparisons were made with *c-fos* standard calibration curves to quantify the levels of *c-fos* mRNA. The data were subjected to a one-way analysis of variance (ANOVA) with subsequent group comparisons using the Dunnett test at the 0.05 level of significance.

## Results

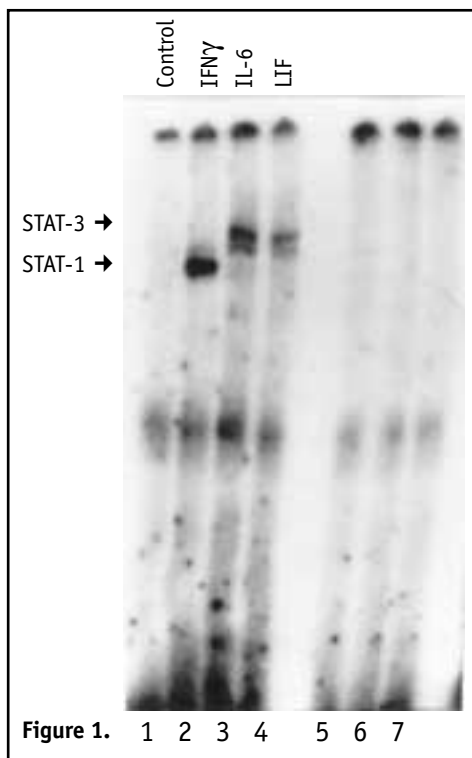
In electromobility shift assay (EMSA) analysis of C6 glioma cell extracts, IL6 and LIF activated STAT3 (and some STAT 1) protein (*Fig. 1*, lanes 3 and 4, respectively) while IFN- $\gamma$  induced STAT1 protein to bind to the m67 oligonucleotide (*Fig. 1*, lane 2) as compared to vehicle treated cultures (*Fig. 1*, lane 1). In competition analysis, the addition of 50X unlabeled oligonucleotide abolished the STAT3/STAT-1 signals (*Fig. 1*, lanes 5–7). The STAT3 proteins were further supershifted by anti-STAT3 antisera (*Fig. 2*, lane 6) but not with anti-STAT1 antisera (*Fig. 2*, lane 5) as compared to control extracts (*Fig. 2*, lane 4). The STAT1 protein complex was slightly supershifted with anti-STAT1 antisera (*Fig. 2*, lane 2) but not with STAT3 antisera (*Fig. 2*, lanes 3) as compared to control extracts (*Fig. 2*, lane 1).

Similarly, in EMSA analysis of astrocyte nuclear extracts, IL-6 and LIF activated STAT3 protein (*Fig. 3*, lanes 3 and 4, respectively) while IFN- $\gamma$  activated STAT1 protein (*Fig. 3*, lane 2) to bind to the m67 oligonucleotide as compared to vehicle treated cells (*Fig. 3*, lane 1). The STAT3 protein complex was further supershifted by anti-STAT3 antisera (\*, *Fig. 3*, lane 5).

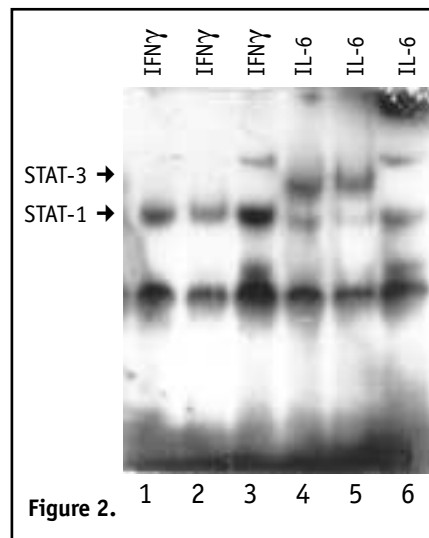
To correlate the activation of STAT proteins with transcriptional regulation, Northern blot and quantitative solution hybridization assays were used to analyze the steady state levels of *c-fos* mRNA after cytokine stimulations. *Figure 4* shows a representative Northern blot analysis of *c-fos* mRNA levels after vehicle (lane 1), IFN $\gamma$  (lane 2), IL6 (lane 3), or LIF (lane 4) treatments that identified a 2.1 kb transcript in the C6 cells. *Figure 5* and *6* show the result of a quantitative solution hybridization assay using the same riboprobe as in the Northern blot analysis. The cytokines differentially induced *c-fos* mRNA levels, whereas IL-6 stimulated *c-fos* mRNA levels by 8.1 and 4.0 folds and LIF increased the levels by 5.4 and 3.2 folds in C6 (*Fig. 5*) and astrocyte culture (*Fig. 6*), respectively. However, IFN $\gamma$  increases in *c-fos* message (by 2.9 folds in both C6 and astrocyte cultures) was not significant in a one way ANOVA statistical analysis.

## Discussion

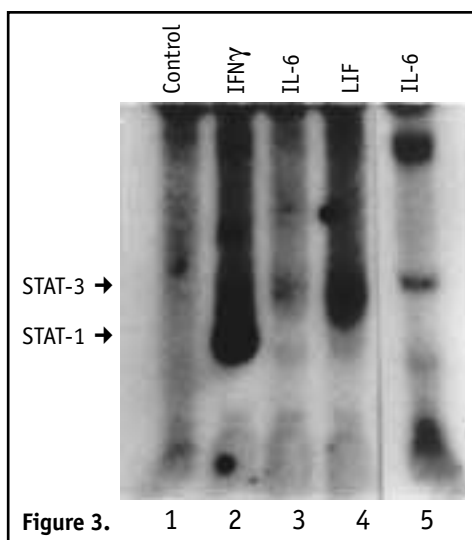
Within the CNS several IL-6 related cytokines modulate astrocyte proliferation, differentiation and survival. IL-6 and CNTF levels rapidly increase in damaged and diseased neural tissues that follows by an increase in astrocyte numbers [9;25]. Through several molecular mechanisms, including the JAK/STAT and the MAP kinase pathways, these cytokines may enable resting astrocytes to proliferating astro-



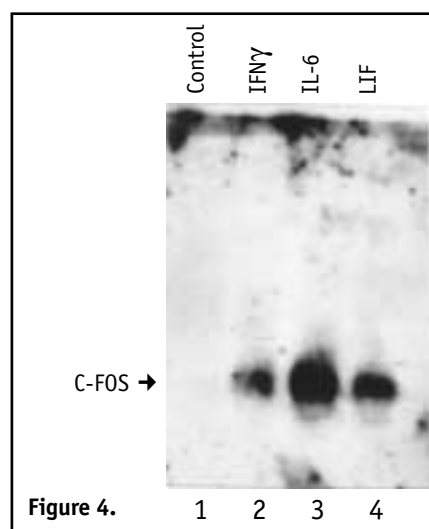
**Figure 1.** Cytokine activation of STAT proteins in C6 cells. C6 cells were treated with vehicle (lane 1), IL-6 (lane 3 and 6), LIF (lane 4 and 7), or IFN $\gamma$  (lane 2 and 5) for 15 min and nuclear extracts prepared for EMSA. IL-6 and LIF activate STAT3 protein, while IFN $\gamma$  activate STAT1 to bind to the m67 oligonucleotide. In competition analysis, the addition of 50X unlabeled oligonucleotide abolished the STAT3/STAT-1 signals (lanes 5-7).



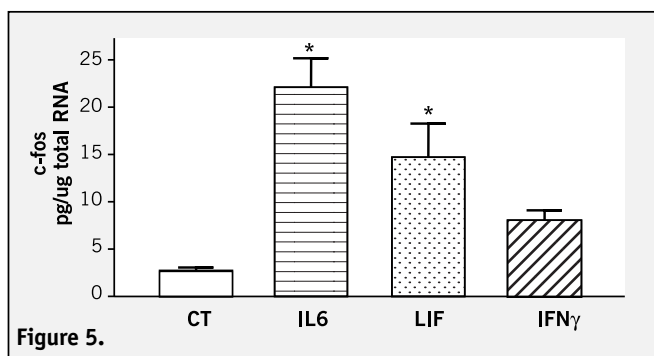
**Figure 2.** Supershift analysis using STAT1 or STAT3 antisera. The STAT3 proteins were supershifted by anti-STAT3 antisera (lane 6) but not with anti-STAT1 antisera (lane 5) as compared to control (lane 4). The STAT1 protein complex was slightly supershifted with anti-STAT1 antisera (lane 2) but not with STAT3 antisera (lane 3) as compared to control (lane 1).



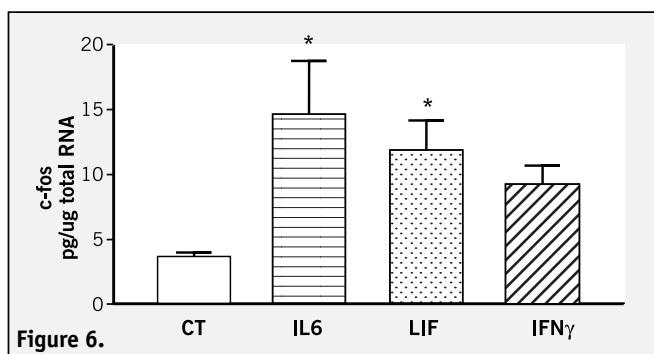
**Figure 3.** Cytokine activation of STAT proteins in astrocyte cultures. EMSA analysis of astrocyte nuclear extracts showing that IL-6 and LIF activated STAT3 protein (lanes 3 and 4, respectively) while IFN- $\gamma$  activated STAT1 protein (lane 2) to bind to m67 oligonucleotide as compared to vehicle treated cells (lane 1). The STAT3 protein complex was further supershifted by anti-STAT3 antisera (asterisks, lane 5).



**Figure 4.** A representative Northern blot analysis of *c-fos* mRNA levels after vehicle (lane 1), IFN $\gamma$  (lane 2), IL-6 (lane 3), or LIF (lane 4) treatments of C6 cells that identified a 2.1 kb transcript.



**Figure 5.** Effects of IL-6, LIF or IFN $\gamma$  on *c-fos* mRNA levels in C6 cells. C6 cells were treated with vehicle, IL-6, LIF, or IFN $\gamma$  for 45 minutes and the mean ( $\pm$ SEM) values of *c-fos* mRNA levels are shown using a quantitative solution hybridization assay. The asterisks indicate significant changes compared to vehicle treated cells using an ANOVA with subsequent group comparisons using the Dunnett test at the 0.05 level of significance (IL-6, LIF).



**Figure 6.** Effects of IL-6, LIF or IFN $\gamma$  on *c-fos* mRNA levels in astrocyte cultures. Astrocyte cultures were treated with vehicle, IL-6, LIF, or IFN $\gamma$  for 45 minutes and the mean ( $\pm$ SEM) values of *c-fos* mRNA levels are shown using a quantitative solution hybridization assay. The asterisks indicate significant changes compared to vehicle treated cells using an ANOVA with subsequent group comparisons using the Dunnett test at the 0.05 level of significance (IL-6, LIF).

cytes [4–6;25]. While recent studies have shown that astrocytes express jak1 and cerebral ischemia induces STAT3 in astrocytes[9], our data directly shows that addition of IL-6, LIF and IFN- $\gamma$  activate STAT3/STAT1/*c-fos* pathways in both primary cortical astrocytes and in C6 glioma cells. The activation of these proteins may function as one of the initiating signals for astrocytic proliferation and differentiation. It is not known if these cytokines also activate the MAP kinase pathway in these cells [5;6].

The members of AP-1 pathway, including *c-fos*, are also regulated by these cytokines in many cells and have been associated with cell cycle, proliferation and differentiation [26]. *c-fos* is induced in the nervous system in response to a large variety of physiological and pathological stimuli, in glia following a hippocampal injury, in focal brain injury of neocortical neurons, and after IL-1 injection to the CNS [27–31]. In astrocytes and endocrine cells several cytokines including IL-1 $\beta$  and IL-6 family members also differentially alter the expression of AP-1, opioids and neuropeptides genes [14–16;18]. Similarly, in our study *c-fos* mRNA levels were differentially effected in astrocytes and C6 cells. Further studies are currently in progress to analyze the effects of these cytokines on AP-1 transcription factors.

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