

Mechanism of Interferon-gamma mediated down-regulation of Interleukin-10 gene expression

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Abbreviations

Stat = signal transducer and activator of transcription

ChIP = chromatin-immuno-precipitation

IFN γ = Interferon-gamma

Abstract

Expression of the anti-inflammatory cytokine IL-10 is suppressed by the pro-inflammatory interferon γ but the mechanism of this action is unknown. We analysed activity of IL-10 promoter luciferase reporter constructs in transfected RPMI 8226.1 B cells that were treated at -2h with IFN γ (1000U/ml) followed by stimulation with LPS (100 ng/ml) at 0h. IFN γ treatment suppressed LPS-induced IL-10 promoter activity in a construct carrying the -1044 promoter and also one containing the -195 promoter. The suppression was independent of the IRF-motif at -182 but involved the Stat-motif at -120. In gelshift analysis this Stat motif did bind LPS-induced Stat3 and with IFN γ treatment it did, in addition, bind Stat1. ChIP analysis for detection of transcription factor binding to chromatin in intact cells demonstrated Stat3 binding to the proximal IL-10 promoter when cells are stimulated with LPS only. Treatment with IFN γ only led to Stat1 binding in ChIP analysis and treatment with IFN γ plus LPS led to reduced Stat3 binding while Stat1 binding remained high. Finally, LPS-induced activity of the trimeric Stat-motif in front of the luciferase reporter was suppressed by IFN γ . These data demonstrate that IFN γ down-regulates expression of the IL-10 gene by a novel mechanism that involves displacement of transactivating Stat3 by IFN γ -induced Stat1.

Introduction

IL-10 is a major immunosuppressive cytokine produced by T-cells, B-cells, dendritic cells and monocytes / macrophages (Fiorentino et al., 1989) (de Waal Malefyt et al., 1992) (Chang et al., 2000). The immunosuppressive activity of IL-10 is highlighted by the findings that IL-10 knock-out mice show spontaneous autoimmune disease and increased resistance to infection (Bettelli et al., 1998) (Dai et al., 1997).

IL-10 acts by binding to the IL-10R1 / IL-10R2 receptor complex, which recruits the kinases Jak1 and Tyk2 and these then phosphorylate and activate Stat3 and -1 transcription factors (Donnelly et al., 1999) (Williams et al., 2004). IL-10 can suppress the immune response via this signalling cascade by decreasing cell surface expression of MHC class II and by down-regulating the expression of cytokines like TNF (de Waal Malefyt et al., 1991). Stat3 appears to be the dominant factor that mediates IL-10 action but how this leads to down-regulation of genes like TNF is still unknown (Williams et al., 2004).

Changes in the expression of IL-10 upon activation of leukocytes are mainly transcriptional and several factors have been implicated in its control. For the murine IL-10 gene an important role of Sp1 in regulating the LPS-stimulated promoter activity was reported (Brightbill et al., 2000). The respective motif is promoter proximal and its mutation reduced both the inducible and the constitutive promoter activity in the murine RAW 264 macrophage cell line (Brightbill et al., 2000) (Tone et al., 2000). Ma et al. (Ma et al., 2001a) noted a Sp1 site at -636 bp of the human promoter, which appeared to be responsible for all the inducible activity in monoblastic cells. For the human gene, transactivation by catecholamines appears to require the action of C/EBP (Brenner et al., 2003) and the most important motif TTGCAAAA, which is 44 bp downstream of the TATA box. Furthermore, there is evidence for a role of c-Maf in IL-10 gene expression, but c-Maf may act indirectly by activating other transcription factors (Cao et al., 2002).

Based on deletion and linker scanning analysis we have identified a Stat motif at -120 that is crucial to gene expression in a human B cell line when stimulated with LPS (Benkhart et al., 2000). This motif was also shown to be essential together with an upstream IRF site for IFN α -induced IL-10 expression (Ziegler-Heitbrock et al., 2003). These data are in conflict with earlier findings of Takeda (Takeda et al., 1999), who

found increased IL-10 in Stat3 knock-out animals. The role of Stat3 in IL-10 gene expression was, however, confirmed in other cell lines and in knock-out mice (Herbeuval et al., 2004) (Cheng et al., 2003) (Maritano et al., 2004). Recently it was shown that SOCS3 deficiency in murine TCR-stimulated T cells led to increased Stat3 activity and IL-10 expression (Kinjyo et al., 2006). Furthermore, constitutive IL-10 expression in T cell lines can be blocked by depletion of Stat3 by RNA interference (Kasprzycka et al., 2006). Hence it appears that the Stat-motif in the human IL-10 gene may be a central trans-element in regulation of this gene.

Expression of the IL-10 gene is controlled by interferons, in that IFN α induces IL-10 while IFN γ can downregulate IL-10 gene expression (Ziegler-Heitbrock et al., 2003). (Chomarat et al., 1993). When looking at the molecular mechanism, the induction by IFN α was shown to be regulated via a IRF-Stat module in the IL-10 promoter (Ziegler-Heitbrock et al., 2003). For IFN γ mediated suppression of gene expression various mechanisms have been proposed including suppression of Sp3 (Hughes et al., 2002) or down-regulation of C/EBP α (Tengku-Muhammad et al., 2000). The main transcription factor used by IFN γ is Stat1 (Schroder et al., 2004). We have shown earlier that over-expression of Stat1 will counter the activation of the human IL-10 promoter (Ziegler-Heitbrock et al., 2003). In the present report we demonstrate that IFN γ -induced Stat1 will compete with and displace Stat3 from the endogenous human IL-10 promoter thus leading to suppression of IL-10 gene expression.

Material and Methods

Cell culture and stimulation

The RPMI 8226.1 B cell line (Ziegler-Heitbrock et al., 1994) was grown in 75 cm² tissue culture flasks (Greiner, Gloucestershire, UK) in RPMI 1640 medium (Biochrom KG/seromed[®], Berlin, Germany) supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin (15140-122, Gibco, Paisley, UK), 2 mM L-glutamine (25030-024, Gibco), 10 x non-essential amino acids (K 0293, Biochrom KG), 1 mM oxal-acetate, 1 mM pyruvate and 9 µg/ml insulin (O-5003, Sigma-Aldrich, Dorset, UK). This medium was passed through a Gambro U-2000 ultrafiltration column (Gambro Medizintechnik GmbH, Planegg-Martinsried, Germany) to deplete contaminating LPS, followed by the addition of 10 % v/v low LPS fetal calf serum (Biochrom). Cells were pre-treated with IFN γ at 1000 U/ml (generously provided by Dr. Wieland Wolf, Rentschler Biotechnologie, Laupheim, Germany) and were stimulated with highly purified LPS from *Salmonella minnesota* (L-6261, Sigma-Aldrich) at 100 ng/ml unless stated otherwise.

ELISA for IL-10 protein

ELISA was performed using the human IL-10 ELISA system (BMS215/2, Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. In brief, cell culture supernatants were added to wells with pre-coated anti-IL-10 antibody and biotin-conjugated anti-IL-10 was added, followed by streptavidin-HRP and substrate. Readings at 450 nm were transformed into pg/ml with reference to recombinant IL-10 standard titration.

RT-PCR analysis of gene expression

For mRNA isolation, 5 x 10⁶ cells were lysed with 1 ml of Tri-Reagent (T9424, Sigma-Aldrich) and processed according to manufacturer's instructions. After addition of chloroform and centrifugation the aqueous upper phase was recovered and mRNA was precipitated with isopropanol, washed with 75% v/v ethanol and dissolved in water. Reverse transcription was done using 1st Strand cDNA Synthesis Kit for RT-PCR (1483188, Roche, East Sussex, UK) with AMV RT and hexanucleotide primers (Amersham Pharmacia, Buckinghamshire, UK) in the presence of nucleotides and RNAsin according to the manufacturer's instructions. The

cDNA was then used for PCR with the LightCycler-FastStart DNA Master SYBR Green I (3003230, Roche) in a LightCycler (Roche). IL-10 amplification was carried out in parallel with amplification of the housekeeping gene β -2-microglobulin for normalisation. Primers used for IL-10 amplification were sense 5'-GCC TAA CAT GCT TCG AGA TC-3', antisense 5'-TGA TGT CTG GGT CTT GGT TC-3' amplifying a 207 bp fragment. The primers used for β -2-microglobulin amplification were sense 5'-GGC TAT CCA GCG TAC TCC AAA-3' and antisense 5'-CAA CTT CAA TGT CGG ATG GAT G-3' amplifying a 116 bp fragment. The PCR conditions were as follows: initial denaturation at 95°C for 10 min; then cycles of 96°C for 10 s, 60°C for 10 s, 72°C for 25 s. mRNA expression levels of IL-10 were normalized to the expression levels of β -2-microglobulin.

Transfection of RPMI 8226 with reporter plasmids and luciferase assay

Promoter luciferase reporter plasmids used were a construct containing -1044 bp upstream of the IL-10 transcription start (pIL-10 (-1044).luci), a construct containing -195 bp upstream of the IL-10 transcription start (pIL-10 (-195).luci), the same construct with a mutation of the IRF-site (pIL-10(-195 IRFmut).luci) and a construct with a trimer of the -120 Stat-site of the IL-10 promoter (p3xLS4.luci) as described in (Benkhart et al., 2000) and (Ziegler-Heitbrock et al., 2003).

RPMI 8226.1 cells were transfected according to (Shakhov et al., 1990). In brief, 10^7 cells in 1 ml RPMI 1640 medium supplemented with 2 mM L-glutamine were admixed with 5 μ g reporter gene plasmid and 125 μ g/ml DEAE-Dextran (E1210, ProFection, Promega, Southampton, UK). After 90 min at 37°C, we added 100 μ l of DMSO (D-5879, Sigma-Aldrich) for 150 sec. Cells were then washed and cultured for 4 days in 6 well plates.

Cells were treated with 1000 U/ml IFN γ and 100 ng/ml LPS and cell lysates were prepared 6 h after LPS treatment using Luciferase Assay System (E1500, Promega), ReportaLightTM Reporter Gene Detection Kit (LT07-316, BioWhittaker, Berkshire, UK) or Enhanced Luciferase Assay Kit (Berthold Detection Systems, Pforzheim, Germany) according to the manufacturer's instructions. Luciferase activity was determined in a Berthold LB 9501 luminometer and the RLU/ μ g protein was calculated for each lysate. Protein concentration of each lysate was determined using BioRad DC Protein Assay Reagents (500-0116).

Gelshift analysis

Nuclear extracts were isolated according to the method of Dignam et al. (Dignam et al., 1983) in the presence of a protease inhibitor cocktail (10 µg/ml aprotinin (A-6279, Sigma-Aldrich), 1 mM PMSF (P-7626, Sigma-Aldrich), 40 µg/ml leupeptin-propionyl (L-3402, Sigma-Aldrich), 20 µg/ml leupeptin-trifluoro-acetate salt (L-2923, Sigma-Aldrich), 20 µg/ml antipain (A-6191, Sigma-Aldrich), 20 µg/ml pepstatin A (P-4265, Sigma-Aldrich), 400 mM N-acetyl-leu-leu-norleu-al (A-6185, Sigma-Aldrich) and 2 mM DTT (443851W, BDH, Dorset, UK)).

Three to 5 µg of nuclear protein was admixed with ³²P-labeled double-stranded LS4 oligonucleotide (sense 5'-ATC CTG TGC CGG GAA ACC-3') in the presence of 0.5 µg poly(dI/dC)/20 µl, 1 mg/ml BSA, 10% v/v nuclear extract buffer D⁺ (20 mM HEPES pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 0.05 mM EDTA pH 8.0, 1% (v/v) Nonidet P40 (NP-40)). After 20 min of incubation at room temperature samples were electrophoresed on non-denaturing polyacrylamide gels in 0.25 x TBE buffer. For supershift analysis nuclear extracts were first incubated with a 1/20 dilution of Ab for 30 min on ice, followed by incubation with the ³²P-labeled oligonucleotide.

The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) as rabbit polyclonal antibodies: Stat1 p84/p91 (E-23) (# sc-346), Stat3 (H-190) (# sc-7179 X). Gels were run with a distance of 10 cm for standard gelshift and 15 cm for supershift analysis.

Western blotting for nuclear factors

Twenty µg nuclear extracts were loaded onto 4-12% Tris-Glycine Novex[®] precast gels (EC60352BOX, Invitrogen Life Technology, Paisley, UK) and separated in 1 x Novex[®] Tris-Gly SDS running buffer (LC2675, Invitrogen) for 90 min at 150 V in the XCell II Minicell electrophoresis and blotting system (Novex San Diego, USA) according to the manufacturer's instructions. Separated proteins were then electroblotted onto Hybond nitrocellulose membranes (RPN.203.N, Amersham). Membranes were then washed three times in 1 x TBS/Tween for 5 min and blocked for 1 h RT in western blot blocking buffer (1 x TBS, 5% w/v skimmed milk powder, 0.1% v/v tween) and then reacted overnight at 4°C with the following rabbit polyclonal

antibodies from Santa Cruz: Stat1 p84/p91 (E-23) (sc-346), p-Stat1 (Tyr701) (sc-7988-R), Stat3 (C-20) (sc-482), p-Stat3 (Tyr 705) (sc79993-R), RNA polymerase II (N20) (sc-899) overnight at 4°C. Membranes were washed followed by incubation with horse-radish peroxidase conjugated anti-Ig antibody (A-0545, Sigma). Finally, filters were incubated with ECLTM western blotting detection reagents (RPN2106, Amersham) and chemiluminescence was detected by exposure to x-ray film.

Chromatin immunoprecipitation

For ChIP 10⁸ cells were resuspended in 1 x PBS with 1% v/v formaldehyde and incubated for 20 min at RT to cross-link the protein to the DNA. The reaction was stopped by addition of glycine to 0.125 M final concentration. The cells were washed twice in 1 x PBS and lysed for 10 min on ice with a protease inhibitor containing lysis buffer (50 mM Tris/HCl pH 8.0, 10 mM EDTA pH 8.0, 1% w/v SDS). All buffers used in ChIP were supplemented with a protease-inhibitor cocktail of 1 mM PMSF, 1 µg/ml leupeptin-propionyl, 1 µg/ml leupeptin-trifluoro-acetate salt, 1 µg/ml aprotinin, 0.1 µg/ml antipain. The chromatin was sheared by sonication of the samples 4 times for 15 s using a Soniprep 150 sonicator (Sanyo, Leicestershire, UK) at a setting of 10 microns, with 1 min incubation on ice between each sonication. Supernatants were precleared by incubation for 30 min at 4°C with 10 µl each of sepharose A beads (50% slurry) (17-0780-01, Amersham) and protein G beads (P-3296, Sigma-Aldrich). Next, the supernatants were diluted 1:2 with dilution buffer (0.01% SDS, 1% Triton, 1.2 mM EDTA pH 8.0, 16.7 mM Tris/HCl pH 8.0, 167 mM NaCl) and immunoprecipitated overnight at 4°C with antibodies against transcription factors Stat3 (sc-482, Santa Cruz) or Stat1 (sc-346, Santa Cruz). Rabbit IgG (sc-2027, Santa Cruz) was used to control for non-specific binding. This material was then admixed with protein A and G sepharose beads for 2 h at 4°C followed by sequentially washing of the beads twice with dilution buffer (0.01% w/v SDS, 1% v/v Triton, 1.2 mM EDTA pH 8.0, 16.7 mM Tris/HCl pH 8.0, 167 mM NaCl), twice with wash buffer 1 (2 mM EDTA pH 8.0, 20 mM Tris/HCl pH 8.0, 1% v/v Triton, 0.1% w/v SDS, 150 mM NaCl) and twice with wash buffer 3 (10 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0). The bead pellet was then resuspended twice in elution buffer (1% w/v SDS, 0.1 M NaHCO₃), the supernatant material was pooled and decrosslinked in the

presence of 0.2 M NaCl for 4 h at 65°C. DNA was isolated by phenol/chloroform extraction following classical procedures and used for PCR amplification.

Amplification of the IL-10 promoter fragment using SYBR[®]Green Taq ReadyMix[™] (S 1816, Sigma-Aldrich) was carried out in a LightCycler (Roche) with the primers sense 5'-GCA GAA GTT CAT GTT CAA CCA A-3' and antisense 5'-AGG CCT CTT CAT TCA TTA AAA A-3' amplifying a 152 bp fragment. The PCR conditions were as follows: initial denaturation at 95°C for 10 min; then cycles of 96°C for 10 s, 60°C for 10 s, 72°C for 25 s. All PCR signals from immunoprecipitated DNA were normalized to PCR signals from non-immunoprecipitated input DNA. The signals obtained with precipitation with the control IgG was subtracted from the signals obtained with the specific anti-Stat antibodies. Results are expressed as percentage of the input.

Bioinformatic analysis

Analysis of mouse promoters for the combined STAT3/STAT1 motif as found in the human IL10 promoter was carried out as follows (All tools were used as provided on the GenomatixPortal, Genomatix, Munich unless other wise stated): The human promoter was analyzed for STAT3 and STAT1 motifs using a weight matrix based approach (MatInspector, GEMS launcher release 4.2.2). Results were used to define a promoter model using the FASTM tool (GEMS launcher release 4.2.2). The distance between the two STAT binding sites found in the human IL-10 promoter was 2 and was set to a range of 2 – 5 in the model. Recognition of the human IL-10 motif was verified using ModelInspector (GEMS launcher release 4.2.2). Then the promoters of genes identified in the microarray study (29) as significantly downregulated (>3) were analyzed for matches of this model as well as the whole database of mouse promoters (EIDorado, release 4.1).

Statistical analysis

Data were analysed using one-tailed paired Student's T-test. Results were considered significant when *p<0.05.

Results

Effect of IFN γ on IL-10 mRNA and protein

In order to demonstrate that the effect of IFN γ on the IL-10 promoter will lead to reduced mRNA synthesis we performed RT-PCR in cells at 6hs post LPS stimulation. Data in Figure 1 A demonstrate a reduction of IL-10 mRNA by factor 2 when cells were pretreated with IFN γ for 2hs followed by LPS stimulation for 6 hours. Also, IL-10 protein levels were significantly reduced with IFN γ pre-treatment at 18hs post LPS stimulation (Figure 1 B).

Effect of IFN γ pre-treatment on IL-10 promoter activity

For the study of the IL-10 promoter activity we transfected RPMI 8226 cells with the pIL-10(-1044).luci plasmid using DEAE Dextran. Cells were then stimulated with LPS and this led to an average 10-fold increase of luciferase activity (see lane 3 and lane 1 in Figure 2). When IFN γ was added at the same time as LPS then the promoter activity was significantly reduced but the strongest effect with a > 2-fold reduction was seen when IFN γ was added 2 hours before stimulation with LPS (lane 4, Figure 2). We therefore chose the -2h addition of IFN γ for all further experiments. Of note, when IFN γ alone was added at time 0 it did induce a 2-fold increase of the low level promoter activity (lane 2 versus 1, Figure 2).

Next we analysed whether a shorter proximal fragment of the human IL-10 promoter would give a similar suppressive effect of IFN γ . Transfection of the cells with a -195 bp fragment of the IL-10 promoter in the luciferase reporter construct also showed robust induction by LPS and a clear suppression with addition of IFN γ (Figure 3A).

The -195 bp fragment contains an IRF and a Stat binding motif as indicated in the promoter scheme in the upper part of Figure 3. The Stat site is required for LPS induced transactivation as shown previously (Benkhart et al., 2000) and the IRF site contributes to IFN α induction of the promoter (Ziegler-Heitbrock et al., 2003). Mutation of the IRF-motif did not affect the ability of IFN γ to suppress the IL-10 promoter activity (Figure 3B, lane 4) indicating that IFN γ is not acting via this site.

The role of the Stat-site could not be tested by the mutation approach since mutation would ablate the LPS signal, such that there would be no activity to be suppressed. We therefore turned to a construct carrying a trimer of the Stat motif from the IL-10 promoter. Using this construct IFN γ could also suppress the LPS-induced promoter activity albeit to a lesser extent (lane 4, Figure 3C). These data suggests that the suppressive effect of IFN γ on the human IL-10 promoter may operate via the Stat site. Taken together, it appears that the regulation of the IL-10 gene by IFN γ operates via the -120 Stat site.

Effect of IFN γ on activation of Stat1 and Stat3

We then analysed the effect of IFN γ on phosphorylation and mobilisation of Stat 1 and Stat3. Analysis of Stat1 in nuclear extracts of RPMI 8226 cells revealed tyrosine phosphorylation of Stat1 α by IFN γ treatment (Figure 4A, upper panel, lane 2), while LPS alone did not affect tyrosine-phosphorylation of any Stat1 protein (lane 3). By contrast, both Stat1 α and Stat1 β were tyrosine phosphorylated when cells were treated with both LPS and IFN γ (Figure 4A, upper panel, lane 4). Total Stat1 protein in the nucleus also was increased most clearly upon treatment with both LPS and IFN γ (Figure 4A, middle panel, lane 4).

Analysis of Stat3 revealed a robust tyrosine phosphorylation with LPS and this was enhanced by co-treatment with IFN γ (Figure 4B, upper panel, lane 3 and 4). This translated into an increased nuclear translocation of total Stat3 protein (Figure 4B, middle panel, lane 3 and 4).

We then asked whether the same pattern would be seen when analysing DNA binding activity. For this we performed gelshift analysis using the radiolabelled Stat motif from the human IL-10 promoter. This motif was reacted with nuclear extracts of RPMI 8226 cells taken at 1 and 4 hours post LPS stimulation as given in the left and right panel, respectively, of Figure 5A.

These studies revealed that Stat1 binding was induced only by IFN γ (lanes 2 and 6 in Figure 5A) but not by LPS. The clear DNA binding induced for Stat1 by IFN γ alone suggests that the faint signal, seen in Figure 4A, upper panel, for IFN γ alone with respect to tyrosine phosphorylation, is functionally relevant.

DNA binding of Stat3 was induced by LPS and not by IFN γ and as reported earlier (14), this was seen most clearly after 4 hours of stimulation (Figure 5A, right panel). The nature of the two DNA binding proteins was confirmed by supershift analysis. Here the low motility upper band was shown to be Stat3 and the high motility lower band was demonstrated to be Stat1 (Figure 5B).

This pattern of DNA binding for Stat1 and Stat3, induced by IFN γ and LPS treatment, matches the tyrosine phosphorylation pattern seen in the Western blots (Figure 4). The crucial finding of the gel shift analysis is seen in lane 8 of Figure 5A, right panel, where with the combination of IFN γ treatment and LPS stimulation there is binding of both LPS induced Stat3 and IFN γ induced Stat 1 to the motif. This suggests that the IFN γ induced Stat1 might be competing with the LPS induced Stat3 for binding to the -120 Stat motif of the human IL-10 promoter.

Effect of IFN γ on binding of Stat1 and Stat3 in the intact nucleus

We then performed ChIP analysis in order to investigate whether the Stat1 and 3 proteins, in fact, compete for binding to the promoter. PCR of a DNA fragment encompassing the Stat-motif confirmed the calculated size of 152 bp. Precipitation with anti-Stat3 antibody revealed good induction of Stat3 binding by LPS (lane 3 in Figure 6A). When cells were pretreated with IFN γ then binding of Stat3 was substantially reduced (lane 4, Figure 6A). A gel of the ChIP samples run with samples taken at 30 cycles does support the findings in that the strongest band is visible in LPS treated cells (Fig6B). Considering that tyrosine phosphorylated Stat3 is higher in IFN γ /LPS cells than in LPS alone cells (Figure 4B, lane 4), the lower binding in ChIP supports the concept that the abundantly available phosphorylated Stat3 is prevented from binding by a mechanism like competition.

When looking at ChIP for Stat1 we observed binding of this transcription factor in IFN γ but not by LPS treated cells (Fig 6 C, lane 2 and 3). When cells were treated with IFN γ plus LPS then Stat1 binding was increased further (Fig 6C, lane 4). Again these quantitative PCR analyses are supported by an analysis of the 32 cycle products in a gel (Fig 6 D). These ChIP analyses suggest that IFN γ treatment induces Stat1 binding, which displaces LPS induced Stat3 from the IL-10 promoter.

Discussion

While IFN γ will enhance various immune defence mechanisms (Boehm et al., 1997), it also can suppress elements of the immune response. This includes the anti-inflammatory cytokine IL-10, which is studied herein. The suppression of IL-10 by IFN γ will thus contribute to the pro-inflammatory action of IFN γ (Chomarat et al., 1993).

We have studied in this report the molecular mechanisms of IFN γ suppression of IL-10 gene expression by looking at the transcriptional control of the IL-10 promoter. In addition to histone acetylation and remodelling of the chromatin at the IL-10 locus (Shoemaker et al., 2006) (Saraiva et al., 2005) several transcription factors have been implicated in the expression of the IL-10 gene and this includes SP1 and c/EBP (Brightbill et al., 2000) (Tone et al., 2000) (Ma et al., 2001a) (Brenner et al., 2003) (Liu et al., 2003). We have provided evidence for a role for Stat3 in driving the human IL-10 promoter in cells stimulated with LPS (Benkhart et al., 2000) (Ziegler-Heitbrock et al., 2003). This is supported by data from Cheng et al, who found absence of IL-10 expression in peritoneal macrophages of Stat3 knock-out mice (Cheng et al., 2003) and by Maritano et al who showed reduced IL-10 in Stat3b knock-out mice (Maritano et al., 2004). Furthermore, a role for Stat3 in IL-10 expression was demonstrated in murine and human T cells (Kinjyo et al., 2006) (Kasprzycka et al., 2006).

Also, studies in human carcinoma cells showed that IL-6 induced IL-10 via the Stat3 transcription factor (Herbeuval et al., 2004). Finally, IRF transcription factors are involved in expression of the gene in cells stimulated with IFN α (Ziegler-Heitbrock et al., 2003).

IFN γ acts via a hetero-dimeric cell surface receptor consisting of one IFNGR1 and one IFNGR2. Ligand binding induces formation of an IFNGR hetero-tetramer followed by activation of JAK-1 leading to phosphorylation, dimerization and translocation of Stat1 (for review see (Schroder et al., 2004)). Additional transcription factor complexes induced are Stat1-Stat2 heterodimers and complexes of Stat1-Stat1 and Stat1-Stat2 with IRF9, the latter complex also termed ISGF3. Furthermore, IRF-1 is transcriptionally induced. Still, Stat1 appears to be the dominant nuclear factor that

mediates IFN γ action, since Stat1^{-/-} are a phenocopy of the IFN γ ^{-/-} mice (Schroder et al., 2004).

Provided that IFN γ acts at the level of transcription, then it might block IL-10 expression by interference with any of these transcription factors. For the lipoprotein lipase gene IFN γ -induced suppression was shown to go along with a reduction in the level of SP3 leading to reduced SP1/SP3 binding to respective motifs in the promoter (Hughes et al., 2002). Also, IFN γ was shown to be able to down-regulate C/EBP α (Tengku-Muhammad et al., 2000). Our previous studies (Benkhart et al., 2000) have, however, shown that these factors are not involved in the regulation of this gene in LPS stimulated human RPMI 8226.1 B cells. Hence, a direct action of IFN γ induced Stat1 on the target gene is likely.

IFN γ was shown to down-regulate the IL-1 gene in murine macrophages but it was unable to do so in Stat1^{-/-} animals (De Boer et al., 2001). Furthermore, IFN γ suppresses MMP-9 via Stat1 (Ma et al., 2001b) and this appears to be due to sequestration of CBP/p300 such that this coactivator is not available for transcription of the MMP-9 gene (Ma et al., 2005).

With respect to IFN γ induced down-regulation of IL-10 Flores et al have studied suppression of this cytokine in CpG stimulated dendritic cells (Flores et al., 2007) They suggested that Stat1 might be involved but the findings were difficult to interpret because IL-10 levels were decreased in Stat1^{-/-} mice such that there was little room for any potential further decrease induced by IFN γ . A role for Stat1 in downregulation of IL-10 in mice was suggested based on the increased IL-10 mRNA and protein levels in LPS treated Stat1^{-/-} animals (VanDeusen et al., 2006) However, IFN γ did not appear to play a significant role in this Stat1 effect, since IFN γ ^{-/-} animals did not show such a pronounced increase of IL-10 expression (VanDeusen et al., 2006). Still, these findings support a critical role of Stat1 in down-regulation of the IL-10 gene.

Additional mechanisms that have been proposed to explain inhibition of Stat3 pathways by IFN γ induced Stat1 include sequestration of Stat3 by Stat1 (Hu et al., 2005) and competition of Stat1 and Stat3 for binding to the cytoplasmic tail of the IFNGRI (Qing and Stark, 2004).

Our studies show that a Stat-motif at -120 in the human IL-10 gene is instrumental for LPS induction and for IFN γ mediated suppression of the gene. We have previously shown that Stat3 over-expression can up-regulate and that Stat1 over-expression can down-regulate the human -195 IL-10 promoter (Ziegler-Heitbrock et al., 2003). Given that IFN γ acts mainly via Stat1 this suggests that IFN γ suppresses the IL-10 gene via Stat1.

Our studies have demonstrated that suppression of the LPS-induced -1044 IL-10 promoter activity can be achieved when IFN γ is added simultaneously with LPS but a clearly stronger effect can be achieved when IFN γ is added at -2hs (see Figure 2).

This finding is surprising given that LPS signalling leads to binding of Stat3 to the promoter only with slow kinetics, while IFN γ can induce strong Stat1 binding already at 1 h (Figure 5A). With this faster kinetics for Stat1 induction an additional pre-culture time would not appear necessary. Hence at this stage the improved suppression with very early addition of IFN γ remains unexplained. Although data in Figure 2 suggest that a longer pre-culture period might give an even stronger effect, we have not tested earlier addition of the cytokine since we wanted to focus on direct actions of IFN γ and avoid indirect, secondary effects.

Suppression by IFN γ was also seen with a shorter -195 fragment of the IL-10 promoter and this contains Stat3 and IRF sites as previously described (Ziegler-Heitbrock et al., 2003). With IFN γ acting both via Stat and IRF (Schroder et al., 2004) we have then evaluated the role of these two sites. Here, mutagenesis of the IRF-site did not affect the ability of IFN γ to suppress IL-10 promoter activity (see Fig 3B). Hence, while it was shown that IFN γ suppresses IL-4 via IRF-1 and -2 (Elser et al., 2002), this mechanism does not appear to be responsible for the suppression of IL-10. Mutagenesis of the Stat-site is not informative for the study of IFN γ suppression of the LPS induction, since with mutation of this site there is no induction by LPS (Benkhart et al., 2000) and hence no activity that can be suppressed. We therefore have taken a reporter gene construct containing a trimer of the Stat-motif. As IFN γ suppressed LPS-induction of this construct we postulate a crucial role of this site for the inhibitory effects of IFN γ action.

We, in fact, could show induction of nuclear Stat1 and Tyr701 phosphorylation by IFN γ , which was more pronounced with the combination of LPS and IFN γ (Fig.4A). As a control protein for Western blotting we have used RNA Polymerase II as a

constitutive nuclear protein. This usually gave weak signals, a finding that can be best explained by our strategy of analysing the same blot first for phosphorylated Stat, then for total Stat and then for Polymerase II such that for this final analysis blots have been stripped twice.

IFN γ did not activate phosphorylation and translocation of Stat3, but it did enhance LPS induction of this transcription factor. Given that Stat3 is driving the human IL-10 promoter such an induction of Stat3 can be expected to counter-act the blocking action of IFN γ .

The Western blotting data are in line with the gel-shift analyses, which demonstrate binding of both transcription factors Stat1 and Stat3 to the same motif, i.e. the -120 sequence of the IL-10 promoter (Fig 5A). Such binding of nuclear proteins to double-stranded DNA motifs in-vitro does not necessarily reflect binding of these proteins to the promoter in intact cells. We therefore have performed ChIP analysis and here we could show binding of Stat3 upon LPS stimulation. With IFN γ pre-treatment this binding was clearly reduced (Fig 6A). Hence, while the Western blot findings showed enhanced presence of phosphorylated Stat3 in IFN γ /LPS treated cells, this did not translate into enhanced binding of Stat3 to the promoter in intact cells. Therefore, a mechanism has to exist that prevents Stat3 binding upon treatment of cells with IFN γ . When performing ChIP for Stat1 we observed robust induction of Stat1 binding in IFN γ /LPS treated cells (Fig 6B) targeting the same element of the IL-10 promoter.

Based on our studies using nuclear translocation, promoter activity analysis, binding in gelshift and ChIP we propose that IFN γ induced Stat1 will bind to the Stat-motif in the human IL-10 promoter and will displace Stat3. This concept is in line with our earlier finding, which demonstrated that overexpression of Stat1 will reduce IL-10 promoter activity (Ziegler-Heitbrock et al., 2003). The Stat1 bound to the site can have no activity or only weak transcriptional activity in order to prevent LPS-induced Stat3 mediated IL-10 gene expression.

The Stat1 competition with Stat3 may operate for many other genes besides IL-10. In earlier studies Ehrt et al (Ehrt et al., 2001) have analysed the transcriptome of murine macrophages in response to IFN γ . Analysis of these data, kindly provided by Drs. Ehrt and Nathan, for genes suppressed by IFN γ revealed 89 of 1299 genes that decreased in expression 3-fold or more. In a bioinformatics search of the promoters of

67 of these 89 genes (annotated genes) for binding motifs similar to the Stat motif in the human IL-10 promoter we found 14 genes to contain such a motif in their promoter. We speculate that these genes (MSR2 GeneID 80891, 2210409B22Rik GeneID 70174, Hpgd GeneID 15446, Mcpt8 GeneID 17231, Ndr1 GeneID 17988, Pmp22 GeneID 18858, Spp1 GeneID 20750, Phlda1 GeneID 21664, Alcam GeneID 11658, Aqp4 GeneID 11829, Bmp1 GeneID 12153, Col4a1 GeneID 12826, Ddef1 GeneID 13196, Ccl9 GeneID 20308) may be regulated in a fashion similar to what we have shown here for the human IL-10 gene.

This mechanism, which operates within hours, may be different from what occurs when cells are primed for 1 or 2 days before adding a stimulus (Hu et al., 2006) (Herrero et al., 2003). When using a system with a 2day priming period then this will alter the entire gene expression program of a cell with many indirect effects (Herrero et al., 2003). The IFN γ effect occurring within a few hours as analysed in the present report is likely to be direct and to act via immediate signalling using transcription factors like Stat1.

Taken together, we can demonstrate herein that IFN γ will suppress IL-10 gene expression via Stat1, which competes in the nucleus with Stat3 for binding to the – 120 Stat element. This may be a paradigm for IFN γ action on other genes controlled by Stat3.

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Legends to Figures

Fig. 1: IFN γ inhibits LPS induced IL-10 mRNA and protein expression.

RPMI 8226.1 cells were treated for 2 h with IFN γ at 1000 U/ml previous to stimulation with LPS at 100 ng/ml for 6h (mRNA) or 18 h (supernatant protein).

A: IL-10 mRNA. The mean of 9 experiments \pm SD is shown.

B: IL-10 protein. The mean of 6 experiments \pm SD is shown. 100% = 31.9 ± 23.7 ng/ml. * = $p < 0.05$ when compared to LPS-stimulated cells.

Fig. 2. IFN γ suppresses LPS induction of the -1044 IL-10 promoter

RPMI 8226.1 cells transfected with pIL-10(-1044).luci (schematic representation of construct above the graph) show a decrease in luciferase-activity when treated with 1000 U/ml IFN γ previous to stimulation with 100 ng/ml LPS for 6h. The mean of 6 experiments \pm SD is shown. 100% = 16.1 ± 12.0 RLU/mg protein. * = $p < 0.05$ when compared to LPS-stimulated cells.

Fig. 3: IFN γ suppresses LPS induction of the -195 IL-10 promoter via the Stat site.

RPMI 8226.1 cells were transfected with pIL-10(-195wt).luci (**A**), pIL-10(-195IRFmut).luci (**B**) or p3xLS4.luci (**C**) and then treated for 2 h with 1000 U IFN γ / ml before stimulation with 100 ng LPS/ml for 6 h. Schematic representation of transcription factor binding sites present in the construct above the graph. **A:** The mean of 6 experiments \pm SD is shown. 100% = 32.5 ± 12.4 RLU/mg protein. **B:** The mean of 5 experiments \pm SD is shown. 100% = 54.2 ± 30.0 RLU/mg protein. **C:** The mean of 6 experiments \pm SD is shown. 100% = 48.6 ± 23.6 RLU/mg protein. * = $p < 0.05$ when compared to LPS-stimulated cells.

Fig. 4: Translocation of phosphorylated Stat 1 and Stat3 into the nucleus

Western blots were performed with nuclear extracts. IFN γ was added at -2h ; LPS was added at 0h. All samples were harvested at 4h after LPS stimulation.

A: IFN γ induces translocation of tyrosine-701 phosphorylated STAT1.

Upper panel: anti-p-STAT1 (Tyr 701) antibody; middle panel: anti-STAT1 p89/91 antibody; lower panel: anti-RNA-polymerase II antibody (Pol II). One of 4 experiments.

B: LPS causes translocation of tyrosine-phosphorylated STAT3 into the nucleus.

Upper panel: anti- p-STAT3 (Tyr 705) antibody; middle panel: anti-STAT3 (C20) antibody; lower panel: anti-RNA-polymerase II antibody (Pol II). One of 4 experiments.

Fig. 5: Gelshift analysis of Stat binding to the -120 motif of the IL-10 promoter.

A: Nuclear extracts obtained from cells treated with LPS at 100 ng/ml and IFN γ at 1000 U/ml were admixed with the DNA motif representing the -120 STAT-binding site of the IL-10 promoter. Nuclear extracts were obtained at 1 and 4 h past stimulation. One of 3 experiments.

B: Nuclear extracts were admixed with anti Stat antibodies before addition of the radio-labelled DNA-motif. Filled arrow = STAT3; open arrow = STAT1. One of 3 experiments.

Fig. 6: ChIP analysis of STAT-binding proteins

Chromatin of RPMI 8226.1 cells treated for 2 h with IFN γ at 1000 U/ml of previous to stimulation with LPS at 100 ng/ml for 4 h was immunoprecipitated using STAT3 (C20) antibody(**A,B**).or STAT1 antibody (**C,D**). Recovery was expressed as % of input. In **A** and **C** the means of 3 or 4 experiments \pm SD is shown for STAT3 and STAT1, respectively. **B** and **D** give an example each of a gel of the PCR products after 30 and 32 cycles, respectively. * = $p < 0.05$.

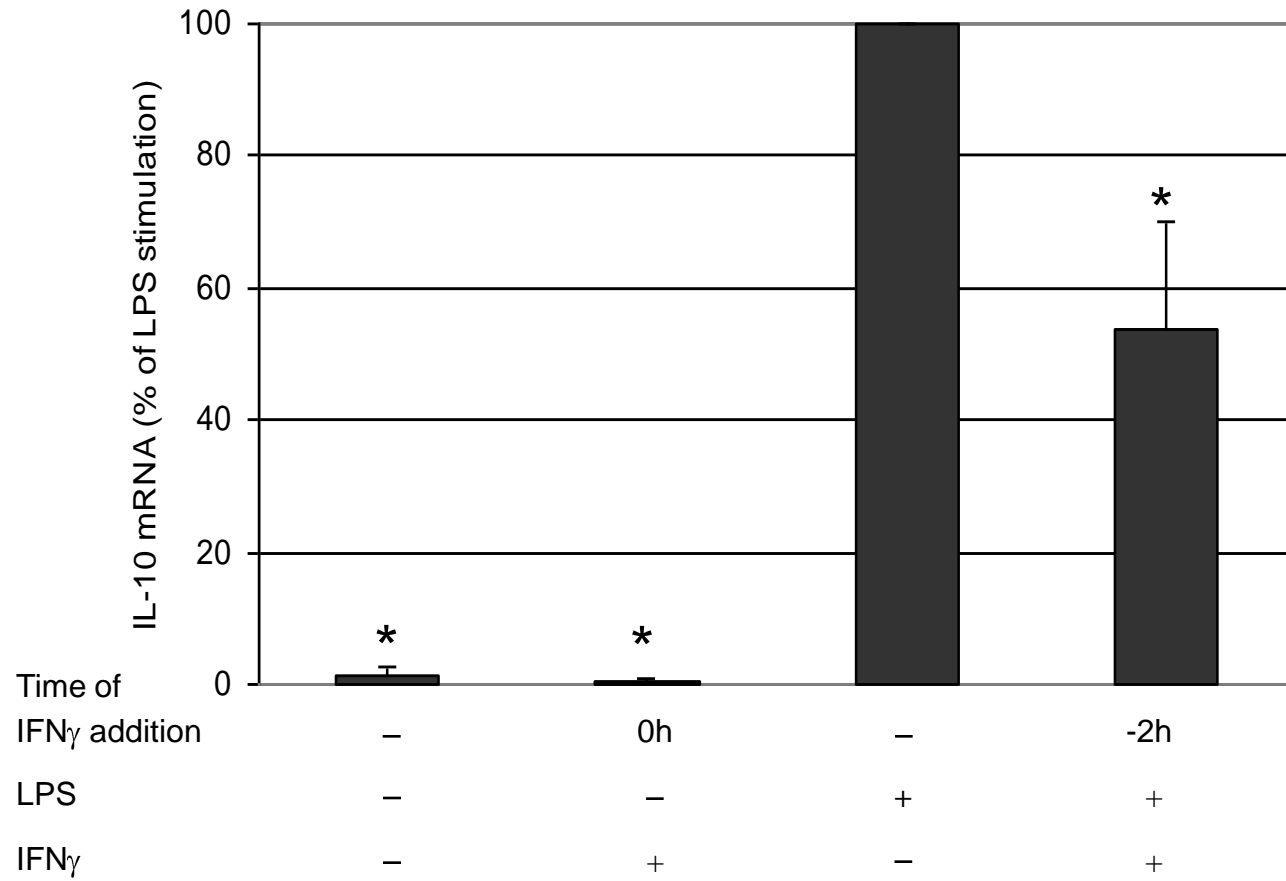
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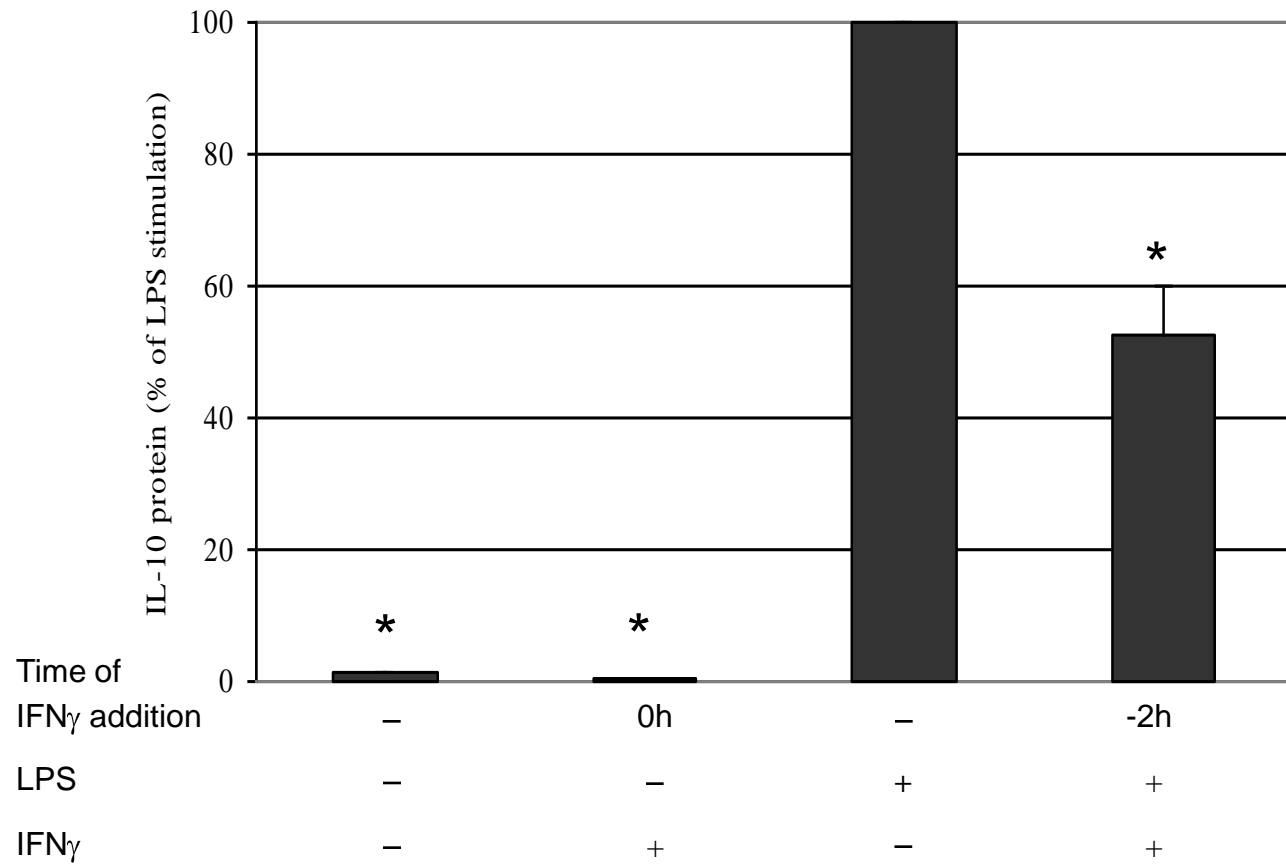
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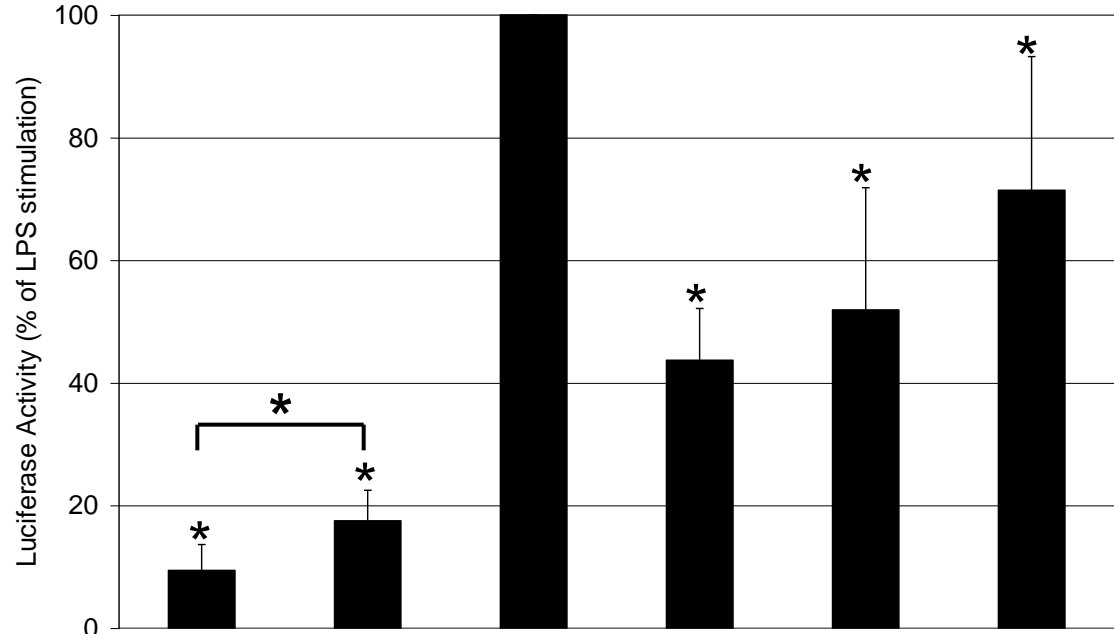
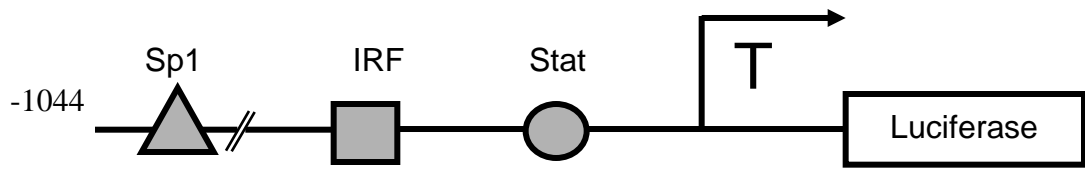
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Schaefer et al., Figure 1 A



Schaefer et al., Figure 1 B

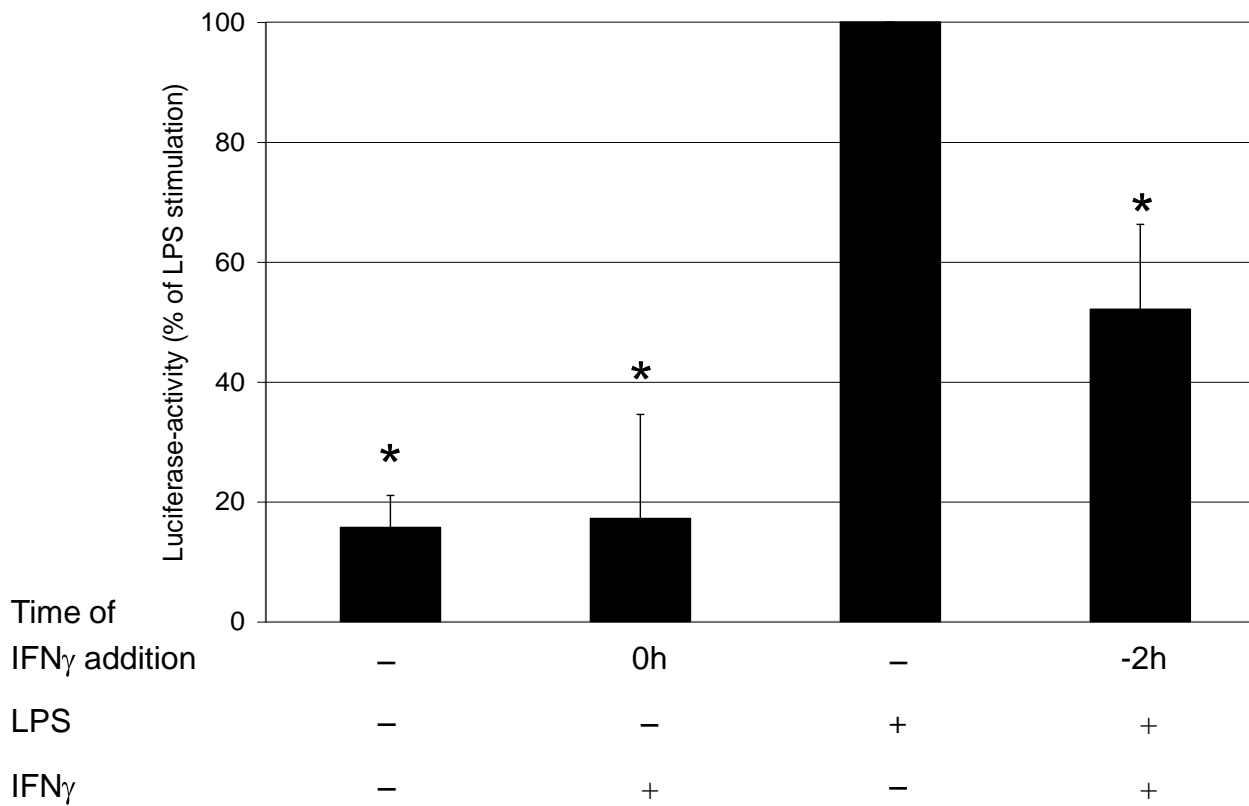
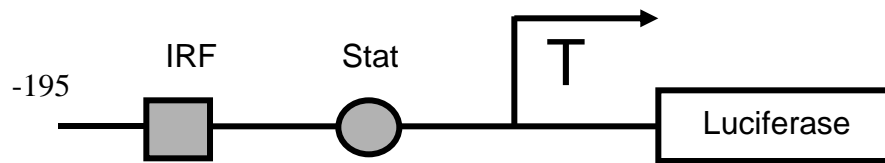


Time of
IFN γ addition

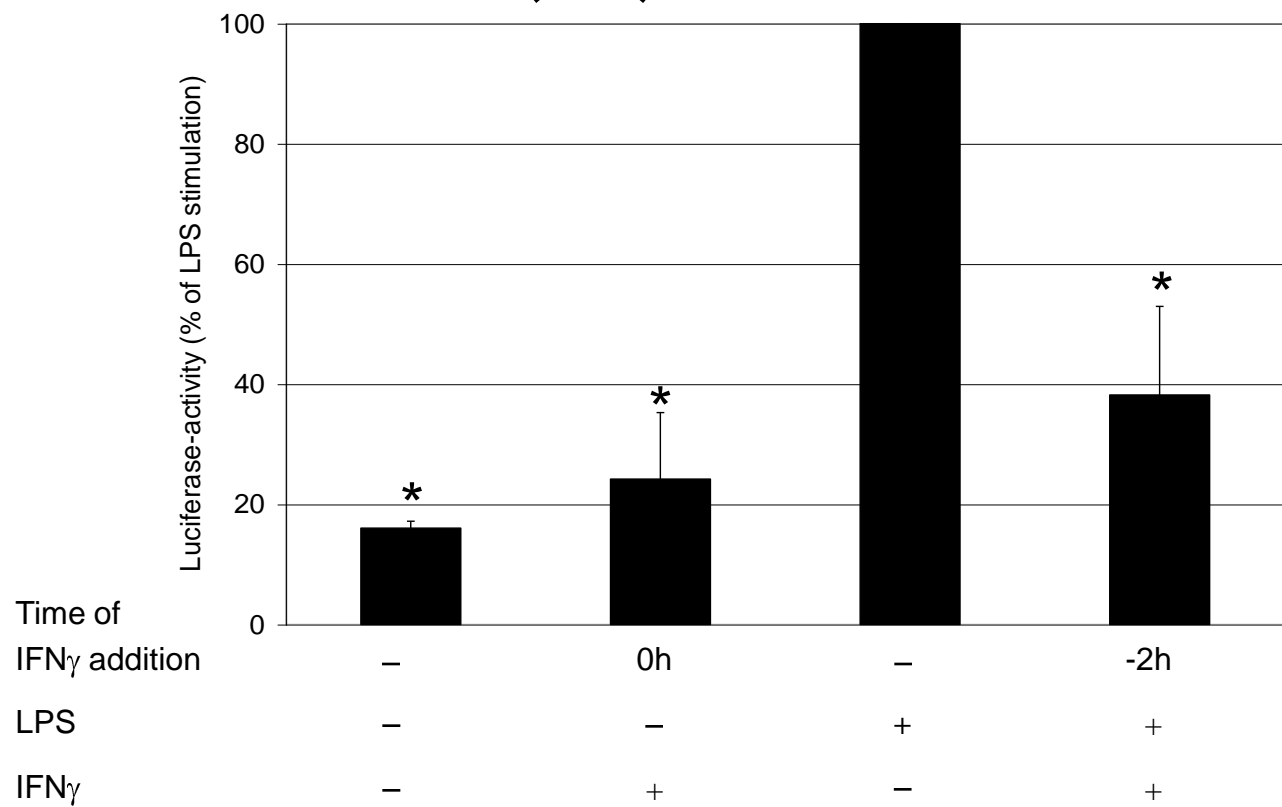
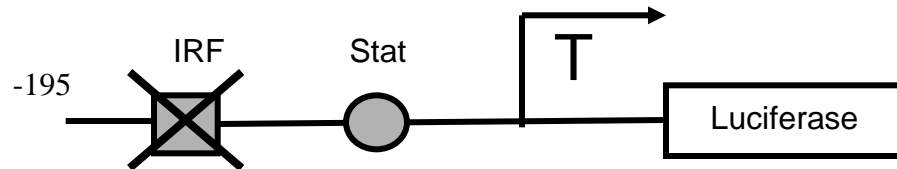
LPS

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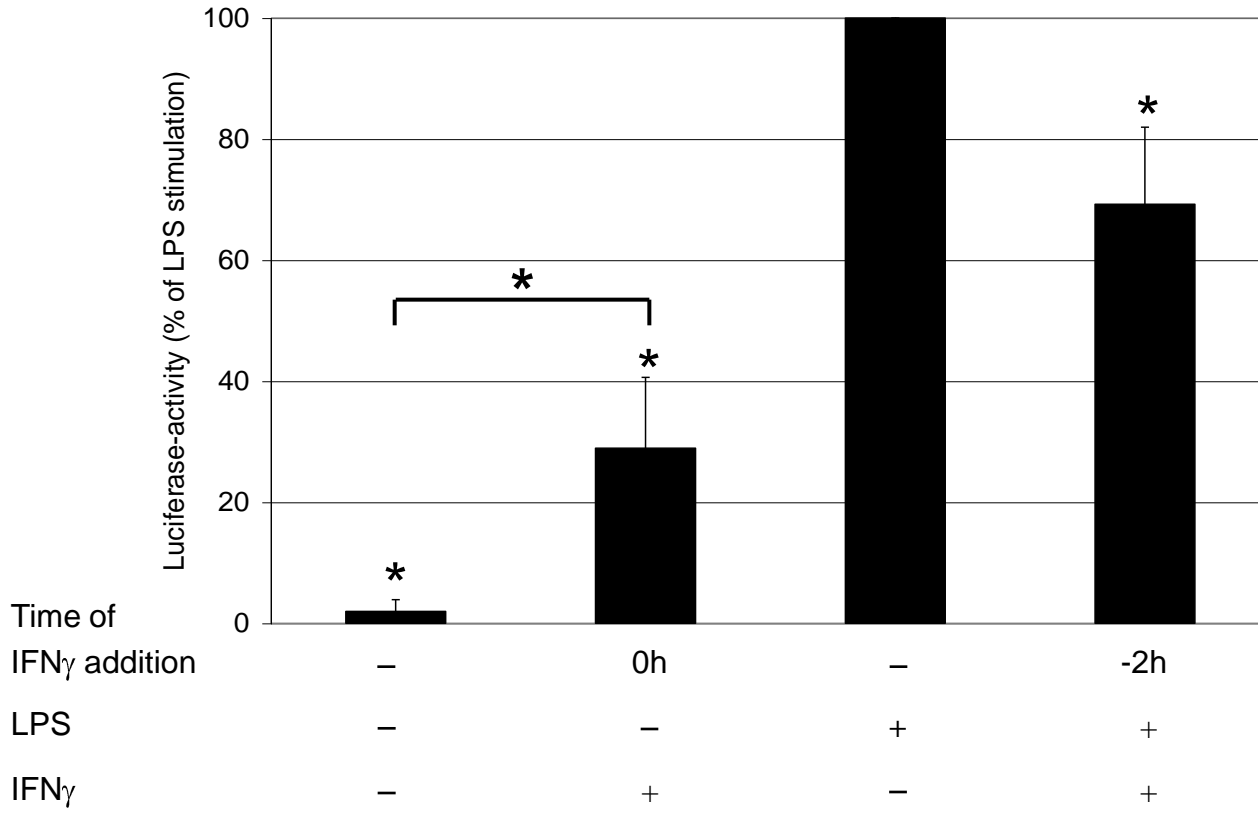
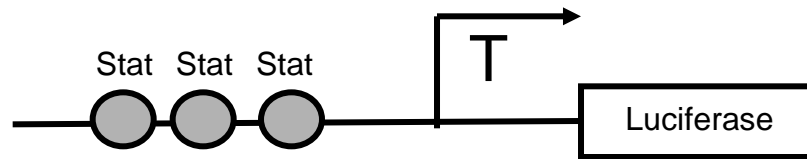
Schaefer et al., Figure 2



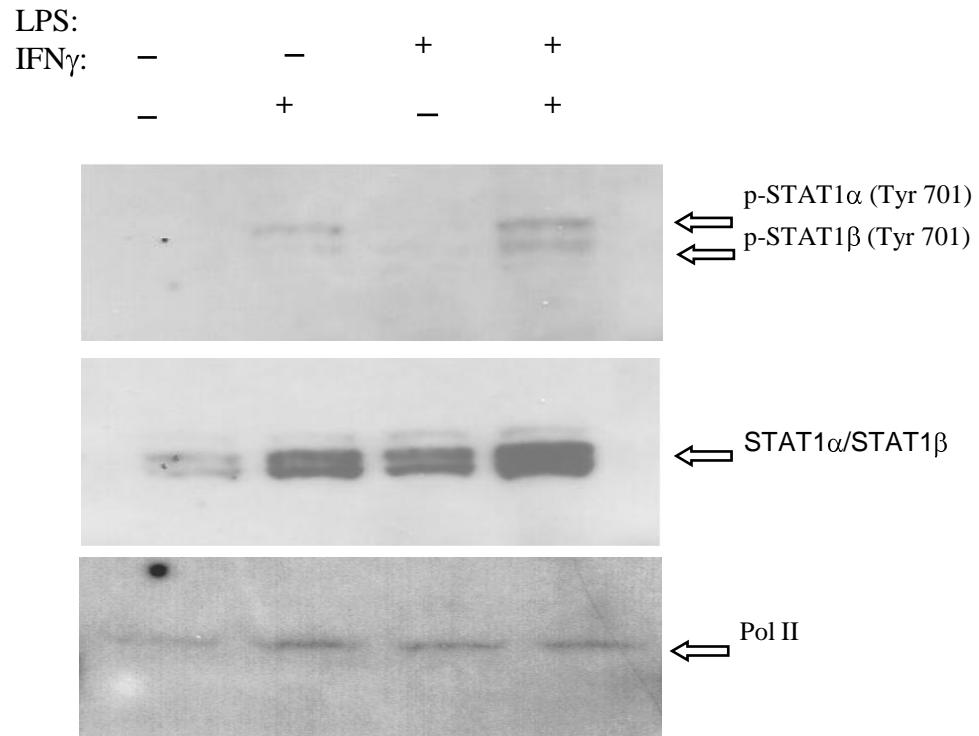
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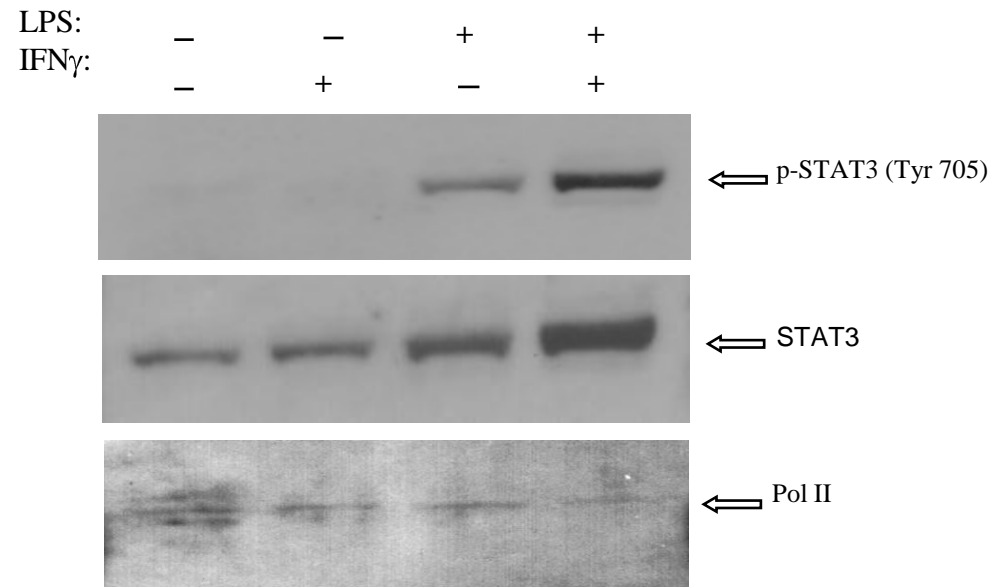
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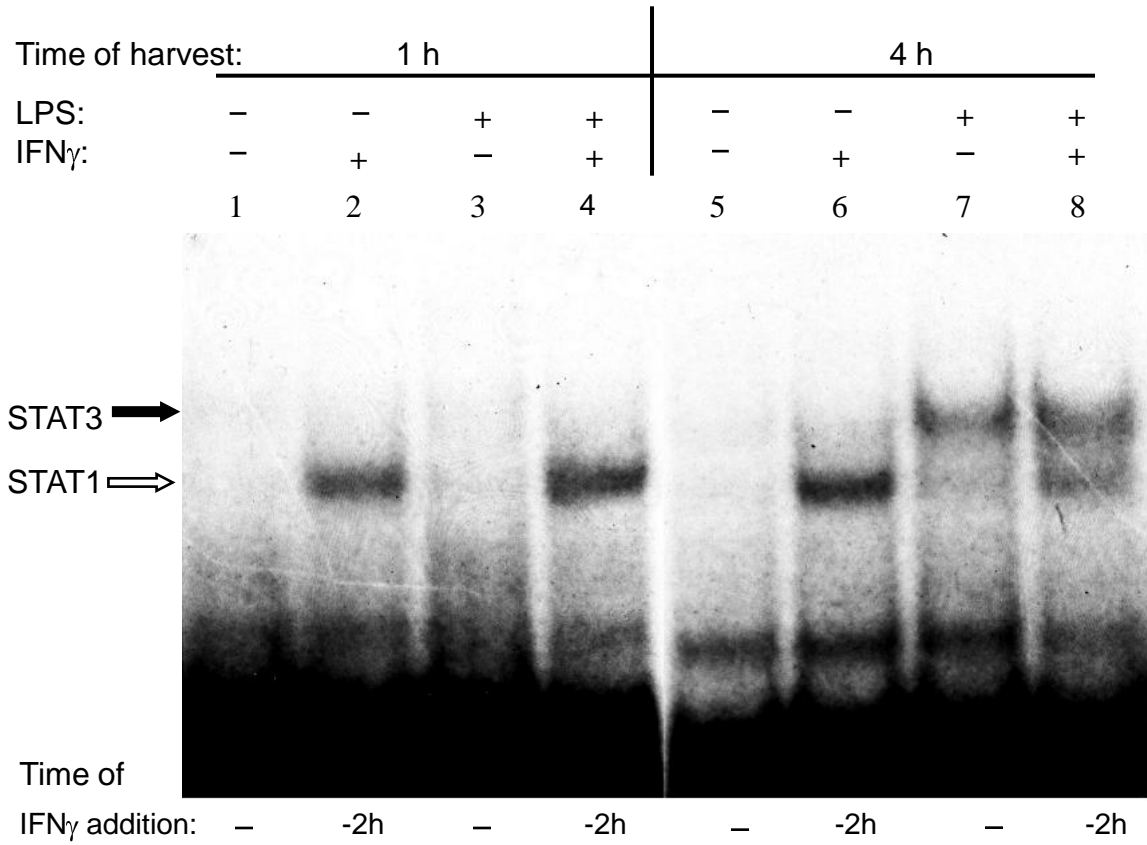
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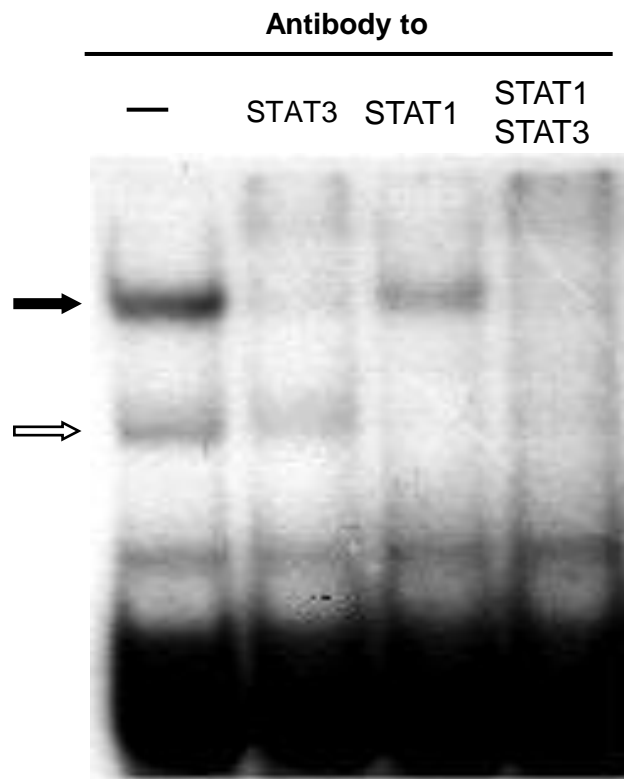
Schaefer et al., Figure 4 A



Schaefer et al., Figure 4 B



Schaefer et al., Figure 5 A



Schaefer et al., Figure 5 B

