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**Reciprocal regulation of the bile acid-activated receptor FXR
and the Interferon- γ -STAT-1 pathway in macrophages**

Running Title: IFN γ and STAT1 regulate FXR

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Summary

Nuclear receptors are a family of ligand regulated factors that exert homeostatic functions at the interface between metabolic and immune function. The farnesoid x receptor (FXR) is a bile acid sensor expressed in immune cells such as macrophages where it exerts counter-regulatory effects. FXR deficient mice demonstrate dysregulated immune response. Expression of FXR is down-regulated in inflamed tissues but the mechanism that leads to FXR down regulation by inflammatory mediators is unknown. In the present study we have investigated the effect of inflammation-related cytokines on macrophages and demonstrated that IFN γ is a potent inhibitor of FXR gene expression/function in macrophages. STAT1 silencing and over-expression experiments demonstrated that FXR repression is mediated by IFN γ dependent activation of STAT1. Since IFN γ is a potent activator of STAT1 we searched for STAT1 binding sites in the human FXR genomic and identified a region of the human FXR gene between the second and third exon that contains three hypothetical STAT1 binding sites. RAW 264.7 transiently transfected with an FXR genomic reporter construct which contained the three STAT binding sites responded to IFN γ with a robust decrease in the reporter activity, demonstrating the potent modulation of FXR transcription by IFN γ . Chromatin immunoprecipitation assay revealed that this region was immunoprecipitated following treatment of macrophage cell lines and supershift assay demonstrated that STAT1 was able to bind one of three identified sites. In summary, these results suggest that IFN γ induced STAT1 homodimers modulate the transcriptional repression of FXR gene in macrophages during inflammation-related cytokines.

Keywords: Farnesoid X Receptor, Interferon- γ , STAT1, Innate immunity, Adaptive immunity.

Introduction

Metabolic nuclear receptors are a family of ligand-activated transcription factors that share a common structure characterized by an highly conserved DNA-binding domain that recognizes specific DNA sequences connected via a linker region to a C-terminal ligand-binding domain (1-3). In addition to their role in metabolism regulation, several members of this family exert counter-regulatory effects on key aspects of the immune system (4,5). Thus in addition to the glucocorticoid receptor, also the peroxisome proliferator-activated receptors (PPARs), the estrogen receptor β , liver-X-receptor α and β (LXR), vitamin D receptor, constitutive androstane receptor and pregnane X receptor, play a role in maintaining immune system homeostasis (6). Deletion of these genes, often results in a un-regulated immune response and a pro-inflammatory phenotype (7). A reciprocal regulation exists between these receptors and inflammatory genes. For example, PPAR γ activation counter-regulates inflammation and inhibits the production of tumor necrosis factor (TNF)- α , but its expression is greatly reduced in inflamed tissues in response to local cytokine production, suggesting that repression of metabolic nuclear receptors might be a mechanism required for inflammation to progress (1,2,8-11).

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily of ligand-activated transcription factors primarily expressed in entero-hepatic tissues (3-5). FXR functions as an endogenous sensor for bile acids, being the primary bile acid, chenodeoxycholic acid (CDCA), the most potent of its endogenous ligands and regulates cholesterol, fatty acid and glucose metabolism (12-17). In its active configuration FXR forms an heterodimer with the retinoid X receptor (RXR). The FXR/RXR heterodimer binds to two AGGTCA half sites separated by one nucleotide, inverted repeat (IR-1), on target genes (3-5). FXR interferes with the activities of transcription factors that activate inflammatory pathways through a mechanism that involves protein-protein interactions with nuclear factor κ B (NF- κ B) and activator protein

1 (AP-1) (trans-repression) (18,19). Several mediators regulates FXR expression. The liver enriched transcription factor (HNF1 α), an essential regulator of bile acid and high density lipoprotein (HDL)-cholesterol metabolism, is a potent activator of FXR gene expression in the liver (20). In contrast, glucose, insulin, activation of toll like receptor (TLR)-4 and endotoxin-regulated cytokines, such as TNF- α and interleukin (IL)-1 β , negatively regulate FXR expression in various tissues (17,21).

The Signal Transducers and Activator of Transcription (STAT) proteins are a family of seven regulatory proteins (1,2,3,4,5A and 5B and STAT6) that play a role in immune tolerance and tumor surveillance (22). The first two STAT proteins were identified in the interferon (IFN) system and STAT1 homodimers mediates type II IFN signalling. Janus activated kinase (JAK) is a non-receptor tyrosine kinase which activates STATs (23).

When IFN γ binds to its receptor on the plasma membrane, JAK phosphorylates the tyrosine 701 residue of STAT1 (23,24). Following tyrosine phosphorylation, the SH2 domain of one STAT1 monomer recognizes the phosphorylated tyrosine residue of the other STAT1 monomer and generates the STAT1 homodimer (22,23). This homodimerization induces the nuclear localization of STAT1, an event that is followed by its binding to the promoter regions of genes containing gamma activated site (GAS) motif, causing the transcription or repression of these genes (22,23,24). Previous studies have shown that exposure of 3T3-L1 adipocytes to IFN γ results in STAT1-dependent repression of PPAR γ transcription and that the PPAR γ 2 promoter contains a highly specific STAT1 binding site (25).

Because the mechanisms that regulate FXR transcription are largely unknown we have investigated whether IFN γ regulates FXR gene expression and activity and whether this effect involves STAT1 activation. The results of these experiments support the notion that a reciprocal regulation exists between the bile acid sensor FXR and IFN γ regulated pathways in macrophages. This mechanism might play a major role in

regulating innate immune response to nutrients and cholesterol-derived mediators.

Materials and methods

Cell Culture

RAW264.7 were grown at 37°C in D-MEM containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. THP-1 were grown at 37°C in RPMI containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. HEPG2 were grown at 37°C in E-MEM containing 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. Cells were regularly passaged to maintain exponential growth. Human peripheral blood derived mononuclear cells (PBMCs) were obtained from normal individual donors to the Blood Bank Service of Perugia University Hospital. PBMCs were isolated by density gradient centrifugation through a Ficoll-Hypaque gradient (Pharmacia Biotech AB, Uppsala, Sweden). Monocytes, were isolated by negative selection using magnetic cell sorting according to the manufacturer instructions (Mylteni Biotec, Milan, Italy). Assessment of FXR expression on purified monocytes was carried-out by qualitative PCR and Western blot analysis.

Western Blotting

Total lysates were prepared by solubilization of cells in NuPage sample buffer (Invitrogen) containing Sample reducing agent (Invitrogen) and separated by polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred to nitrocellulose membranes (Bio-Rad) and probed with primary antibodies FXR (Santa Cruz, H-130), phosphoSTAT1(Tyr701) (Cell Signaling), STAT1 (Cell Signaling) or Tubulin (Sigma). The anti-

immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody, and specific protein bands were visualized using Super Signal West Dura (Pierce), following the manufacturer's suggested protocol.

RNA Extraction

Total RNA was isolated from cells using the TRIzol reagent according to the manufacturer's specifications (Invitrogen, Milan, Italy). One μ g RNA was purified of the genomic DNA by DNase-I treatment (Invitrogen) and random reverse-transcribed with Superscript II (Invitrogen) in 20 μ l reaction volume.

Qualitative and Quantitative PCR

For qualitative and quantitative PCR conditions were as previously described (26). All PCR primers were designed using software PRIMER3-OUTPUT using published sequence data from the NCBI database. Quantification of gene expression was performed using the following sense and antisense primers:

mGAPDH: ctgagtatgtcgtggagtctac	and	gttggtggtgcaggatgcattg;
mFXR: tgtgagggctgcaaaggtht	and	acatccccatctctctgcac;
mTNFα: acggcatggatctcaaagac	and	gtgggtgaggagcacgtagt;
mIL1β: tcacagcagcacatcaacaa	and	tgtcctcatcctcgaaggtc;
mIFNγ: gctttgcagctcttcctcat	and	gtcaccatccttttgccagt;
mIRF1: gcaaaaccaagaggaagctg	and	gagactgctgctgacgacac;
mMCP1: cccaatgagtaggctggaga	and	tctggacccattccttcttg;
mIP10: tcacctgctgggtctgagt	and	gtggcaatgatctcaacacg;
mICAM-1: gggaatgtcaccaggaatgt	and	

tgagttttatggcctcctcct; **mSTAT1:** cttgacgaccctaagcgaac and
 ccgggacatctcatcaaact; **hGAPDH:** gaaggtgaaggtcggagt and
 catgggtggaatcatattggaa; **hFXR:** tacatgcgaagaagtgtaaga and
 actgtcttcattcacgggtctgat; **hSTAT-RE (used for ChIP experiment):**
 catgaccaaggtagatcatgac and cccaagatacgtgcttgcat.

Construction of Reporter Plasmids

The genomic DNA fragment containing the Hind-III site, the HNF1 α response element, the STATP1, STATP2 and STATP3 putative binding sites was synthesized at MWG-Eurofins. The following sense and antisense primers were then oligo annealed and phosphorylated at 5' : HindIII-HNF1 α RE-STATREs sense, 5'-AAGCTTATTGTTAATGACTAATCTGTTTCCTCTAAATTCTAATAAA

TTTCAGATAAA-3'; HindIII-HNF1 α RE-STATREs antisense, 5'-TTTATCTGAAA
 TTTATTAGAAA

TTTAGAGGAAAACAGATTAGTCATTAACAATAAGCTT-3'. After 3' dATP attachment using Taq DNA Polymerase (Invitrogen), double strand oligo was cloned into pCR2.1 by using TOPO TA cloning kit (Invitrogen). From pCR2.1 vector, the genomic fragment was extracted and then subcloned into pGL3 basic vector using the restriction enzymes Kpn-I and Xho-I (pGL3HNF1 α RE/STAT1REs). The same cloning strategy was used for the cloning of the STATP2 mutated binding site into pGL3 vector (pGL3HNF1 α RE/STAT1RE_{S_{P2mut}}) by using the following sense and antisense primers: HindIII-HNF1 α RE-STATREs-P2mut sense,

5'-
 AAGCTTATTGTTAATGACTAATCTGTTTCCTCTAAAT**GTATCGTAC**ATTTCAGATAAA-
 3'; HindIII-HNF1 α RE-STATREs-P2mut antisense, 5'-

TTTATCTGAAAT**TGTACGATACA**TTTAGAGGAAAACAGATTAGTCATTAACAATAAGC
TT-3'.

Transactivation Assay

All transfection experiments carried out using Fugene HD according to manufacturer specifications (Roche). RAW264.7 cells were transfected with 1 μ g of pGL3 or 1 μ g of FXR genomic luciferase reporter vector containing the HNF1 α and STAT responsive elements (pGL3HNF1 α RE/ STAT1REs) or 1 μ g of FXR genomic luciferase reporter vector containing the HNF1 α and STAT responsive elements containing the mutated STAT-P2 binding site (pGL3HNF1 α RE/STAT1Re_{SP2mut}), with 200 ng pCMV- β gal, as internal control for transfection efficiency, with 100 ng of pSG5-HNF1 α or 100 ng of pSG5-STAT1 or with the combination of pSG5-HNF1 α and pSG5-STAT1. The pGEM vector was added to normalize the amounts of DNA transfected in each assay (2.5 μ g/well). Forty-eight hours post-transfection, cells were stimulated with 100 ng/ml IFN γ for 18 hours. Control cultures received vehicle (0.1% DMSO) alone. Cells were lysed in 100 μ l diluted reporter lysis buffer (Promega), and 5 μ l cellular lysates were assayed for luciferase activity using Luciferase Assay System (Promega) and luminescence measured by an automated luminometer. Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β -galactosidase activity. All experiments were done in triplicate and were repeated at least once.

Electrophoretic Mobility Shift Assay (EMSA)

Preparation of nuclear extract from THP-1 cells not treated or stimulated with IFN γ at 100 ng/ml for 18 hours were done using NE-PER (PIERCE). The probes used for electrophoretic mobility shift assay (EMSA) were labelled with biotin using Biotin 3' end DNA labelling kit (PIERCE) according to the manufacturer's instructions. The sequences of probes used were: P1: TTTCCTCTAAA; P2: TTCTAATAAAA and P3: TTTCAGATAAAA. For EMSA, 5 μ g of nuclear extract were incubated with 15 fmol of the indicated biotin-end-labelled probes in a total volume of 20 μ L of binding buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 10% glycerol, 1 μ g of poly dI-dC) for 20 minutes at room temperature. For competition assays, excess unlabeled oligonucleotides were preincubated with nuclear extract for 15 minutes prior to the addition of the biotin-labelled probe. The reactions were loaded on a 6% polyacrylamide non-denaturing gel in 0.5X Tris-borate-EDTA and electrophoresed for 1 hours at 100 V. The protein/DNA complexes were then transferred to positively charged nylon membrane (PIERCE) and the supershift was detected using Chemiluminescent Nucleic Acid Detection Module (PIERCE).

Chromatin Immunoprecipitation (ChIP)

A ChIP assay was performed according to the manufacturer's protocols (Abcam Ltd, Cambridge, UK) with minor modifications. In brief, THP1 cells not treated or stimulated with IFN γ at 100 ng/ml for 18 hours were cross-linked with 1% formaldehyde at room temperature and the reaction was terminated by the addition of glycine to a final concentration of 0.125 M. Cells were washed in ice-cold PBS and lysed with SDS lysis buffer (1% SDS,

10 mM EDTA, and 50 mM Tris-HCl, pH 8). Cellular lysates were diluted with ChIP dilution buffer, sonicated, and immunoprecipitated with specific antibody: anti-STAT1 or anti-phosphoSTAT1(Tyr701) from Cell Signaling. Immunoprecipitates were collected with protein A beads (Amersham Bioscience) and washed sequentially first with a low-salt wash buffer and then with high-salt wash buffer using manufacturer's recommended procedures. DNA was eluted by addition of 1% SDS and 0.1 M NaHCO₃, and the cross-linking reactions were reversed by heating the mixture to 65°C overnight. The DNA was recovered from immunoprecipitated material by proteinase K treatment at 65°C for 1 h followed by phenol/chloroform (1:1) extraction, ethanol precipitation and dissolved into 50 μ l of water. Five microliters was used for quantitative real-time PCR (qRT-PCR).

Transient transfection of STAT1 and HNF1 α constructs

All the transfections were making using Fugene HD according to manufacturer specifications (Roche). 2×10^6 RAW264.7 cells were transfected with 5 μ g of expression vector pCMVHNF1 α alone or in combination with 5 μ g of expression vector pCMVSTAT1 or 5 μ g of silencing vector psiRNASTAT1 (Origene). Forty-eight hours post-transfection, cells were stimulated with 100 ng/ml IFN γ for 18 hours. Control cultures received vehicle (0.1% DMSO) alone. Cells were lysed in Tryzol and RNA was extracted as above.

Statistical Analysis

All values are expressed as mean \pm SE of n observations per group. Comparisons of more than two groups were made with a one-way ANOVA with post-hoc Tukey's test. Differences were considered statistically significant if P was < 0.05 .

Results.

FXR is expressed in macrophages and regulates macrophages activation induced by IFN γ

We have first examined whether macrophage cell lines and peripheral blood derived mononuclear cells (PBMCs) express FXR. HepG2, an hepatocarcinoma cell line expressing high levels of FXR mRNA, was used as a positive control. Expression of FXR mRNA was detected by qualitative PCR in both human (THP1) and mouse (RAW264.7) macrophage cells lines. (Figure 1A). FXR protein (\approx 55 kDa) was detected by Western blot analysis in HepG2 cell line (positive control) and, though to a lower level, in RAW264.7 and THP1 cells lines (Figure 1B). In addition, FXR expression was detected by qualitative RT-PCR and Western blot analysis in PBMCs (Figure 1C and D).

To investigate whether FXR acts as a regulatory gene in the context of macrophages activation induced by IFN γ (100 ng/ml for 18 hours), RAW264.7 cells were exposed to 6E-CDCA 1 μ M, a semi-synthetic bile acid derivative that activates FXR with an EC₅₀ of ~300 nM (being the EC₅₀ of CDCA, the most potent of naturally occurring bile acid ligands, ~10 μ M). As shown in Figure 2, FXR activation with 6-ECDCA, caused a significant downregulation of the expression of several IFN γ -responsive genes, including MCP-1 and ICAM-1 (n=4; *p<0.05 versus not treated cells, °p<0.05 versus IFN γ treated cells). Because cultured macrophages express low levels of endogenous FXR, we have then investigated whether FXR over-expression results in a more robust regulation of IFN γ activity. For this purpose RAW264.7 were transiently transfected with pCMVSPORT6-FXR, serum starved 18 hours, and then stimulated with IFN γ (100 ng/ml for 18hours). Over expression of FXR was confirmed by RT-PCR (Fig 3A, n=3; *p<0.05 versus not transfected cells). FXR over-expression was sufficient to cause a robust inhibition of the expression of IFN γ regulated genes (Figure 3B,C,D and E n=4; *p<0.05 cells stimulated with IFN γ versus cells stimulated with IFN γ and over-expressing FXR). This effect was further enhanced by exposure to 6-ECDCA at 1 μ M. Thus, the combination of FXR over-expression and its activation by 6-ECDCA resulted in an almost complete abrogation of the stimulatory effect exerted by IFN γ on IRF1, IP10, MCP1 and ICAM-1 (Figure 4A,B,C and D n=4; *p<0.05 versus not treated cells, #p<0.05 versus IFN γ stimulated cells).

IFN γ treatment results in a decrease of FXR expression

Because FXR seems to act as an endogenous counter-regulatory gene, one would speculate that macrophages activation should regulate its

expression in order to allow the progression of an immune response. As shown in Figure 5A exposure to IFN γ (100 ng/ml), results in a significant decrease of FXR expression as assessed by RT-PCR (Figure 5A, n=3; *p<0.05 versus not treated cells) and Western blot analysis (Figure 5B).

STAT1 mediates the repression of FXR expression in vitro

Since STAT1 is a critical mediator in the propagation of IFN γ -mediated signals in macrophages, we have examined whether exposure of RAW264.7 cells to IFN γ 100 ng/ml causes STAT1 phosphorylation on Tyrosine 701. As shown in Figure 6A an increase in STAT1 phosphorylation was observed between 30' and 180' after treatment with IFN γ , while Western blotting analysis of total STAT1 protein failed to show any increase in total protein level.

To investigate whether STAT1 mediates the FXR transcriptional repression induced by IFN γ , RAW264.7 cells were co-transfected with plasmids containing a coding sequence for HNF1 α and STAT1 (pCMVHNF1 α and pCMVSTAT1) and then stimulated with IFN γ , 100 ng/ml, for 18 hours. pCMVHNF1 α was used as a positive regulator to induce the FXR transcription in these cells. Quantitative RT-PCR demonstrated that the relative expression of STAT1 in RAW264.7 cells transfected with pCMVSTAT1 was significantly enhanced in comparison with RAW264.7 cells not transfected or transfected with pCMVHNF1 α only (Figure 6B and 6C, n=4; *p<0.05 versus not transfected cells; #p<0.05 versus HNF1 α alone expressing cells). FXR mRNA expression in RAW264.7 over-expressing HNF1 α and STAT1 was reduced by 50% in comparison to cells

transfected with pCMVHNF1 α only (Figure 6D, columns 1 and 2; n=4; *p<0.05 versus not treated HNF1 α alone expressing cells). In addition, the FXR mRNA expression was further down-regulated by treating STAT1 over-expressing RAW264.7 cells with IFN γ (Figure 6D, columns 3 and 4 n=4; *p<0.05 versus not treated HNF1 α alone expressing cells, n=4; #p<0.05 versus not treated HNF1 α and STAT1 expressing cells).

To further investigate the role of STAT1 in regulating FXR gene expression, STAT1 mRNA expression in RAW264.7 was silenced using a commercial plasmid containing a siRNA for STAT1 (psiRNASTAT1; Origene). As show in Figure 6E transfection of STAT1 siRNA attenuated STAT1 mRNA expression by 50% in comparison to cells not transfected or transfected with pCMVHNF1 α alone (Figure 6E and 6F, n=4; *p<0.05 versus not transfected cells #p<0.05 versus HNF1 α alone expressing cells). To investigate whether STAT1 abrogation interfere with the ability of IFN γ to modulate the expression of FXR, STAT1-deficient RAW264.7 were co-transfected with a plasmid containing the coding sequence for HNF1 α (pCMVHNF1 α and psiRNASTAT1). While exposure of RAW264.7 transfected with the pCMVHNF1 α to IFN γ (100 ng/ml for 18 hours) resulted in a robust downregulation of FXR mRNA expression, this inhibitory effect was completely abrogated by STAT1 silencing (Figure 6G, n=4; *p<0.05 versus not treated HNF1 α alone expressing cells).

IFN γ modulates the transcription of the FXR gene

We have then examined whether FXR genomic sequences contains responsive elements for IFN γ . We focused our search on GAS like

elements (consensus GAS sequence: TTCNNNNAAA) (25) that are known to mediate IFN γ sensitive genes in a STAT1 dependent manner. Our analysis revealed that a region in the human FXR genomic between the second and the third exon contains three putative STAT1 response elements (Figure 7A). To determine if this region of the human FXR genomic was sensitive to IFN γ regulation we generated a luciferase reporter construct (pGL3HNF1 α RE/STAT1REs) which contains the HNF1 α responsive element cloned from the human FXR 5' flanking region and the region containing the three putative STAT1 responsive elements (Figure 7B). This vector was then transiently transfected into RAW264.7 cells. In each experiment cells were co-transfected with a β -galactosidase vector as a control for the transfection efficiency. In addition, RAW 264.7 were also co-transfected with HNF1 α expression vector (pCMVHNF1 α) alone or in combination with STAT1 expression vector (pCMVSTAT1). As expected co-transfection of pGL3HNF1 α RE/STAT1REs with HNF1 α expression vector resulted in a substantial increase in luciferase activity compared to transfection of the luciferase reporter construct alone (Figure 7C, columns 1 and 3, n=3, *p<0.05 versus pGL3HNF1 α RE/STAT1REs alone transfected cells). Co-transfection of pGL3HNF1 α RE/STAT1REs with STAT1 expression vector alone resulted in a greater down-regulation of luciferase activity compared to transfection of pGL3HNF1 α RE/STAT1REs alone (Figure 7C, columns 1 and 5, n=3 *p<0.05 versus pGL3HNF1 α RE/STAT1REs alone transfected cells). Co-transfection with both HNF1 α and STAT1 expression

vectors decreased luciferase activity in comparison with pCMVHNF1 α alone transfected cells (Figure 7C, columns 3 and 7, $^{\circ}p < 0.05$ versus pCMVHNF1 α expressing cells). The treatment of all transfected cells with IFN γ (100 ng/ml for 18 hours), resulted in a robust reduction of luciferase activity compared to not stimulated cells (Figure 7C, lanes 2, 4, 6 and 8, $\#p < 0.05$ versus not stimulated cells). These results suggest that modulation of FXR transcription by IFN γ could be mediated by a region of FXR genomic between the second and third exon containing putative STAT1 binding sites.

STAT1 binds to the FXR regulatory region in vitro

To investigate the interaction between STAT1 and FXR genomic, chromatin immunoprecipitation (ChIP) was performed using serum starved THP1 cells exposed to IFN γ (100 ng/ml for 18 hours). As shown in Figure 8A, quantitative Real-Time PCR of the FXR genomic between the second and third exon confirmed the binding of both the proteins STAT1 and STAT1 phosphorylated on the FXR genomic. (Figure 8A, $n = 4$; $*p < 0.05$ versus not treated cells immunoprecipitated with STAT1 antibody, $\#p < 0.05$ versus not treated cells immunoprecipitated with phosphoSTAT1 antibody).

To characterize whether the three GAS like responsive elements of the human FXR genomic region between the second and third exon binds STAT1 we performed an EMSA using three different probes designed P1, P2 and P3. Each probe matches a single GAS like response element. These probes were biotin labelled and incubated with nuclear extracts

prepared from THP1 cells untreated or treated with IFN γ 100 ng/ml for 18 hours. As shown in Figure 8B, while the first putative STAT1 response element, P1, failed to bind nuclear extracts from THP1 treated with IFN γ , a robust binding was observed with the second probe, P2. Finally, the third probe, P3, was also able to bind nuclear extracts from THP1 both in the absence and in presence of IFN γ indicating that the binding of probe P3 to the FXR genomic is independent to IFN γ activation. DNA binding and supershift was almost completely abrogated using a mutated STAT1 P2 probe as well as an anti STAT1 antibody, while when STAT1 binding probe (IRF1-GAS: TTTCCCCGAAA) was used as positive control we observed the same supershift of the probe P2 (Figure 8C lanes 5, 6 and 7). Finally, to confirm that only the P2 STAT1 binding site is necessary to down-regulate FXR gene expression, we performed a luciferase experiment with a vector containing a mutation of the P2 site (pGL3HNF1 α RE/STAT1Res_{P2mut}). As shown in Figure 8D, the mutation of P2 binding site failed to down-regulate the FXR transcription in presence of IFN γ treatment (Figure 8D, n=3; *p<0.05 versus pGL3HNF1 α RE/STAT1REs alone transfected cells; #p<0.05 versus pGL3HNF1 α RE/STAT1REs, pCMVHNF1 α and pCMVSTAT1 transfected cells not stimulated with IFN γ ; °p<0.05 versus pGL3HNF1 α RE/STAT1Res_{P2mut} alone transfected cells).

All together, these results suggest that modulation of FXR transcription by IFN γ was mediated only by the STAT1 binding site P2.

Discussion

Inflammatory host responses to foreign challenge involve a complex network of mediators that establish both an innate and acquired immune response. Although beneficial in the setting of defence of the host against infectious invaders, this network of immune mediators can become unchecked, contributing to the pathogenesis of common chronic inflammatory diseases such as atherosclerosis (27), obesity-induced insulin resistance(28), arthritis (29), inflammatory bowel disease (30) and multiple sclerosis (31). At its basic cellular level, many of the signalling mediators that are produced by these chronic inflammatory events are under the transcriptional control of early transcription factors including NF- κ B and AP-1 (32,33).

Recent attention has been directed at several members of the nuclear receptor superfamily because of their physiological roles as negative regulators of inflammatory responses (34,35). For example glucocorticoid receptor (GR), the prototypical receptor of this class of agents, senses endogenous steroids generated by cholesterol metabolism and inhibits inflammation. GR-mediated inhibition of inflammation involves the transrepression of inflammatory mediators through different mechanisms: i) direct interaction between GR and transcription factors such as AP-1 and NF- κ B (11,36,37); ii) Inhibition of the signalling of mitogen-activated protein kinase pathways that mediates the expression of inflammatory genes (38,39) and iii) competition with co-activator complexes (40,41). Similar to GR, PPAR γ ligands exert anti-

inflammatory activities in macrophages by competition for transcriptional co-activators, including NF-kappaB (42) and/or by inhibition of IFN γ stimulated Janus kinase-STAT1 signalling pathway (43).

A growing body of evidences support the notion that a mutual inhibition between pro-inflammatory mediators (cytokines and early transcription factors) and nuclear receptors exists. Thus, while Toll like receptor 4 activation by LPS leads to a robust up-regulation of steady-state mRNA of several transcription factors, such as AP-1, NF-kB, STAT1 and STAT3, and cytokines, including TNF α , IL1 β and IFN γ (44); it also downregulates the expression of members of nuclear receptor superfamily such as RXR α , PXR, FXR, LXR, PPAR α , PPAR γ , PXR and CAR (44). Similarly to LPS, IFN γ , a Th1 type cytokine secreted by CD4 $^+$ and CD8 $^+$ T cells, NK cells and dendritic cells, decreases the expression of PPAR γ in adipocytes by blocking the synthesis and increasing the degradation of this transcription regulator (25,45).

In the present study we demonstrated a novel cross talk between the bile acid sensor FXR and the IFN γ network. In fact, we observed that while FXR exerts an anti-inflammatory and immuno-regulatory activities, treatment of macrophages with IFN γ results in a STAT1-dependent repression of FXR mRNA and protein expression. This conclusion is based on the following results: i) FXR activation caused a robust down-regulation of the expression of several IFN γ regulated genes, including IRF1, IP10, MCP1 and ICAM1; ii) FXR gene expression is downregulated following treatment of macrophages with IFN γ ; iii) IFN γ mediated

repression of FXR is mediated by STAT1 activation. Our findings suggest that the IFN γ induced repression of FXR mRNA is due to a direct interaction of STAT1 with the FXR genomic. Thus we found that the region between the second and the third exon of the FXR gene contains three putative GAS/ISRE like elements that function as STAT1 binding sites (consensus GAS sequence: TTCNNNAAA) (25). Transfection of macrophages with a vector expressing these three GAS/IRE elements resulted in a robust inhibition of FXR transcription in response to IFN γ treatment. The interaction of STAT1 on GAS/ISRE elements on the FXR gene was further investigated by CHIP experiments carried out with anti-STAT1 and anti-phospho-STAT1 antibodies in THP1 cells exposed to IFN γ . The results of these experiments confirmed that the region between the second and third exon of FXR genomic effectively binds STAT1. The specificity of these STAT1 binding sites was further investigated by the following experiments: i) EMSA assay showed that only the second STAT1 binding site (**TTC**TAAT**AAA**) was capable of binding STAT1 to the FXR genomic following IFN γ stimulation and that this interaction resulted in a repression of FXR transcription; ii) luciferase assay showed that mutation of STAT1 response element P2 failed to reduce the transactivation in presence of IFN γ .

Hence, the tightly controlled regulation of FXR gene expression we observed in presence of IFN γ is due to interaction of STAT1 to GAS element found in the region of FXR genomic between second and third exon. Because FXR functions as a counter-regulatory gene, and mice

lacking FXR demonstrate a pro-inflammatory phenotype (46), it is not surprising that FXR gene expression is negatively regulated during inflammation (21). Several studies have shown that LPS, TNF α and IL1 β exert a negative regulatory role on FXR, and that FXR gene expression is downregulated in the liver during the acute phase of inflammation (21). Although this negative modulation is of critical importance in the context of regulation of early phase response, little is known regarding the modulation of FXR in response to mediators located at the interface between innate and immune response. In the current study we have described for the first time the molecular mechanism of the IFN γ mediated repression of nuclear receptor FXR. In conclusion, we have shown that FXR and IFN γ exert a mutual regulatory effects and that FXR is regulated in response to IFN γ activation in macrophages. This study highlight the potential co-regulatory effect that mediators of inflammation exert on genes involved in bile acid homeostasis.

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Figure Legends

Figure 1. FXR expression in macrophages. **Panel A.** Qualitative PCR showing the expression of FXR mRNA in macrophages cell lines RAW264.7 and THP-1. HEPG2 is the positive control in this experiment. **Panel B.** Western Blotting anti FXR showing the expression of FXR protein in macrophages cell lines RAW264.7 and THP-1. HEPG2 is the positive control in this experiment. **Panel C.** Qualitative PCR Western blot analysis of FXR mRNA expression by PMBC-derived CD14⁺ and HepG2 cells. **Panel D.** Western blot analysis of FXR protein expression by PMBC-derived CD14⁺ and HepG2 cells.

Figure 2. FXR activation regulates macrophage activation induced by IFN γ . **Panel A, B, C and D.** RAW264.7 cells serum starved were stimulated with IFN γ 100 ng/ml in presence or in absence of 6ECDCA 1 μ M for 18 hours. Quantitative RT-PCR showing the modulation of IRF1, IP10, MCP1 and ICAM1 mRNA expression by 6E-CDCA. (n=4; *p<0.05 versus not treated cells, ^op<0.05 versus IFN γ treated cells)

Figure 3. FXR over-expression modulates the IFN γ induced target genes. **Panel A.** Quantitative RT-PCR showing the FXR over-expression in RAW264.7 transfected with pCMVSPORT6-FXR. (n=3; *p<0.05 versus not transfected cells) **Panel B, C, D and E.** RAW264.7 cells over-expressing FXR, serum starved, were stimulated with IFN γ 100 ng/ml for 18 hours. Quantitative RT-PCR showing the modulation of IRF1, IP10, MCP1 and ICAM1 mRNA expression following FXR over-expression. (n=4; *p<0.05

cells stimulated with IFN γ versus cells stimulated with IFN γ and over-expressing FXR).

Figure 4. FXR over-expression and its activation by 6-ECDCA resulted in an almost complete abrogation of the stimulatory effect exerted by IFN γ . RAW264.7 cells were transiently transfected with pCMVSPORT6-FXR, serum starved 18 hours and then stimulated with IFN γ 100 ng/ml in presence or in absence of 6ECDCA 1 μ M for 18 hours. Quantitative RT-PCR showing the almost complete down-regulation of IRF1 (**Panel A**), IP10 (**Panel B**), MCP1 (**Panel C**) and ICAM1 (**Panel D**) mRNA expression following FXR over-expression and activation by 6ECDCA. (n=4; *p<0.05 versus not treated cells, #p<0.05 versus IFN γ stimulated cells).

Figure 5. IFN γ modulates FXR expression. **Panel A.** Quantitative RT-PCR showing the reduction of FXR mRNA in macrophages RAW264.7 serum starved and stimulated with IFN γ 100 ng/ml 18 hours. (n=3; *p<0.05 versus not treated cells). **Panel B.** Western Blotting anti FXR showing FXR protein down-regulation in RAW 264.7 serum starved and stimulated with IFN γ 100 ng/ml for 24, 48 and 72 hours.

Figure 6. **Panel A.** Western Blotting analysis of STAT1 and phospho-STAT1(Tyr701) proteins following IFN γ treatment of RAW264.7 for 5-15-30-60 and 180 minutes. **Panel B.** Quantitative PCR showing STAT1 over-expression in RAW264.7 cells transfected with pCMVSTAT1 (n=4; *p<0.05 versus not transfected cells) **Panel C.** Quantitative PCR showing STAT1 expression in RAW264.7 cells transfected with pCMVSTAT1 and pCMVHNF1 α (n=4; #p<0.05 versus HNF1 α alone expressing cells) **Panel D.**

Quantitative PCR showing FXR mRNA relative expression in RAW264.7 cells transfected with pCMVHNF1 α (white bars) or with both pCMVSTAT1 and pCMVHNF1 α (black bars) stimulated with IFN γ 100 ng/ml for 18 hours. (n=4; *p<0.05 versus not treated HNF1 α alone expressing cells, n=4; #p<0.05 versus not treated HNF1 α and STAT1 expressing cells). **Panel E.** Quantitative PCR showing STAT1 silencing in RAW264.7 cells transfected with psiRNASTAT1. (n=4; *p<0.05 versus not transfected cells). **Panel F.** Quantitative PCR showing STAT1 expression in RAW264.7 cells transfected with psiRNASTAT1 and pCMVHNF1 α . (n=4; *p<0.05 versus not transfected cells; #p<0.05 versus HNF1 α alone expressing cells). **Panel G.** Quantitative PCR showing FXR mRNA relative expression in RAW264.7 cells transfected with pCMVHNF1 α (white bars) or with both psiRNASTAT1 and pCMVHNF1 α (black bars) stimulated with IFN γ 100 ng/ml for 18 hours. (n=4; *p<0.05 versus not treated HNF1 α alone expressing cells).

Figure 7. Panel A. Analysis of the human FXR gene, showing three putative STAT1 binding sites between second and third exon. **Panel B.** Luciferase reporter vector containing both HNF1 α responsive element and STAT1 putative binding sites (pGL3HNF1 α RE/STAT1REs). **Panel C.** RAW264.7 cells were transfected with luciferase reporter vector alone (white bars: pGL3HNF1 α RE/STAT1REs), with luciferase vector plus HNF1 α expression vector (black bars: pCMVHNF1 α), with luciferase vector plus STAT1 expression vector (grey bars: pCMVSTAT1), or with luciferase vector and both HNF1 α and STAT1 expression vectors (striped bars: pHCMVNF1 α + pCMVSTAT1). 24 hours post-transfection cells were stimulated with IFN γ

100 ng/ml for 18 hours. Luciferase activity is shown as the ratio of luciferase to β -galactosidase activities. Data represent the mean \pm S.D. of 3 experiments. * $p < 0.05$ versus pGL3HNF1 α RE/STAT1REs alone transfected cells; $^{\circ}p < 0.05$ versus pCMVHNF1 α expressing cells; # $p < 0.05$ versus not stimulated cells.

Figure 8. Panel A. STAT1 binds to FXR genomic in the context of chromatin structure. ChIP experiments were performed in THP1 serum starved not treated and treated with IFN γ 100 ng/ml for 18 hours. Chromatin was prepared, immunoprecipitated with antibodies directed against STAT1 and phospho-STAT1 (Tyr 701) proteins and quantitative Real-Time PCR of the FXR genomic between second and third exon was performed. Data represent the mean \pm S.D. of 4 experiments. * $p < 0.05$ versus not treated cells immunoprecipitated with STAT1 antibody, # $p < 0.05$ versus not treated cells immunoprecipitated with phosphoSTAT1 antibody. **Panel B.** STAT1 binds to FXR genomic in vitro. Electrophoretic mobility shift assays were performed using three different probes designed P1, P2 and P3. Each probe matches a single GAS like response element. Probes were biotin labeled and incubated with nuclear extracts from THP1 not treated or treated with IFN γ 100 ng/ml for 18 hours. Competition experiments were performed using a 50 fold excess of unlabeled oligo. **Panel C.** STAT1 P2 probe biotin labeled was not incubated or incubated with nuclear extracts from THP1 cells not treated or treated with IFN γ 100 ng/ml for 18 hours (lanes 1,2 and 3). Competition experiments were performed using a 50 fold excess of unlabeled oligo or

1 μ g of STAT1 antibody (lanes 4 and 5). Mutated P2 probe and positive control STAT1 probe biotin labeled were incubated with nuclear extracts from THP1 stimulated cells (lanes 6 and 7). **Panel D.** Mutated STAT1 response element P2 failed to reduce the transactivation following IFN γ treatment. RAW264.7 cells were transfected with the luciferase reporter vector pGL3HNF1 α RE/STAT1REs alone (white bar), with pGL3HNF1 α RE/STAT1REs plus pCMVHNF1 α and pCMVSTAT1 expression vectors (black bars), with luciferase reporter vector pGL3HNF1 α RE/STAT1RE_{SP2mut} alone (grey bar), or with pGL3HNF1 α RE/STAT1RE_{SP2mut} and both pCMVHNF1 α and pCMVSTAT1 expression vectors (striped bars). 24 hours post-transfection cells were stimulated with IFN γ 100 ng/ml for 18 hours. Luciferase activity is shown as the ratio of luciferase to β -galactosidase activities. Data represent the mean \pm S.D. of 3 experiments. *p<0.05 versus pGL3HNF1 α RE/STAT1REs alone transfected cells; #p<0.05 versus pGL3HNF1 α RE/STAT1REs, pCMVHNF1 α and pCMVSTAT1 transfected cells not stimulated with IFN γ ; °p<0.05 versus pGL3HNF1 α RE/STAT1RE_{SP2mut} alone transfected cells.

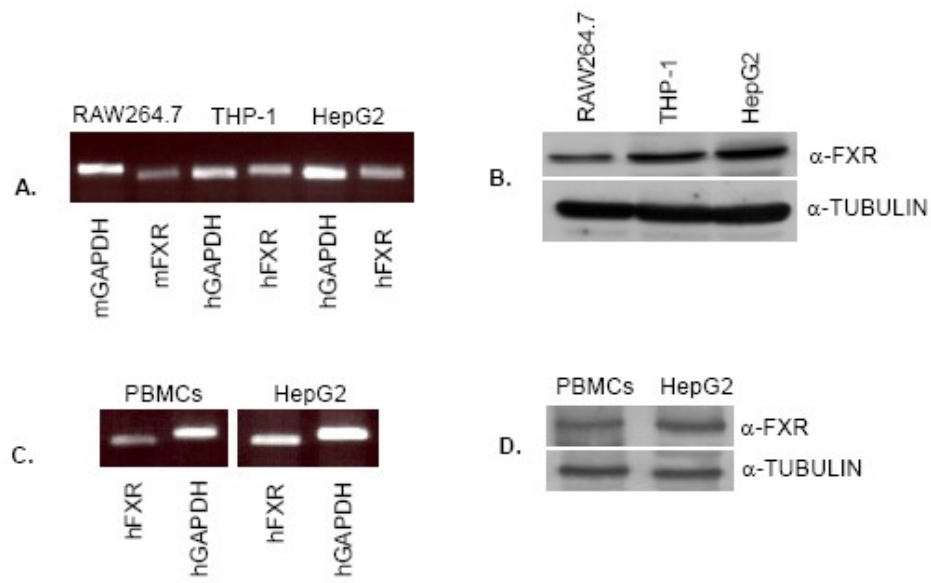


Figure 1

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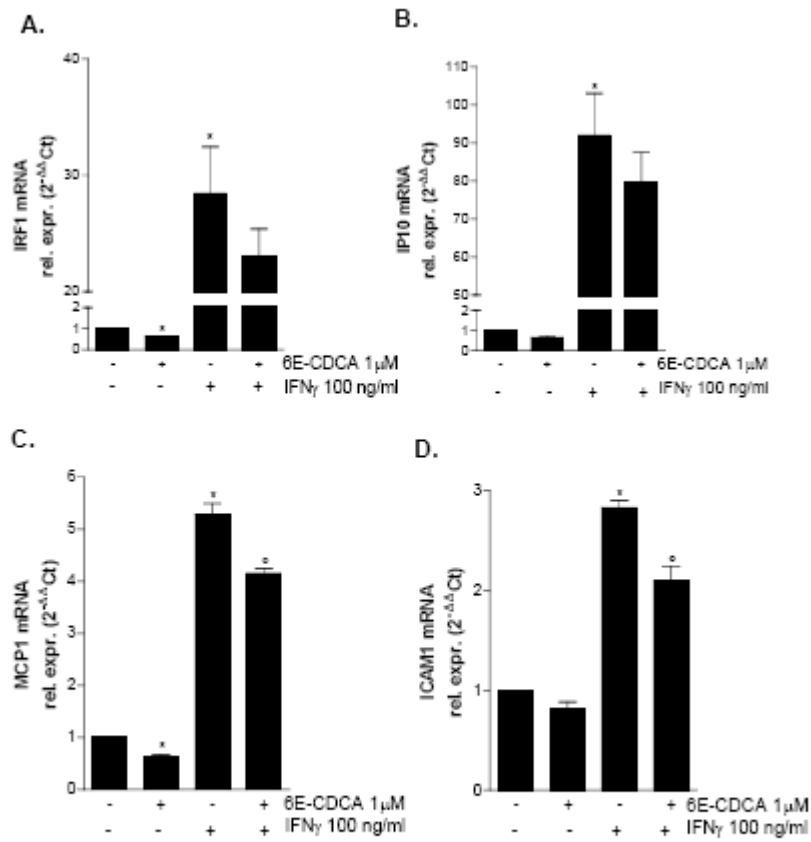


Figure 2

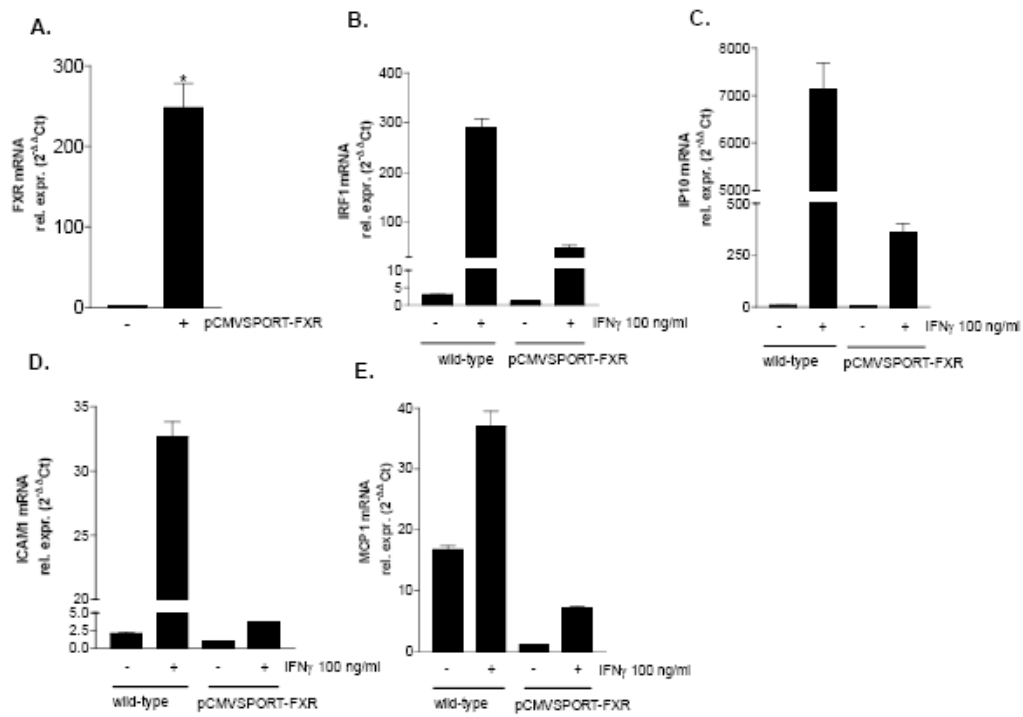


Figure 3

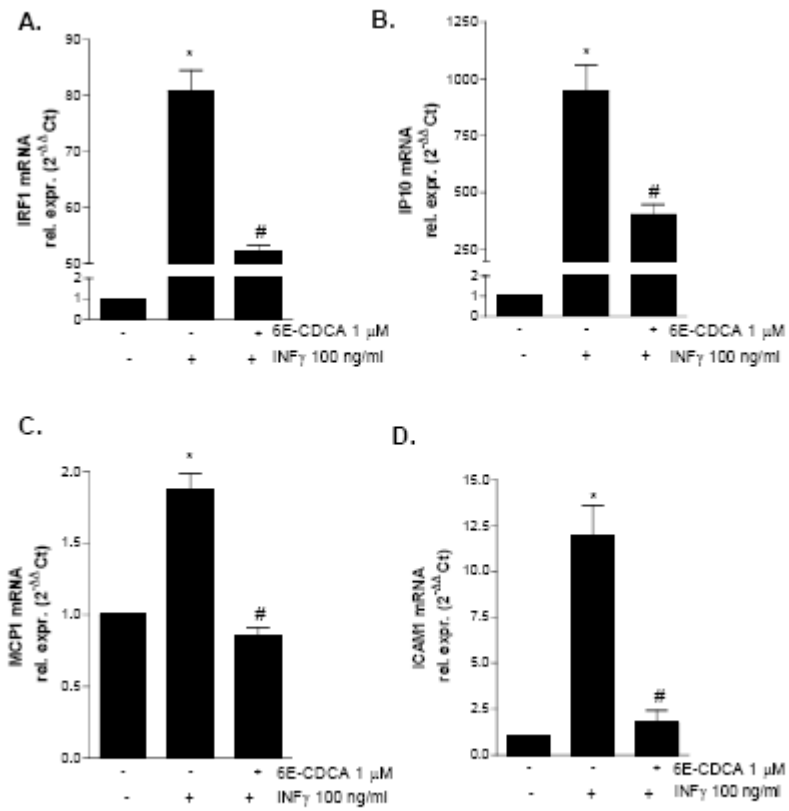


Figure 4

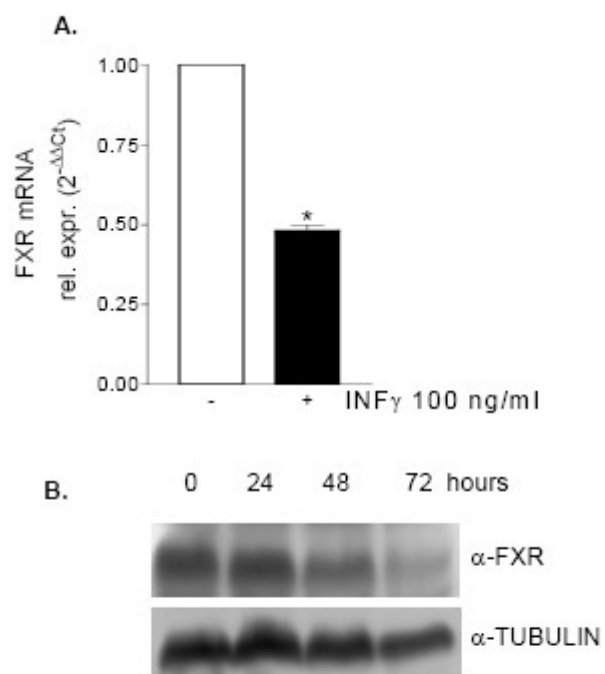


Figure 5

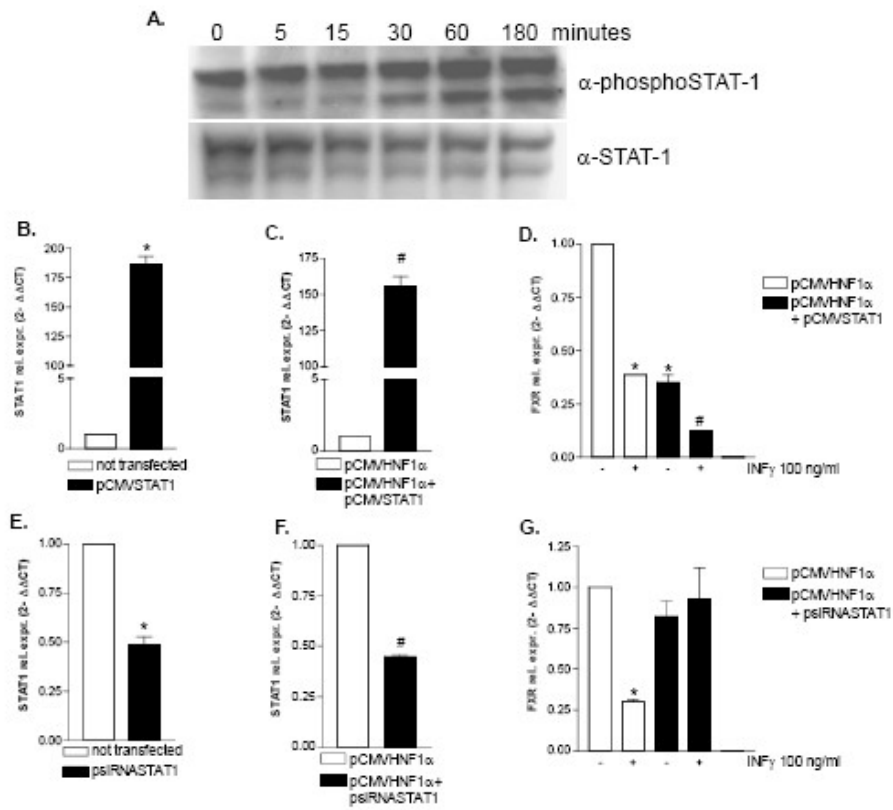


Figure 6

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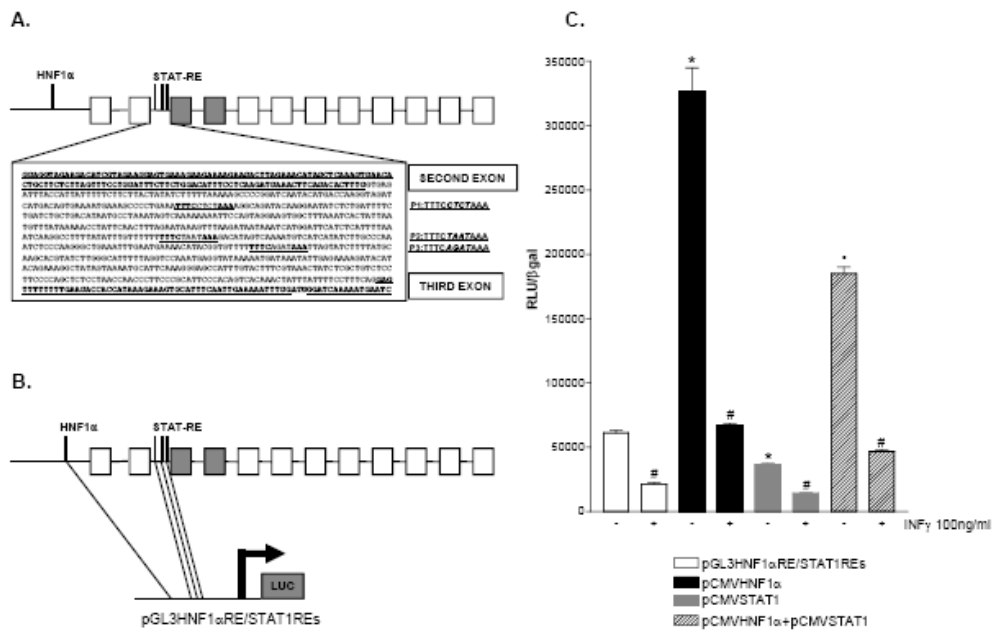


Figure 7

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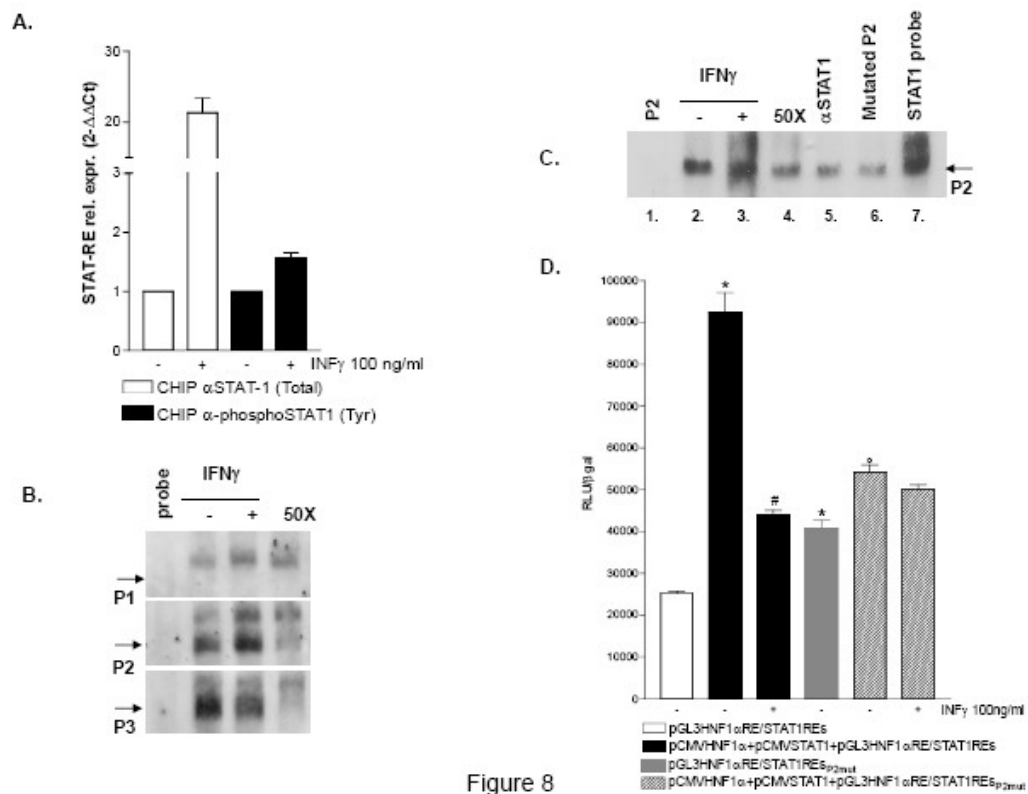


Figure 8

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