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# Molecular Mechanisms Underlying the Inhibition of IFN-γ-Induced, STAT1-Mediated Gene Transcription in Human Macrophages by Simvastatin and Agonists of PPARs and LXRs

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## ABSTRACT

PPARs and LXRs are ligand-activated transcription factors that are emerging as promising therapeutic targets for limiting atherosclerosis, an inflammatory disorder orchestrated by cytokines. The potent anti-atherogenic actions of these nuclear receptors involve the regulation of glucose and lipid metabolism along with attenuation of the inflammatory response. Similarly, cholesterol-lowering drugs, statins, inhibit inflammation. Unfortunately, the mechanisms underlying such inhibitory actions of these agents in human macrophages are poorly understood and were therefore investigated in relation to IFN-γ, a key pro-atherogenic cytokine, which mediates its cellular effects mainly through STAT1. Simvastatin and PPAR agonists had no effect on the IFN-γ-induced, phosphorylation-mediated activation of STAT1 and its DNA binding but attenuated its ability to activate gene transcription. On the other hand, LXR activators attenuated both DNA binding and trans-activation potential of STAT1 induced by IFN-γ. These studies reveal differences in the mechanism of action of agonists of PPARs (and simvastatin) and LXRs on the IFN-γ-induced, STAT1-mediated gene transcription in human macrophages. J. Cell. Biochem. 112: 675–683, 2011. 

**KEY WORDS:** IFN-γ; MACROPHAGES; STAT1; PPAR; LXR; TRANSCRIPTIONAL INHIBITION; SIMVASTATIN

PPARs and LXRs are emerging as promising targets for therapeutic intervention of atherosclerosis, an inflammatory disorder orchestrated by key cytokines such as IFN-γ [Hansen and Connolly, 2008; McLaren and Ramji, 2009; Li et al., 2010]. The potent anti-atherogenic actions of these nuclear receptors (NRs) involve both the activation and the inhibition of gene transcription [Hansen and Connolly, 2008; Hong and Tontonoz, 2008]. Activation requires the binding of heterodimers of these receptors with retinoid-X- receptor (RXR) to recognition sequences in the promoter regions of several genes implicated in the control of glucose and lipid homeostasis [Hansen and Connolly, 2008; Hong and Tontonoz, 2008]. On the other hand, transrepression of inflammatory gene transcription is independent of such direct DNA-binding and involves interference with the activities of major signalling pathways/transcription factors involved in regulating the expression of these genes [Hong and Tontonoz, 2008]. Unfortunately, the molecular mechanisms underlying such trans-repression are poorly understood, particularly in relation to IFN-γ signalling due to limited studies to date. Further studies are required both to improve our knowledge of the molecular basis of NR-mediated transrepression of IFN-γ signalling and for the development of novel therapeutic strategies to combat atherosclerosis.

IFN-γ controls a number of steps in the pathogenesis of atherosclerosis, including the recruitment of inflammatory cells to the activated endothelium, foam cell formation, apoptosis and plaque stability [McLaren and Ramji, 2009; Li et al., 2010]. A marked attenuation of atherosclerosis is observed in mouse models of this disease that lack either IFN-γ or its receptors, whereas administration of this cytokine or its induced expression potentiates the development of this disease in such models [McLaren and Ramji, 2009]. The JAK-STAT pathway plays a key role in IFN-γ signalling [McLaren and Ramji, 2009]. Binding of IFN-γ to its cognate receptors leads to their oligomerisation and the subsequent activation of associated JAK1 and JAK2 by trans-phosphorylation. Activated JAKs then phosphorylate a critical tyrosine residue in the cytoplasmic domain of the receptors, which then serves as a docking site for STAT1α (called STAT1 hereafter) leading to the phosphorylation of this protein on tyrosine 701. STAT1 then dimerises, translocates to the nucleus and interacts with γ-activated sequence elements (GAS) in the regulatory regions of target genes

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[McLaren and Ramji, 2009]. In addition to tyrosine 701, STAT1 phosphorylation at serine 727 is required for maximal activation [McLaren and Ramji, 2009]. The purpose of this study was to investigate the mechanisms underlying the anti-inflammatory actions of agonists of PPARs and LXRs on IFN-γ signalling through STAT1 in human macrophages. Simvastatin was included for comparative purposes because such HMG-CoA reductase inhibitors also attenuate the inflammatory response [Jain and Ridker, 2005].

## MATERIALS AND METHODS

### MATERIALS

The human monocytic THP-1 cell line and the human hepatoma Hep3B cell lines were from the European Collection of Animal Cell Cultures. The antisera were obtained from Sigma (β-actin), Santa-Cruz [STAT1 p89/94 used for electrophoretic mobility shift assays (EMSA)] and Cell Signalling Technology [phospho-STAT1 (Tyr701 or Ser727) and STAT1 p84/91 used in Western blot analysis]. Other specific reagents were obtained from Peprotech (IFN-γ), Sigma [phorbol 12-myristate 13-acetate (PMA), 22-(*R*)-hydroxycholesterol (22-(*R*)-HC), 22-(*S*)-hydroxycholesterol (22-(*S*)-HC), 9-cis-retinoic acid (9cRA)]; Calbiochem (activated simvastatin, sodium salt; TO901317); Qiagen (SuperFECT$^{TM}$); BIOMOL [15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$)]; Cayman Chemicals (rosiglitazone BRL49653, GW7647); and Active Motif (Nuclear Extract Kit).

### CELL CULTURE

Human monocyte-derived macrophages (HMDMs) (isolated from buffy coats as reported previously [Li et al., 2010; McLaren et al., 2010a,b]) and the cell lines were maintained in Dulbecco modified Eagle's medium (DMEM) (Hep3B) or RPMI 1640 (THP-1, HMDMs) supplemented with heat-inactivated (56°C, 30 min) foetal calf serum [5% (v/v) for primary cultures and 10% (v/v) for all other cells], penicillin (100 U/ml) and streptomycin (100 μg/ml). THP-1 monocytes were first differentiated to macrophages by incubation with 0.16 μM PMA for 24 h, and then treated with the mediators for the requisite time. For experiments involving the use of NR agonists, the cells were incubated in medium containing delipidated HI-FCS, which was prepared essentially as described by Cham and Knowles [1976]. The cultures were maintained at 37°C in a humidified atmosphere containing 5% (v/v) $CO_2$ in air.

### PREPARATION OF CELL EXTRACTS, SDS–PAGE AND WESTERN BLOT ANALYSIS

Whole cell extracts were made in a buffer containing both phosphatase and protease inhibitors in order to maintain the proteins in an intact, phosphorylated state as previously described [Irvine et al., 2005; Harvey et al., 2007; Ali et al., 2010]. SDS–PAGE and Western blot analysis was carried out as previously described [Irvine et al., 2005; Harvey et al., 2007; Ali et al., 2010]. The relative molecular weight of the immunoreactive proteins was determined by comparison to rainbow protein size markers (GE Healthcare), which were subjected to SDS–PAGE and transfer to PVDF membranes but not incubation with the various antisera and detection using the Enhanced Chemi-Luminescent (ECL) detection kit from GE Healthcare (i.e., alignment of the autoradiogram with the excised lane of the PVDF membrane containing the size-fractionated markers).

### EMSA

Nuclear extracts were prepared using the Nuclear Extract Kit according to the manufacturer's protocol (Active Motif). The radiolabelling of oligonucleotides and EMSA were carried out as previously described [Irvine et al., 2005; Harvey et al., 2007; Ali et al., 2010]. In competition assays, the binding reaction mixture was incubated for 10 min on ice with 250-fold molar excess of unlabelled competitor oligonucleotides prior to the addition of the radiolabelled probe. The sequences of the oligonucleotides used were: 5′-GATCGCTTCCCTTTCCTACTTCCTGGAAA-3′ and 5′-TGGATTTCCAGGAAGTAGGAAAGGGAAG-3′ (MCP-GAS); 5′-CATGTTATGCATATTCCTGTAAGTG-3′ and 5′-CACTTACAGGAA-TATGCATAACATG-3′ (STAT1 consensus); and 5′-GGAGTTGAGGG-GACTTTCCCA-3′ and 5′-GGCCTGGGAAAGTCCCCTCAA-3′ (NF-κB consensus).

### TRANSFECTION OF CELLS

Hep3B cells were transfected using the SuperFect$^{TM}$ transfection reagent [Irvine et al., 2005]. The DNA/SuperFect$^{TM}$ complex was prepared by diluting DNA (1–2μg) in 50 μl DMEM followed by incubation for 10 min at room temperature after mixing with SuperFect$^{TM}$ (3.5 μl/μg DNA). The complex was then resuspended in 500 μl DMEM complete medium and added drop wise to the cells. The cells were then incubated for 1 h as normal prior to further treatment with vehicle or IFN-γ for 6 h. The luciferase activity in cell extracts was determined using a commercially available kit (Promega) and normalized to the total protein concentration, which was measured using the Micro BCA protein assay reagent kit (Pierce).

### STATISTICAL ANALYSIS

The intensity of bands from immunoblots and EMSA were analysed using GeneTools$^{TM}$ (Syngene). Statistical analyses of the data was carried out using the Student's *t*-test with $P < 0.05$ considered as statistically significant. Identical conclusions were obtained when the data for some figures was analysed by other tests (one-way ANOVA for Fig. 7, one-way ANOVA with Dunnett's post hoc test for Fig. 2A).

## RESULTS

### THE ACTION OF LIPID LOWERING DRUGS ON THE IFN-*γ*-MEDIATED PHOSPHORYLATION OF STAT1

There are three PPAR isoforms (α, β/δ and γ) and two LXRs (α and β) [Hansen and Connolly, 2008]. Our studies focussed on PPAR-α and –γ along with the LXRs because the exact role of PPARβ/δ in atherosclerosis is less clear [Hansen and Connolly, 2008]. The majority of the studies employed the PPARα agonist GW7647, the PPARγ agonist BRL49653 (rosiglitazone) and the LXR agonist TO901317 (activates both the α and β isoforms) [Bensinger and Tontonoz, 2008]. In addition, the endogenous ligands for PPARγ, 15d-$PGJ_2$, and LXRs, 22-(*R*)-HC, either alone or in

combination with the ligand for RXR, 9cRA [Bensinger and Tontonoz, 2008], were included in some experiments. Furthermore, activated simvastatin (carboxylate form that is active in whole cells and in cell-free preparations) was included for comparative purposes because statins also inhibit inflammatory gene expression [Jain and Ridker, 2005]. All these agents are used widely to delineate the actions of PPARs, LXRs or statins in various physiological and pathophysiological processes [Jain and Ridker, 2005; Bensinger and Tontonoz, 2008 and references therein].

The human THP-1 cell line has been employed extensively to investigate the cytokine-mediated regulation of macrophage gene expression relevant to atherogenesis due to its paralleled conservation of mechanisms with primary HMDMs [Auwerx, 1991; Irvine et al., 2005; Harvey et al., 2007; Li et al., 2010; McLaren et al., 2010a,b]. This cellular system was therefore used initially to delineate the impact of the different pharmacological agents on STAT1 actions in human macrophages. PPAR and LXR agonists along with simvastatin have previously been shown to inhibit inflammation and associated changes in gene expression *in vitro* and *in vivo*, including the actions of IFN-γ [Jain and Ridker, 2005; Leon and Zuckerman, 2005; Bensinger and Tontonoz, 2008; Hong and Tontonoz, 2008]. As expected, semi-quantitative RT-PCR analysis showed that BRL49653, GW7647, TO901317 and simvastatin inhibited the IFN-γ-induced expression of monocyte chemoattractant protein-1 (MCP-1) mRNA expression in these cells (data not shown). MCP-1 plays a key role in atherosclerosis and both promoter dissection and DNA-protein interaction studies have shown that STAT1 is critical for its IFN-γ-induced gene transcription [Zhou et al., 2001; Harvey et al., 2007].

The IFN-γ-mediated activation of STAT1 is associated with the phosphorylation of the protein at both tyrosine 701 and serine 727 [Harvey et al., 2007]. The effect of the mediators on such phosphorylation was therefore investigated in THP-1 macrophages by Western blot analysis using antisera against phospho-STAT1 Tyr701, phospho-STAT1 Ser 727 and total STAT1. The cells were pre-treated with the mediators for the requisite time and then incubated in the absence or the presence of IFN-γ for 30 min, a time point corresponding to a marked IFN-γ-mediated phosphorylation at both these sites without an effect on the total levels of the protein [Harvey et al., 2007]. The concentrations of the various effectors were based on previous studies [Jain and Ridker, 2005; Leon and Zuckerman, 2005; Bensinger and Tontonoz, 2008; McLaren and Ramji, 2009 and references therein]. It should, however, be noted that the concentration of simvastain used was higher than the plasma concentrations of this drug in patients. The STAT1 protein exists as two isoforms, STAT1α (91 kDa) and the splice variant STAT1β (84 kDa). These two isoforms are collectively referred to as STAT1; however, it should be noted that STAT1α is the major isoform expressed in macrophages and activated by IFN-γ. Figure 1 shows that the IFN-γ-induced phosphorylation of STAT1 at both tyrosine 701 and serine 727 was not affected by BRL49653, 15d-$PGJ_2$, GW7647, simvastatin or TO901317. This was confirmed by statistical analysis of the relative phosphorylation level of STAT1 at Tyr 701 or Ser727 normalized to the expression of the total STAT1 protein, as determined by densitometric analysis, from three independent experiments (data not shown).

In contrast, such phosphorylation was attenuated in a statistically significant manner by the endogenous LXR/RXR ligands 22-(*R*)-HC and 9cRA (Fig. 2A). 22-(*S*)-HC, an inactive enantiomer of 22-(*R*)-HC that binds to the LXRs but does not activate it, had no effect (Fig. 2A).

To rule out the possibility that the observed effects of the ligands were peculiar to THP-1 macrophages, the action of BRL49653, 15dPG$J_2$ and 22-(*R*)-HC/9cRA on STAT1 phosphorylation in HMDMs was investigated. A statistically significant attenuation of the IFN-γ-induced STAT1 phosphorylation at both these sites was only obtained with 22-(*R*)-HC and 9cRA (Fig. 2B). Because of such conservation, further experiments were carried out on THP-1 macrophages.

Studies on gene regulation by 22-(*R*)-HC and 9cRA have shown that whereas either ligand can act in isolation, a marked, often synergistic response is observed when they are together. To evaluate whether a similar action occurs at the level of IFN-γ-induced STAT1 phosphorylation, THP-1 macrophages were pre-treated with 22-(*R*)-HC and 9cRA alone or together and then incubated in the absence or the presence of IFN-γ. A statistically significant attenuation of the IFN-γ-induced STAT1 phosphorylation was observed with each of these ligands either alone or in combination (Fig. 3). Combinations of 22-(*R*)-HC and 9cRA were used as endogenous ligands for subsequent studies.

The Western blot analysis detailed above used whole cell extracts. Because the activation of STAT1 via phosphorylation of tyrosine 701 is associated with nuclear translocation of the protein, the effect of the various mediators on the levels of phospho-STAT1 701 in nuclear extracts was determined. Nuclear enrichment was assessed by probing with antibodies against nuclear and cytoplasmic proteins (nucleolin and β-actin, respectively). Consistent with the data from whole cell extracts, an attenuation of phospho-Tyr 701 levels in nuclear extracts from IFN-γ treated cells was only observed with 22-(*R*)-HC and 9cRA and not with the other agents (data not shown).

### AGONISTS OF PPARs OR LXRs ALONG WITH SIMVASTATIN ATTENUATE THE IFN-*γ*-INDUCED, STAT1-MEDIATED TRANSACTIVATION

Transcriptional activation by STAT1 can be monitored by transfection assays using a luciferase-based plasmid containing three copies of STAT1 responsive elements (3xly6e) [Harvey et al., 2007]. The effect of the PPARγ and LXR agonists along with simvastatin on the activity of this promoter was therefore investigated. Because THP-1 macrophages are difficult to transfect with exogenous DNA, the analysis was carried out on the human hepatoma Hep3B cell line, which has been employed for numerous studies on IFN-γ signalling and the actions of NRs or statins [e.g., Jain et al., 2006; Kim et al., 2007]. The kinetics of IFN-γ-induced STAT1 phosphorylation at both Tyr701 and Ser727 in these cells are similar to that in THP-1 macrophages (data not shown). The IFN-γ-induced activation of this promoter was attenuated by pre-treatment of the cells with simvastatin, BRL49653, 15d-PGJ2, TO901317 and 22-(*R*)-HC plus 9cRA (Fig. 4A). In addition, representative experiments were carried out using the human MCP1 gene promoter [MCP(213)Luc plasmid] containing the IFN-γ responsive elements [Zhou et al., 2001], which we have shown

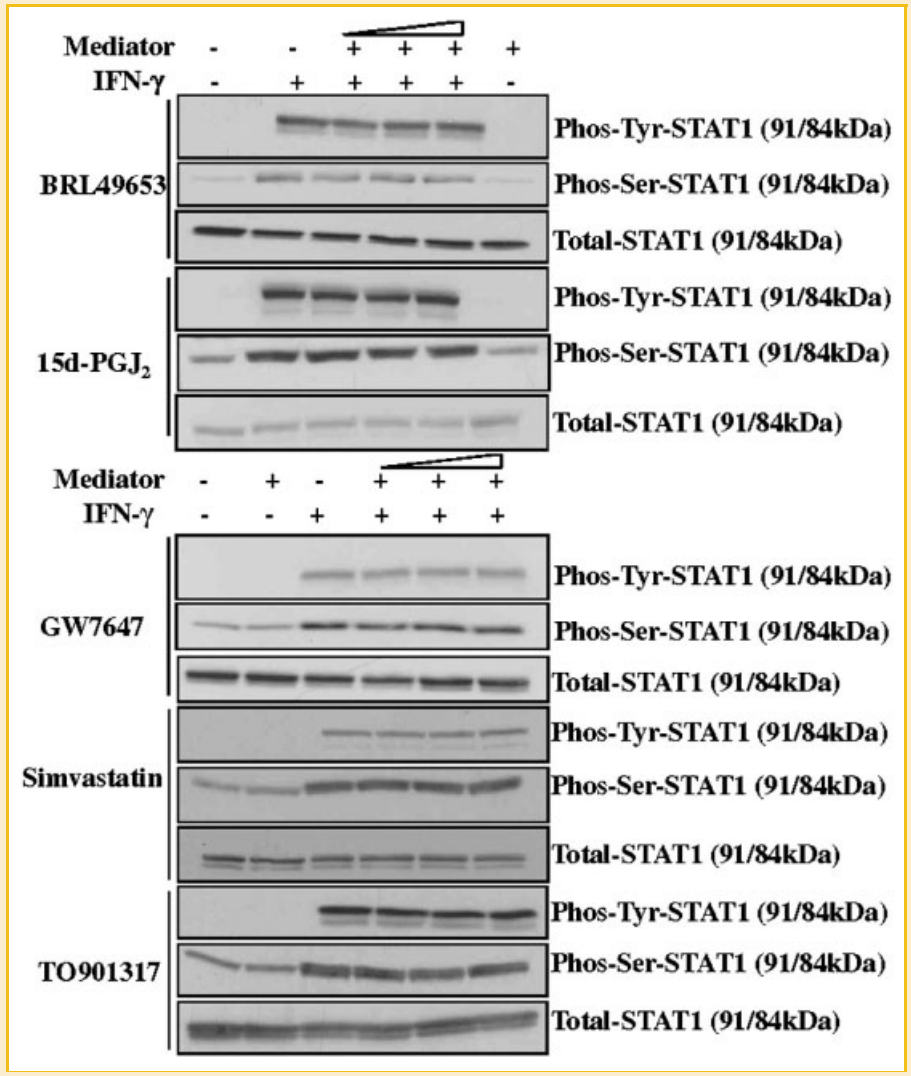


Fig. 1. IFN-γ-mediated STAT1 phosphorylation is not affected by BRL49653, 15d-$PGJ_2$, GW7647, simvastatin or TO901317. THP-1 macrophages were pre-treated for 2 h with increasing concentrations of BRL49653 (5, 10 and 20 μM), 15d-$PGJ_2$ (1, 5 and 10 μM), GW7647 (0.5, 1 and 5 μM); TO901317 (1, 5, or 10 μM), for 1 h with simvastatin (1, 5 and 10 μM), or the DMSO vehicle control (−) (the amount corresponding to the highest concentration of these agents was used for the −IFN-γ + mediator sample). The cells were then incubated for 30 min in the absence or the presence of 1,000 U/ml IFN-γ. Western blot analysis was performed using antibodies against pSTAT1 Tyr701 (Phos-Tyr-STAT1), pSTAT1 Ser727 (Phos-Ser-STAT1) and total STAT1. The images shown are representative of three independent experiments. Statistical analysis showed that none of these agents affected the IFN-γ-induced STAT1 phosphorylation on tyrosine 701 or serine 727 (data not shown).

previously to bind STAT1 [Harvey et al., 2007]. The IFN-γ induced activity of this natural promoter was also attenuated by incubation of the cells with activators of PPARγ (BRL49653 and 15d-PGJ2) and LXR/RXR heterodimer [22-(*R*)-HC plus 9cRA] along with simvastatin (Fig. 4B).

To further confirm the requirement of the corresponding NR for transrepression, representative experiments were performed in relation to the LXRs. For this, co-transfection experiments were performed using expression plasmids specifying for LXR-α or -β with the vector pSG5 used as a control [Lehmann et al., 1997]. 22-(*R*)-HC and 9cRA attenuated the IFN-γ-induced activity of this promoter in cells transfected with the control pSG5 vector (Fig. 4C). In addition, the expression of LXR-α or -β inhibited the IFN-γ-activated promoter activity in the absence or the presence of the ligands (Fig. 4C).

Direct interaction between the co-activator p300/CBP and STAT1 has been shown to play an important role in transcriptional activation [Wojciak et al., 2009]. Indeed, models involving competition between STAT1 and p300/CBP have been found to be involved in the PPARγ-dependent repression of the inducible nitric oxide synthase (iNOS) expression [Li et al., 2000]. In order to investigate whether this was generally applicable to STAT1 responsive promoters, co-transfection experiments were therefore carried out using the 3xly6e plasmid and an expression vector specifying for p300. The PPARγ agonists BRL49653 and 15d-$PGJ_2$ were used with 22-(*R*)-HC and 9cRA included for comparative purposes. Although, as expected, transfection of a p300 expression plasmid enhanced the IFN-γ-induced transactivation by STAT1 in cells treated with the DMSO vehicle, it could not overcome the

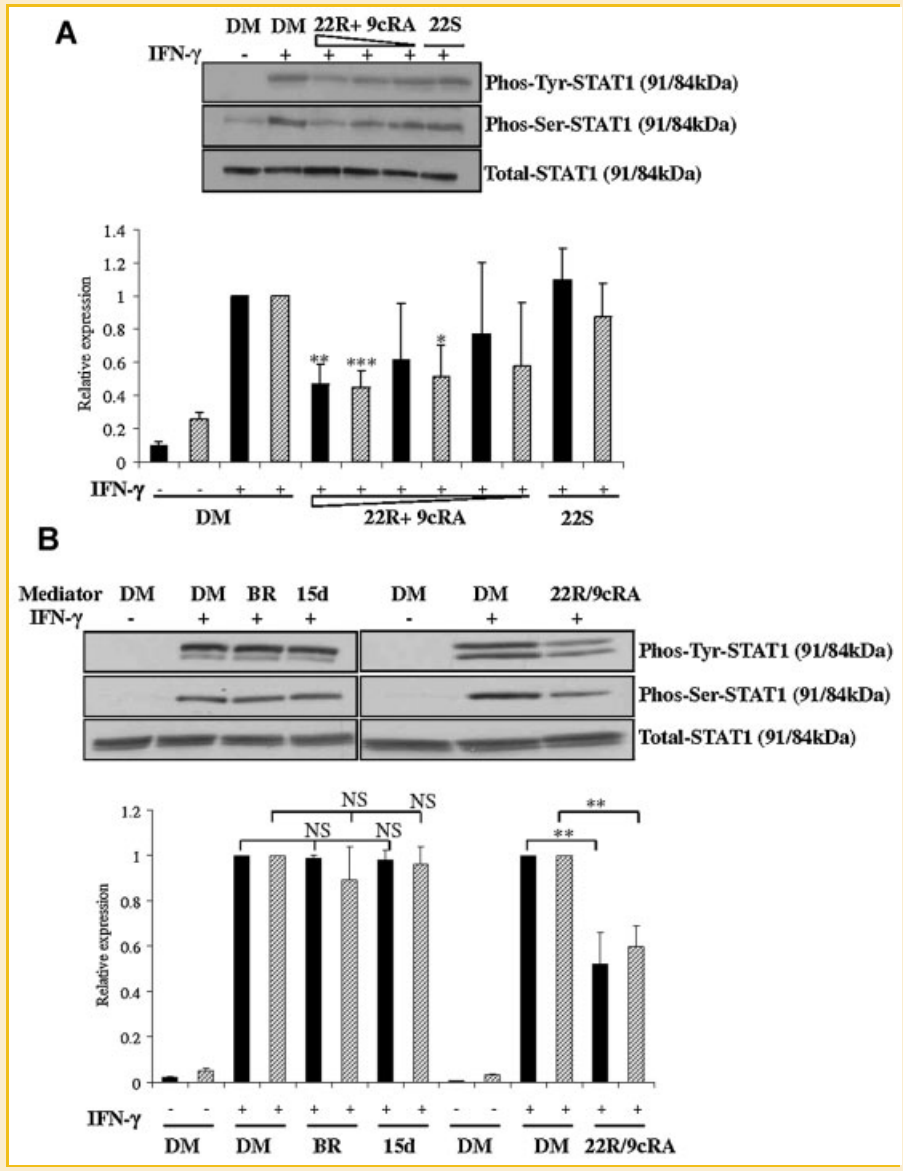


Fig. 2. IFN-γ-induced STAT1 phosphorylation in THP-1 macrophages and HMDMs is attenuated by 22-(*R*)-HC and 9cRA. THP-1 macrophages (A) or HMDMs (B) were pre-treated for 24 h with 22-(*R*)-HC plus 9cRA (22R + 9cRA) (1, 2, or 5 μg/ml 22-(*R*)-HC in the presence of 10 μM 9cRA in panel (A) and 2 μg/ml 22-(*R*)-HC/10 μM 9cRA for panel (B) or 22-(*S*)-HC (22S) (2 μg/ml), or, for 2 h with BRL49653 (20 μM) or 15d-$PGJ_2$ (10 μM) [DMSO (DM) was used as a vehicle control)]. The cells were then incubated for 30 min in the absence or the presence of 1,000 U/ml IFN-γ. Western blot analysis was performed using antibodies against pSTAT1 Tyr701 (Phos-Tyr-STAT1), pSTAT1 Ser727 (Phos-Ser-STAT1) and total STAT1. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at Tyr 701 or Ser727 normalized to the expression of the total STAT1 protein (filled and hatched histograms, respectively), as determined by densitometric analysis, from three independent experiments (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1) ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant).

transrepression by the PPARγ ligands BRL49653 or 15dPGJ$_2$, or, 22-(*R*)-HC plus 9cRA (Fig. 5).

## AGONISTS OF LXRs BUT NOT PPARs ALONG WITH SIMVASTATIN ATTENUATES STAT1 DNA BINDING

We have previously shown that IFN-γ induces the binding of STAT1 to its recognition sequence in the MCP-1 gene promoter (MCPGAS) in the murine macrophage J774.2 cell line [Harvey et al., 2007]. However, such studies have not been carried on human THP-1 macrophages and were therefore performed using EMSA. IFN-γ treatment of THP-1 macrophages resulted in the formation of at least six DNA-protein complexes (C1–C6 in Fig. 6), which were induced to varying extent following treatment of the cells with IFN-γ for 30 and 180 min. The specificity of DNA-protein interactions was confirmed by competition assays (Fig. 6). Pre-incubation of the extracts with the anti-STAT1 antibody resulted in the formation of a slower migrating antibody-protein-DNA supershift complex and inhibition of complexes C1–C2, thereby indicating that they contain STAT1 whereas complexes 3–6 contain other DNA binding proteins. Complexes 1–2 probably

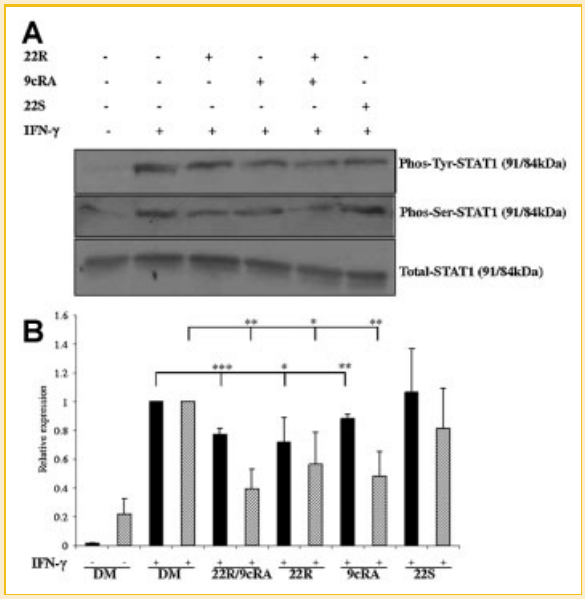


Fig. 3. IFN-γ-induced phosphorylation of STAT is attenuated by 22-(*R*)-HC or 9cRA, either alone or in combination. THP-1 macrophages were pre-treated for 24 h with 22-(*R*)-HC (22R; 2 μg/ml) or 9cRA (10 μM), either alone or together, or 22-(*S*)-HC (22S; 2 μg/ml) [DMSO (DM) was used as a vehicle control)]. The cells were then incubated for 30 min in the absence or the presence of 1,000 U/ml IFN-γ. Western blot analysis was performed using antibodies against pSTAT1 Tyr701 (Phos-Tyr-STAT1), pSTAT1 Ser727 (Phos-Ser-STAT1) and total STAT1. The graphs show the relative phosphorylation level (mean ± SD) of STAT1 at Tyr 701 or Ser727 normalized to the expression of the total STAT1 protein (filled and hatched histograms, respectively), as determined by densitometric analysis, from three independent experiments (the value from IFN-γ-treated DMSO control sample was arbitrarily assigned as 1) (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

represent DNA binding by STAT1 splice variant-α (p91) and -β (p84). As the binding of STAT1 (C1-C2) to MCPGAS reached the highest level at 30 min and declined at 180 min, the 30 min time point was selected for further EMSA.

The IFN-γ-induced STAT1 binding was not affected by incubation of the cells with BRL49653, 15d-PGJ2, GW7647 or simvastatin (Fig. 7). In contrast, such binding was attenuated when the cells were incubated with 22-(*R*)-HC plus 9cRA or TO901317 (Fig. 7).

## DISCUSSION

Inhibition of inflammatory gene expression by statins and PPAR/LXR activators plays a potentially important role in their anti-atherogenic activities [Jain and Ridker, 2005; Bensinger and Tontonoz, 2008; Hansen and Connolly, 2008]. Unfortunately, the mechanisms underlying the inhibitory actions of these agents on IFN-γ signalling are poorly understood despite the pivotal role of this cytokine in atherosclerosis [McLaren and Ramji, 2009]. We show here that agonists of PPARs and LXRs along with statins attenuate the IFN-γ-induced, STAT1-mediated gene transcription in human macrophages by different mechanisms. Agonists of PPARs, along with simvastatin, inhibit STAT1-mediated transactivation without affecting its DNA binding. On the other hand, LXR activators attenuate both STAT1 DNA binding and transactivation.

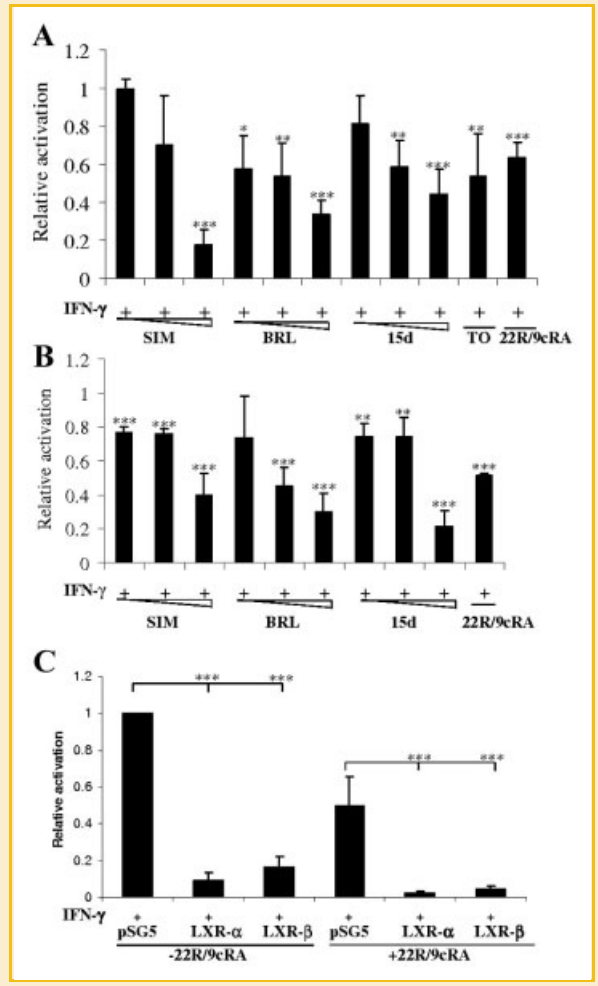


Fig. 4. PPAR and LXR activators, along with simvastatin, attenuate STAT1-mediated transactivation induced by IFN-γ. A,B: Hep3B cells were transfected with the 3xIy6e (three STAT1 responsive elements) or the MCP(213)Luc plasmids (panels A and B, respectively) and pre-treated for 1 h with simvastatin (SIM; 1, 5 and 10 μM), for 2 h with BRL49653 (BRL; 5, 10 and 20 μM), 15d-$PGJ_2$ (15d; 1, 5 and 10 μM), TO901317 (TO; 10 μM), or for 24 h with 22-(*R*)-HC plus 9cRA (22R/9cRA; 2 μg/ml/10 μM). In each case, cells incubated with an equivalent amount of DMSO were included as a vehicle control. C: the cells were co-transfected with 3xIy6e and the pSG5 vector (control) or expression plasmids for LXR-α and -β, and pre-treated for 24 h with 22R-(*R*)-HC (1 μg/ml) plus 9cRA (5 μM) (+22R/9cRA) or DMSO vehicle control (−22R/9cRA). Transfected cells were incubated for 6 h in the absence or the presence of 100 U/ml IFN-γ and the luciferase activity in cell extracts were normalised to protein concentration. The fold activation by IFN-γ was determined with those from vehicle-treated cells in the presence of IFN-γ (panels A,B) or cells transfected with pSG5 and then treated with vehicle in the presence of IFN-γ arbitrarily assigned as 1 (not shown in panels A,B). The data is from three to five independent experiments (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

An emerging theme from studies on PPARs and LXRs is the existence of species-specific mode and mechanism of action [see Rigamonti et al., 2008 for a review]. Indeed, the LXR-α gene is subject to autoregulation in human macrophages but not those of mouse origin [Laffitte et al., 2001]. Such species-specific differences

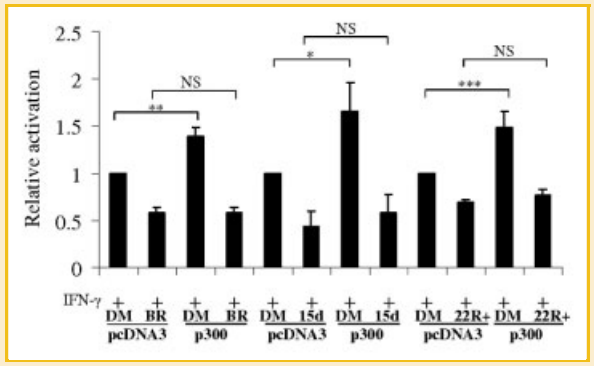


Fig. 5. Effect of transfection of p300 expression plasmid on ligand-mediated inhibition of IFN-γ-stimulated activity of STAT1 responsive elements. Hep3B cells were co-transfected with 3xIy6e and the pcDNA3 vector (control) or the p300 expression plasmid, and pre-treated for 24 h with 22R-(*R*)-HC (2 μg/ml) plus 9cRA (10 μM) (22R+), or, for 2h with BRL49653 (BR; 20 μM) or 15d-$PGJ_2$ (15d; 10 μM) [DMSO (DM) was used as a vehicle control]. Transfected cells were incubated for 6 h in the absence or the presence of 100 U/ml IFN-γ and the luciferase activity in cell extracts were normalised to protein concentration. The fold activation by IFN-γ was determined with those from cells transfected with pcDNA3 and then treated with vehicle in the presence of IFN-γ arbitrarily assigned as 1 and the others represented relative to this value. The data are from three independent experiments ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant).

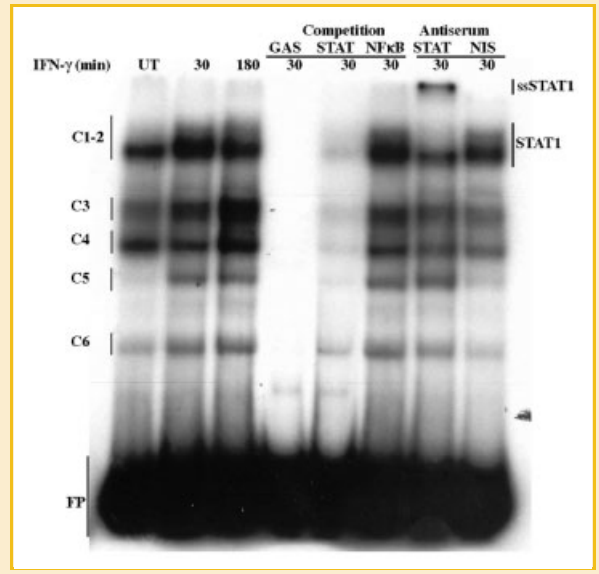


Fig. 6. IFN-γ induces the binding of STAT1 to its recognition sequence in the MCP-1 gene promoter in THP-1 macrophages. The cells were either untreated (UT) or incubated with 1,000 U/ml of IFN-γ for 30 and 180 min. Nuclear extracts were prepared and used for EMSA with radiolabelled probe MCPGAS. The nuclear extracts prepared from the IFN-γ-treated cells (30 min) were employed for competition and antibody interference/supershift EMSA. Competition assays were carried out using a 250× molar excess of unlabelled GAS, STAT1 and NFκB consensus sequences. Antibody interference/supershift EMSA was carried out using STAT1 specific antibody with non-immune serum (NIS) serving as a negative control. The position of the different DNA-protein complexes (C1–C6), STAT1-DNA protein complex (STAT1), STAT1-DNA-protein supershift complex (ssSTAT1) and free probe (FP) are shown. The data are representative of three independent experimental series.

could be responsible, at least in part, for the differences between our findings and those in the limited studies on IFN-γ carried out previously, most of which used cells of murine origin. For example, the PPARγ ligand 15d-$PGJ_2$ has been found to attenuate the IFN-γ-induced phosphorylation of STAT1 in mouse mesangial cells [Panzer et al., 2008], murine RAW264.7 macrophages [Chen et al., 2003] and rat RINm5F cells [Weber et al., 2004]. It should also be noted that some of these effects of 15d-$PGJ_2$ were found to be PPARγ-independent as they were not observed with the synthetic ligands or remained unaffected following inhibition of the action of this NR [Chen et al., 2003; Weber et al., 2004; Panzer et al., 2008]. Indeed, 15d-$PGJ_2$ has been shown to activate intracellular signalling pathways that then impact on gene expression [Chen et al., 2003; Weber et al., 2007; Panzer et al., 2008]. In contrast to these studies, we have obtained identical results with both 15d$PGJ_2$ and the synthetic ligand BRL49653 (Figs. 1,4,5 and 7).

p300 is a key co-activator for STAT1 and indeed transfection of a p300 expression plasmid augmented the IFN-γ-induced, STAT1-mediated transactivation (Fig. 5). Previous studies have suggested that the PPARγ-dependent repression of the iNOS gene involves competition for a limiting amount of p300 [Li et al., 2010]. However, co-transfection assays suggest that this is unlikely to be a general mechanism for IFN-γ-induced, STAT1-mediated trans-activation (Fig. 5). Although the exact mechanisms downstream of STAT1 remain to be determined, it is possible that, as reported for transrepression through NF-κB [Pascual et al., 2005], a mechanism involving the prevention of clearance of co-repressors at target gene promoters by activated, sumoylated PPARs may be involved in the inhibition of IFN-γ/STAT1-mediated transactivation.

Simvastatin has been shown to repress the transcription of the CIITA and CD40 genes by inhibiting the expression of STAT1 [Lee et al., 2007, 2008]. In contrast, our results show that simvastatin has no effect on STAT1 expression, phosphorylation or DNA binding but instead inhibits its transactivation (Figs. 1,4 and 7). The precise reasons for these differences are currently unclear but species-specific mechanisms might again have contributed given that the previous studies were carried out on cells of mouse origin [Lee et al., 2007, 2008]. Indeed, consistent with our findings, simvastatin has been found not to affect the IFN-γ-induced STAT1 phosphorylation, nuclear translocation or DNA binding in human microvascular endothelial cells in relation to MHC class II gene activation by this cytokine [Sadeghi et al., 2001]. Such findings therefore argue for the need to carry out studies on the anti-inflammatory actions of agents used to limit atherosclerosis on human cells.

Using cultured rat astrocytes, LXR ligands have been shown recently to inhibit STAT1 binding to target gene promoters via a sumoylation-dependent mechanism without affecting STAT1 phosphorylation or nuclear translocation [Lee et al., 2009]. This is therefore similar to our findings in human macrophages, thereby suggesting a common action for LXRs on STAT1 DNA binding in different cell types. Interestingly, whilst LXR ligands had no effect on the IFN-γ-induced STAT1 phosphorylation in primary astrocytes, this was affected in microglia cells [Lee et al., 2009]. Interestingly, our studies show that whilst TO901317 and 22-(*R*)-HC

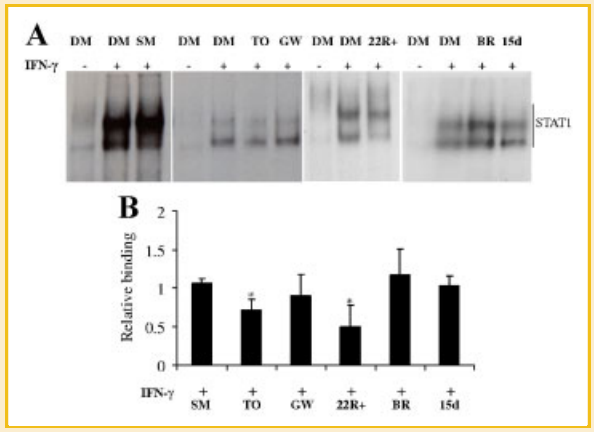


Fig. 7. LXR activators attenuate the IFN-γ-induced STAT1 binding. THP-1 macrophages were pre-treated for 1 h with simvastatin (SM; 10 μM), 2 h with TO901317 (TO; 10 μM), GW7647 (GW; 5 μM), BRL49653 (BR; 20 μM), 15d-$PGJ_2$ (15d; 10 μM), or 24 h with 22-(*R*)-HC plus 9cRA (22R+; 2 μg/ml 22-(*R*)-HC and 10 μM 9cRA) [DMSO (DM) was used as vehicle-control]. The cells were incubated for 30 min in the absence or the presence of 1,000 U/ml IFN-γ. Nuclear extracts were prepared and used for EMSA using the radiolabelled MCPGAS consensus probe (A). The free probe has been run out of the gel. The histograms in panel (B) show the outcome of densitometric analysis of STAT-1:DNA complexes from three independent experiments with the value from IFN-γ-treated cells in the presence of vehicle alone being arbitrarily assigned as 1 (not shown). The results are the mean± SD from three independent experimental series ($^*P < 0.05$).

plus 9cRA inhibited the IFN-γ-induced STAT1 DNA binding and trans-activation (Figs. 4 and 7), TO901317 had no effect on STAT1 phosphorylation whereas this was inhibited by 22R plus 9cRA (Figs. 1–3). The exact reason for this difference is currently unclear though differences in the affinity of the ligands for the receptors could have contributed. Overall, these findings suggest that subtle cell-type and ligand-specific mechanisms might operate on the actions of activated LXRs on STAT1 phosphorylation even though the ultimate outcome is inhibition of STAT1 DNA binding and trans-activation.

Because NRs are present in the nucleus, the precise mechanism through which they affect components in the cytoplasm (e.g., 22-(*R*)-HC and/or 9cRA-mediated inhibition of STAT1 phosphorylation and activation) remains to be determined. It should be noted that NR such as PPARγ have been found to shuttle from the nucleus to the cytoplasm [Von Knethen et al., 2007, 2010]. It is therefore possible that such NR in the cytoplasm affects the IFN-γ-induced STAT1 phosphorylation or there is transcriptional activation of a cytoplasmic protein that mediates the inhibitory action of the NR. Many NRs have also been found to mediate both genomic and non-genomic effects, such as modulation of signal transduction pathways, with the latter attributed in some cases to receptors different to that mediating gene transcription [Ordonez-Moran and Munoz, 2009]. Most evidence indicates that the latter result from a population of NR molecules acting outside the cell nucleus.

As mentioned above some of the reagents such as 15d-$PGJ_2$ have been found to have receptor-independent effects [Chen et al., 2003; Weber et al., 2007; Panzer et al., 2008]. Because of this, we had used both synthetic and endogenous ligands for PPARγ and LXRs. In addition, we had used 22-(*S*)-HC, an inactive enantiomer of 22-(*R*)-HC that binds to the LXRs but does not activate it. Furthermore, we had confirmed the requirement of LXRs by co-transfection experiments (Fig. 4C). However, experiments by knockdown approaches will be required for further confirmation of receptor-dependent mechanism.

In conclusion, our studies show that activators of PPARs (and simvastatin) and LXRs inhibit IFN-γ-induced, STAT1-mediated gene transcription in human macrophages via different mechanisms. Simvastatin and activators of PPAR-α and -γ inhibit STAT1-mediated transactivation without affecting its DNA binding. On the other hand, LXR activators attenuate both STAT1 DNA binding and transactivation.

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