Peroxisome proliferator-activated receptor-γ activation induces 11β-hydroxysteroid dehydrogenase type 1 activity in human alternative macrophages.

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Abstract
Objectives

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) catalyses the intracellular reduction of inactive cortisone to active cortisol, the natural ligand activating the glucocorticoid receptor (GR). Peroxisome Proliferator-Activated Receptor gamma (PPARγ) is a nuclear receptor controlling inflammation, lipid metabolism and the macrophage polarization state. In this study, we investigated the impact of macrophage polarization on the expression and activity of 11β-HSD1 and the role of PPAR therein.

Methods and Results

11β-HSD1 gene expression is higher in pro-inflammatory M1 and anti-inflammatory M2 macrophages than in resting macrophages (RM), whereas its activity is highest in M2 macrophages. Interestingly, PPARγ activation induces 11β-HSD1 enzyme activity in M2 macrophages, but not in RM or M1 macrophages. Consequently, human M2 macrophages displayed enhanced responsiveness to the 11β-HSD1 substrate cortisone, an effect amplified by PPAR -induction of 11β-HSD1 activity, as illustrated by an increased expression of GR target genes.

Conclusions

Our data identify a positive cross-talk between PPARγ and GR in human M2 macrophages via the induction of 11β-HSD1 expression and activity.

MESH Keywords 11-beta-Hydroxysteroid Dehydrogenase Type 1 ; biosynthesis ; genetics ; Cells, Cultured ; Cortisone ; metabolism ; Enzyme Induction ; Genes, Reporter ; Humans ; Hydrocortisone ; metabolism ; Inflammation ; enzymology ; genetics ; immunology ; Interleukin-4 ; metabolism ; Macrophages ; drug effects ; enzymology ; immunology ; PPAR gamma ; agonists ; genetics ; metabolism ; RNA Interference ; Receptors, Glucocorticoid ; metabolism ; Thiazolidinediones ; pharmacology ; Time Factors ; Transfection

Author Keywords 11beta-HSDI ; PPARgamma ; human alternative macrophages ; inflammation ; GR

Introduction

Macrophages are heterogeneous cells [1 ,2]. A pro-inflammatory cytokine-rich environment triggers an activation profile yielding classically activated macrophages (M1) which are involved in the Th1 immune response. In the presence of Th2 cytokines, such as IL-4 and IL-13, monocytes orient toward an alternative activation state (M2) and are involved in the Th2 immune response [1]. M1 macrophages mainly produce pro-inflammatory mediators, express MHC class II molecules and have a high microbicidal activity, whereas M2 macrophages produce high amounts of anti-inflammatory molecules (IL-10, TGFβ and IL-1Ra), promote cell growth and tissue repair and display a high endocytic capacity [1].

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) catalyses the intracellular reduction of inactive cortisone and 11-dehydrocorticosterone to, respectively, active cortisol and corticosterone which are ligands of the glucocorticoid receptor (GR) [3]. Glucocorticoid-activated GR regulates numerous metabolic and homeostatic processes, including carbohydrate, protein and lipid metabolism, and exerts anti-inflammatory and immunosuppressive properties [4]. 11β-HSD1 is expressed in several tissues, including liver, adipose tissue and brain [5 ,6]and myeloid cells, such as dendritic cells and macrophages [7]. 11β-HSD1 is not expressed in human monocytes, but is induced upon differentiation into macrophages, and its expression is further enhanced by the anti-inflammatory Th2 cytokines IL-4 and IL-13 [8].

The inflammatory status of macrophages is controlled by nuclear receptors, such as the Peroxisome Proliferator-Activated Receptors (PPARs) α, β/δ and γ [9]and the GR. Indeed, GR activation results in the polarization of monocytes to a specific subtype of M2 macrophages, termed M2c [10]. PPARγ is activated by natural (15-deoxy-Δ12, 14-prostaglandin J2 (15d-PGJ2)) [11]and synthetic ligands, including the antidiabetic glitazones (rosiglitazone and pioglitazone) and the GW1929 compound [12]. In activated M1 macrophages, the anti-inflammatory PPARγ-dependent trans-repression pathway is initiated by sumoylation of the liganded-PPARγ ligand-binding domain maintaining the co-repressor complex on NF-κB response elements [13]. PPARγ also enhances the alternative activation and differentiation of monocytes into macrophages [14 ,15]thus leading to a more pronounced anti-inflammatory M2 phenotype [15]. However, it is not known whether PPARγ and GR cross-talk in the control of macrophage polarization and functions.

Here, we show that 11β-HSD1 enzyme activity is highest in M2 macrophages and that PPARγ ligands specifically induce 11β-HSD1 enzyme activity in anti-inflammatory M2, but not in RM and M1 macrophages. This novel mechanism may contribute to the anti-inflammatory activities of PPARγ in M2 macrophages and could have consequences in inflammatory diseases, such as atherosclerosis.

Methods

Cell culture

Human peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation [16]. Monocyte differentiation to RM occurred after 7 days of culture in RPMI1640 medium (Invitrogen, France) supplemented with gentamicin (40μ g/mL), L-glutamine (2mM) (Sigma-Aldrich, France) and 10% human serum (Abcys, France). M2 macrophages were obtained by differentiating freshly isolated monocytes with human IL-4 (15ng/mL, Promocell, Germany) for 7 days. M1 macrophages were obtained by activating RM macrophages with LPS (100ng/ml) during 4h. Agonists for PPAR (GW1929, rosiglitazone), PPARα (GW7647), PPARβ/ δ (GW501516), the PPAR antagonist (GW9662), RU486, cortisone or dexamethasone were added as indicated.

Bone marrow-derived macrophages (BMDM) prepared from C57BL/6J mice [17], were treated with GW1929 (0.5, 1, 5μM) or rosiglitazone (1, 5, 10μM) for 24h.

RNA extraction and analysis

RNA was isolated from macrophages using Trizol (Invitrogen), reverse transcribed and cDNAs quantified by Q-PCR on a MX4000 apparatus (Agilent Biotechnologies) using the Brilliant II SYBR® Green Q-PCR Master Mix (Agilent Biotechnologies) with specific primers (table 1). TNFα and IL-6 were measured using Brillant Multiplex® Q-PCR Master Mix kit (Agilent Biotechnologies) with the primers/probes: TNFα: 5′-CCCATGTTGTAGCAAACCCTCA-3′ (forward), 5′-ATCTCTCAGCTCCAGGCCATTG-3′ (reverse) and 5′ Cy5 TTGGCCCGGCGGTTCAGCCACT 3′DDQ2 (probe); IL-6: 5′CAATAACCACCCCTGACCCAAC3′ (forward), 5′ -AAGCTGCGCAGAATGAGATGAG-3′ (reverse) and 5′FAM TGCCAGCCTGCTGACGAAGCTGCA 3′BHQ1 (probe) and cyclophilin: 5′-TGGTCAACCCCACCGTGTTC-3′ (forward), 5′-TGCAAACAGCTCAAAGGAGACG-3′ (reverse) and 5′Yakima Yellow TTGCCGTCGACGGCGAGCCCTT 3′EDQ (probe).

Electrophoretic Mobility Shift Assay (EMSA)

PPARγ and RXRα were *in vitro* transcribed from the pSG5-hPPARγ and pSG5-hRXRα plasmids (Promega, Madison, WI). For the preparation of nuclear extracts, RM, M1 and M2 macrophages were collected in hypotonic buffer (15mM Hepes pH 7.8, 10mM KCl, 2 mM $MgCl_2$, 0.1mM EDTA, 3mM DTT and protease inhibitors). After 15min incubation at 4°C, NP40 was added before centrifugation. The cell pellet was dissolved in hypertonic buffer (30mM Hepes, 50 mM KCl, 300mM NaCl, 0.1mM EDTA, 3mM DTT, 10% glycerol); after 30min incubation at 4°C, nuclear extracts were collected. Proteins were incubated for 10min in binding buffer (Hepes 10mM pH 7.8, NaCl 100mM, EDTA 0.1mM, 10% glycerol, 1mg/ml BSA) containing poly(dI-dC) (1μg) and herring sperm DNA (1μg). Double stranded oligonucleotides containing the wild-type DR1-PPARγ response element (PPRE) present between nucleotides −2406/−2393 of the human 11β-HSD1 promoter, end-labeled using T4 polynucleotide kinase and γ^{32} P-ATP, was added as probe to the binding reaction. For supershift assays, monoclonal anti-human PPARγ antibody (Santacruz Biotechnology) was added to the binding reaction. DNA/protein complexes were resolved by non-denaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-EDTA. Classical DR1-PPARγ response element was used as positive control.

Plasmid cloning and transient transfection experiments

The reporter plasmid $(11\beta\text{-HSD1-PPREwt})_6$ -TK-pGL3 was generated by inserting 6 copies of the double-strand oligonucleotides (for 5′-CTT GAA GGG TTG AAA GGT CAA AAC TAT -3′; rev 5′-ATA GTT TTG ACC TTT CAA CCC TTC AAG-3) into the pTK-pGL3 plasmid. Macrophages were transfected overnight in RPMI medium containing 10% human serum with reporter plasmids and expression vectors (pSG5-empty or pSG5-hPPARγ) using jetPEI (Polyplus transfection, France). Subsequently, cells were incubated for additional 24h with GW1929 (600nM) or DMSO and luciferase and β-galactosidase activities were measured.

Chromatin Immunoprecipitation (ChIP) and ChIP-seq assays

Macrophages were cross-linked with 1% formaldehyde/PBS for 10min [18], collected in PBS and nuclear extracts were prepared. The chromatin was fragmented to ~500 bp in a 10mM Tris buffer pH 7.5 containing 1mM EDTA, 0.5mM EGTA, 100mM NaCl, 0.1% Na-Deoxycholate, 0.5% N-lauroyl sarcosine and 0.4% SDS using a bioruptor sonication bath (Diagenode, Belgium). Half of the chromatin was immunoprecipitated overnight in a 10 mM Tris buffer pH 7.5 containing 1mM EDTA, 0.5mM EGTA, 100mM NaCl, 0.1% Na-Deoxycholate, 0.5% N-lauroyl sarcosine and 0.08% SDS and 1% Triton X100 using either PPARγ antibodies or control IgG (Santacruz Biotechnology) in combination with Protein A/G Dynabeads (Invitrogen). Precipitates were washed with RIPA buffer. Eluates were obtained with 1% SDS in 50mM Tris pH8.0/1mM EDTA and the cross-linking was reversed at 65°C overnight. Immunoprecipitated DNA was purified by phenol-chloroform-isoamilyc alcohol extraction and ethanol precipitation. Then, either a 150 bp region of the 11β-HSD1 promoter area was amplified by PCR, using the following primers 5′-GACACAAAGTGGATTAGATGTTTATT and 5′-ACATCCTGTAGCCTCTGGAA, or a 77 bp region of β-actin was amplified using 5′-AGTGTGGTCCTGCGACTTCTAAG and 5′-CCTGGGCTTGAGAGGTAGAGTGT.

Chromatin was used for immunoprecipitation using antibody against H3K9ac (Millipore). ChIP-seq was performed and analysed as described [19], including total tag number equalization before data visualization. H3K9ac ChIP-seq data were visualized using the University of California at Santa Cruz (UCSC) genome browser. H3K9ac ChIP-QPCR was performed on chromatin from several different donors using the following primers: 11β-HSD1promoter 1: 5′-CAGGACCACTTCCAAGCATT-3′ and 5′-CCAAAGAGAAGCCAGAGTGG-3′; 11β-HSD1promoter 2: 5′CTTGGCCATCTGGAAGTCTC-3′ and 5′-TGCTAGCCAATTTCCCTGTC-3′; negative control 5′-CAGGATATACACCCCCGTGA-3′ and 5′-CAAAGTGCGTACACCTTGGTA-3′.

Short-interfering (si)RNA assays

Differentiated RM macrophages were transfected with siRNA specific for human PPARγ and non-silencing control scrambled siRNA (Ambion), using the transfection reagent DharmaFECT4 (Dharmacon). After 16h, cells were incubated with GW1929 (600nM) or vehicle (DMSO) and harvested 24h later.

Adenovirus generation

The recombinant adenovirus (Ad)-GFP (Green Fluorescent Protein) and Ad-PPARγ were obtained as described [20]. $1.5x10^6$ macrophages were infected at a multiplicity of 100 viral particles/cell by adding virus stocks directly to the culture medium. After 16h, cells were incubated for 24h in the absence or presence of GW1929 (600nM).

Protein extraction and western blot analysis

Cells were harvested in ice-cold lysis buffer (RIPA), proteins collected by centrifugation, separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (Amersham) and revealed using goat polyclonal antibodies against 11β-HSD1 (Abcam) or β-actin (Santacruz Biotechnology). After incubation with a secondary peroxidase-conjugated antibody (Santacruz Biotechnology), immunoreactive bands were revealed using ECL detection (Amersham) and band intensity quantified by QuantityOne software.

11β-HSD1 enzyme activity assay

Cellular 11β-HSD1 reductase activity was determined as described [21]. Cells were incubated for the indicated time periods (from 90min to 8h) in serum-free medium containing cortisone (200nM) as substrate and [^{3} H]-cortisone (10 nM; 50–60 Ci/mmol; ARC, USA) as tracer. After incubation, medium was collected and steroids extracted with ethyl acetate. The extracts were dried under nitrogen, reconstituted with isopropanol containing a mixture of cortisol (F) and cortisone (E) (1.5mg/ml each) and applied to silica gel TLC plates. Cortisol and cortisone were separated in a chloroform/methanol solvent system (9:1,vol:vol) and steroids visualized under iodine, scraped off the plate and radioactivity counted. The conversion of cortisone to cortisol was calculated as the radioactivity index of (F/E+F) and reflects 11β-HSD1 enzyme activity. Each analysis was performed in triplicate.

Statistical analysis

Statistically differences between groups were analyzed by ANOVA and Student's t test and considered significant when $p<0.05$.

Results

11β-HSD1 reductase expression and activity is highest in M2 macrophages

To determine whether 11β-HSD1 expression and activity are dependent on the macrophage polarization states, its expression was analyzed and compared between human RM, M1 and M2 macrophages. Expression of the M2 markers MR, AMAC1 (figure 1B,C), IL-1Ra, IL-10 and TGFβ (supplemental figure 1), measured as positive control of alternative polarization, was elevated in M2 macrophages. 11β-HSD1 mRNA levels were higher in M1 and, most pronouncedly, in M2 compared to RM macrophages (figure 1A). By contrast, in mouse BMDM, 11β-HSD1 expression was not induced by IL-4 during differentiation, indicating that 11β-HSD1 regulation is species-specific and that is not secondary to cellular differentiation (data not shown).

To investigate whether M1 and M2 macrophages also exhibit increased 11β-HSD1 reductase activity compared to RM macrophages, cells were incubated with radio-labeled cortisone as substrate and conversion into cortisol was measured at different times. Surprisingly, the percentage of cortisone conversion to active cortisol was approximately 12% after 90min and 37% after 8h incubation of RM macrophages with cortisone, whereas it was 10% after 90min and 25% after 8h in M1 macrophages (figure 2A,B), indicating that the elevated 11β-HSD1 mRNA in M1 macrophages does not translate in higher enzyme activity. By contrast, cortisone to cortisol conversion was already 50% after 90min of cortisone incubation and reached 80% within 8h in M2 macrophages (figure 2C). Thus, 11β-HSD1 reductase activity is higher in M2 compared to RM and M1 macrophages. Moreover, expression of hexose-6-phosphate-deydrogenase (H6PDH), a cofactor for 11β-HSD1 [22], was also higher in M2 compared to RM macrophages (data not shown). This increased conversion of exogenous cortisone into active cortisol conferred a higher transactivation response to cortisone of a GR response element (GRE) driven-reporter vector in M2 compared to RM macrophages, despite lower GR mRNA levels (supplemental figure 2). In addition, cortisone inhibited LPS-induced expression of IL-6, TNFα and IL-1β in M2 but not RM macrophages, indicating that cortisone to cortisol conversion results in anti-inflammatory effects only in M2 macrophages (figure 2D,E,F). Dexamethasone, a GR-specific agonist which does not require metabolism by 11β-HSD1 to be active, exerted anti-inflammatory properties both in RM and M2 macrophages (figure 2).

Cortisone induces the expression of GR target genes more pronouncedly in M2 macrophages

To determine the functional consequences of the higher 11β-HSD1 activity in M2 macrophages, the expression of known GR target genes was measured in RM and M2 macrophages after cortisone activation for different time periods (figure 3). Cortisone induces the expression of glucocorticoid-induced leucine zipper (GILZ) (A), angiopoietin-like 4 (ANGPTL4) [23](B) and pyruvate dehydrogenase kinase 4 (PDK4) [24](C) more strongly in M2 compared to RM macrophages.

11β-HSD1 gene expression is induced by PPARγ activation in RM, M1 and M2 macrophages

Since PPAR plays a role in macrophage functions and polarization, we next investigated whether PPAR regulates 11β-HSD1 gene expression. Treatment with the PPARγ ligand GW1929 significantly increased 11β-HSD1 gene and protein expression both in RM, M1 and M2 macrophages (figure 4A,B). This regulation occurred in a dose-dependent manner with rosiglitazone (supplemental figure 3A). 11 β-HSD1 gene induction by GW1929 was already detectable within 12h after activation and further increased up to 48h (supplemental figure 3B). By contrast, treatment with PPARα or PPARβ/δ specific agonists did not modify 11β-HSD1 mRNA (supplemental figure 3C). Expression of the 11β-HSD1 gene was significantly reduced upon PPARγ activation in mouse BMDM, indicating the existence of species-specific regulatory mechanisms (supplemental figure 3D).

The increase of 11β-HSD1 gene expression by GW1929 was abolished in the presence of GW9662, a potent and selective PPARγ antagonist [25](figure 4C). In line, 11β-HSD1 induction upon PPARγ activation was completely lost in PPARγ siRNA- compared to scrambled siRNA-transfected macrophages (figure 4D). Moreover, infection of macrophages with an adenovirus coding for PPARγ (Ad-PPARγ) increased 11β-HSD1 expression by approximately 3.5-fold compared to macrophages infected with an adenovirus coding for GFP (Ad-GFP) and addition of GW1929 resulted in a further 2.5-fold induction of 11β-HSD1 mRNA levels (figure 4E).

PPAR regulates 11β-HSD1 gene expression at the transcriptional level

To determine whether 11β-HSD1 is a direct PPAR target gene, the human 11β-HSD1 promoter was analyzed *in silico* . A putative PPRE was identified between nucleotides 2406–2393. EMSA were performed to examine whether the PPAR -RXR heterodimer binds to this 11β-HSD1-PPRE. Incubation of this labeled PPRE oligonucleotide with *in vitro* translated PPARγ and RXRα (indicated as RXR) resulted in the formation of a retarded complex (figure 5A ; indicated by the arrow). Incubation with an anti-PPARγ antibody prevented complex formation, indicating binding-specificity of PPARγ to the PPRE site (figure 5A). To test whether PPARγ activates transcription of the (−2406–2393)11β-HSD1-PPRE, 6 copies of this element were cloned in front of the herpes simplex virus thymidine kinase promoter yielding the $(11\beta\text{-HSD1-PPRE})_6$ -Tk-Luc reporter vector which was transfected into differentiated primary human macrophages. Co-transfection of the pSG5-PPARγ expression vector led to a significant induction of $(11\beta\text{-HSD1-PPRE})_6$ transcriptional activity, an effect further enhanced by GW929 (600nM) (figure 5B). The consensus DR1- PPRE site cloned in 6 copies, used as positive control, was also induced (figure 5C).

Incubation of the 11β-HSD1 labelled PPRE oligonucleotide with nuclear extracts from RM, M1 and M2 macrophage resulted in a weak retarded complex visible only in M2 macrophages. The presence of *in vitro* translated RXRα amplified the formation of the complex, which was again stronger with extracts from M2 macrophages and which was blocked by an anti-PPARγ antibody (figure 5D). To evaluate whether PPARγ binds to the 11β-HSD1 promoter *in cell* , a ChIP assay was performed on RM and M2 macrophages. The genomic DNA region encompassing the PPRE of the 11β-HSD1 gene was immunoprecipitated with an anti-PPARγ antibody. Q-PCR analysis revealed that PPARγ binding was stronger in M2 compared to RM macrophages (figure 5E). Since active promoters are marked with elevated levels of acetylation of histone H3 lysine 9 (H3K9ac) around their transcription start site [26], we monitored the presence of H3K9ac at both known 11β-HSD1 promoters [27]in RM and M2 macrophages. ChIP followed by high-throughput sequencing (ChIP-seq) indicated that H3K9ac was specifically present at the P2 promoter, which contains the identified PPRE, but not at the P1 promoter, in both RM and M2 macrophages (figure 5F). Moreover, H3K9ac levels at promoter P2 were higher in M2 macrophages than in RM as confirmed using ChIP-qPCR performed on chromatin from 2 different donors (figure 5F). Finally, activity of the 11β-HSD1-TK-Luc PPRE reporter vector was compared in RM and M2 macrophages in the absence or presence of GW1929 (600nM). Basal transcriptional activity was higher in M2 macrophages (figure 5G), an effect further enhanced by GW1929. Overall, these data suggest that 11β-HSD1 is expressed from the P2 promoter in human macrophages and that this promoter confers a stronger 11β-HSD1 expression and response to PPAR activation in M2 macrophages.

11β-HSD1 reductase activity is induced by PPARγ ligands in M2 macrophages

To assess whether the induction of 11β-HSD1 expression by PPARγ agonists results in an increased enzyme activity, differentiated RM, M1 and M2 macrophages were treated for 24h in the presence or absence of GW1929 and subsequently incubated with radio-labeled cortisone. Surprisingly, 11β-HSD1 activity was not influenced by GW1929 in RM or M1 macrophages (figure 6A,B), despite induction of 11β-HSD1 mRNA expression (figure 4A). However, GW1929-treated M2 macrophages displayed a 1.6-fold higher cortisone to cortisol conversion within 60min, indicative of increased 11β-HSD1 activity (figure 6C). Thus, PPARγ activation induces 11β-HSD1 enzyme activity specifically in M2 macrophages. To determine the functionality of PPARγ-induced 11β-HSD1 enzyme activity in M2 macrophages, the expression of GR target genes was measured. Cortisone treatment more pronouncedly increased GILZ, PDK4 and ANGPTL4 expression in M2 compared to RM macrophages, an effect amplified by pre-treatment with GW1929 (figure 6D,E,F) and inhibited by the GR antagonist RU486 [28](supplemental figure 4). These data further indicate that 11β-HSD1 enzyme activity is higher and induced by PPARγ only in M2 macrophages.

Discussion

In this paper we report that expression of 11β-HSD1, an enzyme which amplifies intracellular GC action, is dependent on the macrophage phenotype. M2 macrophages express higher gene and protein levels of 11β-HSD1 than RM and M1 macrophages. In line with the expression levels, 11β-HSD1 enzyme activity is higher in M2 than in RM and M1 macrophages, resulting in a more pronounced conversion of cortisone into active cortisol. Moreover, we demonstrate that PPARγ activation increases the expression and activity of 11β-HSD1 in alternative human macrophages. *In vitro* experiments show that different PPARγ ligands, rosiglitazone and GW1929, increase the expression of 11β-HSD1 mRNA in RM, M1 and M2 macrophages. The induction of 11β-HSD1 expression by PPARγ appears species-specific, since PPARγ inhibits its expression in mouse adipocytes [29]. Our data indicate that PPARγ ligands exert their effects via a PPARγ-dependent mechanism. Indeed, both the PPARγ antagonist GW9662 and silencing of PPARγ expression abolished 11β-HSD1 induction by GW1929. In addition, over-expression of PPARγ in macrophages induced 11β-HSD1 gene expression, an effect enhanced by GW1929. PPARγ activates 11β-HSD1 transcription *via* a PPRE located in the 11β-HSD1 P2 promoter. Binding of PPARγ to this site occurs also *in cell* , as demonstrated by ChIP experiments, and is enhanced in M2 compared to RM macrophages.

The regulation of 11β-HSD1 enzyme activity by PPARγ activation is dependent on the macrophage phenotype. Surprisingly, PPARγ activation induced 11β-HSD1 enzyme activity selectively in M2 macrophages, but not in RM nor M1 macrophages, although mRNA levels of 11β-HSD1 were induced, albeit not to the same extent, by PPARγ ligands in all three cell types. Concomitantly, PPARγ induced cortisone conversion into cortisol in M2 macrophages, which was accompanied by an increased expression of the GR-target genes GILZ, PDK4 and ANGPTL4.

We have previously demonstrated that PPARγ activation promotes M2 macrophage polarization [15]. On the basis of our results, 11β-HSD1 activity appears to be a marker of macrophage M2 activation. More importantly, this high expression level of 11β-HSD1 could contribute to the functions of M2 macrophages by converting cortisone to cortisol and thus activating GR target genes. Interestingly, it has been reported that ANGPTL4 binds to the extra-cellular matrix in ischemic tissues and reduces endothelial cell adhesion, prevents the organisation of focal adhesion molecules and actin stress fibers and decreases cell migration [30]. Therefore, M2 macrophage ANGPTL4 can exert paracrine effects on endothelial cells thus participating in the modulation of angiogenesis in a hypoxic environment. M2 macrophages play also a role in tissue remodelling and repair and hence contribute to the resolution of inflammation [1]. Similarly, a role of 11β-HSD1 in the resolution of inflammation has also been reported [31]. Using macrophages isolated from 11β-HSD1-deficient mice, it has been shown that 11β-HSD1-mediated intracellular active GC formation enhances phagocytosis of neutrophils undergoing apoptosis during the resolution of inflammation [31]. Also macrophages from 11β-HSD1-deficient mice overproduce pro-inflammatory cytokines and are hyper-responsive to LPS stimulation, suggesting that such macrophages exhibit an M1 phenotype [32].

Our data indicate that the activity 11β-HSD1 is tightly related to the inflammatory status of human macrophages. Elevated 11β-HSD1 activity in polarized M2 macrophages might contribute to their function in the anti-inflammatory response and the resolution of inflammation, with potential consequences on inflammatory diseases, such as atherosclerosis. M2 macrophage 11β-HSD1 could thus contribute to the anti-inflammatory action of these cells within the atherosclerotic plaques. Finally, our findings demonstrate a novel cross-talk between the nuclear receptors PPARɣ and GR.

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Figure 1

11β-HSD1 gene expression is higher in M2 macrophages

Primary human macrophages were cultured for 7 days in the absence (RM) or in the presence of IL-4 (15 ng/ml) (M2). Pro-inflammatory M1 macrophages were obtained by activating RM macrophages with LPS (100ng/ml) during 4h. 11β-HSD1 **(A)** , MR **(B)** and AMAC1 **(C)** mRNA levels measured by Q-PCR and normalized to those of cyclophilin. Results are expressed as the mean value ± SD of triplicate determinations, representative of three independent experiments. Statistically significant differences are indicated (**p<0.01,***p<0.001).

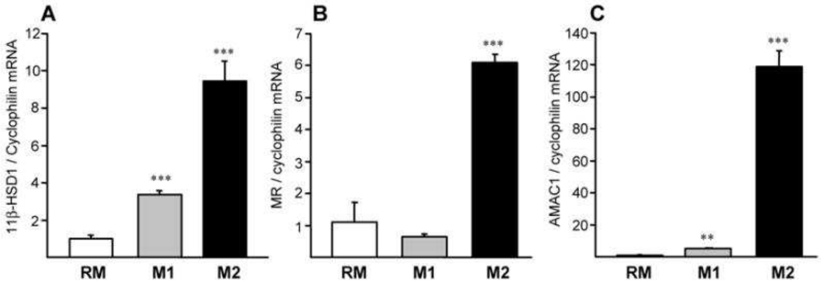

Figure 2

11β-HSD1 enzyme activity is highest in M2 macrophages leading to pronounced anti-inflammatory effects of cortisone

RM **(A)** , M1 **(B)** and M2 macrophages **(C)** were incubated with radio-labeled cortisone (E) for the indicated time periods. Production of cortisol (F) was then measured. 11β-HSD1 reductase activity was determined as the percentage conversion of cortisone to cortisol. Results are representative of two independent experiments. Statistically significant differences are indicated (*p<0.05;**p<0.01,***p<0.001). RM and M2 macrophages were treated with cortisone or dexamethasone (Dexa, 1μM) for 2h before stimulation with LPS (100ng/ml) for 4h. mRNA levels of IL-6 **(D)** , TNFα **(E)** and IL-1β **(F)** were measured by Q-PCR. Results are representative of three independent experiments. Statistically significant differences between control and treated cells are indicated (*p<0.05,**p<0.01).

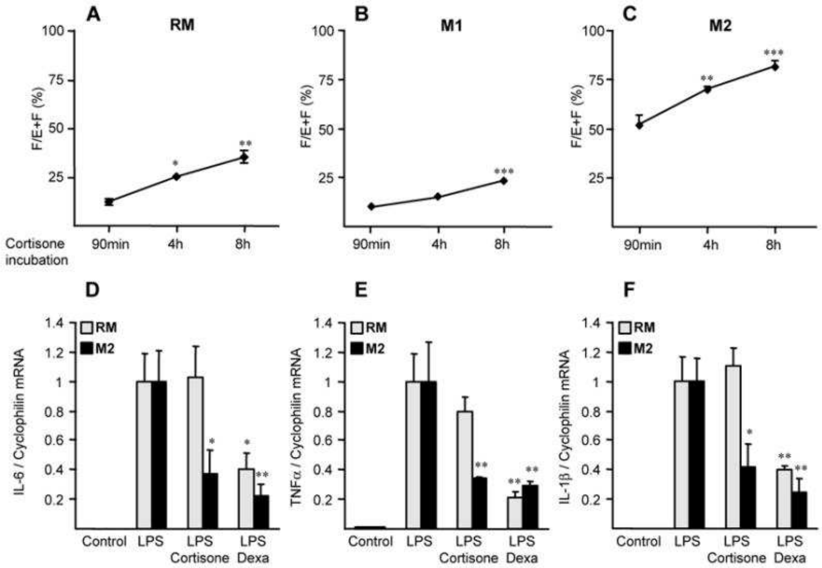

Figure 3

Cortisone more pronouncedly induces the expression of GR target genes in M2 macrophages

RM and M2 macrophages were incubated with cortisone (1 μM) for different time points. GILZ **(A)** , ANGPTL4 **(B),** and PDK4 **(C)** mRNA levels were measured by Q-PCR. Statistically significant differences between control and treated cells are indicated (*p<0.05;**p<0.01;*** p<0.001).

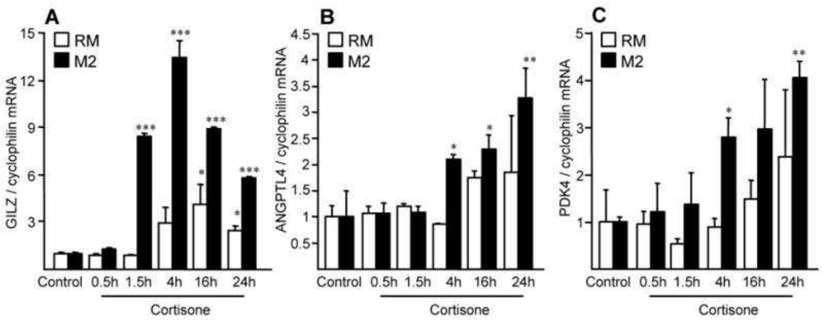

Figure 4

11β-HSD1 expression is induced by PPARγ in RM, M1 and M2 macrophages

(A) RM, M2 and M1 macrophages were treated in the absence or in the presence of GW1929 (600nM); 11β-HSD1 mRNA level was measured by Q-PCR. Results are representative of three independent experiments. Statistically significant differences between control and treated cells (*p<0.05;**p<0.01;***p<0.001) and basal RM or M1 and M2 macrophages are indicated (§ p<0.05;§§ p<0.01). **(B)** Intracellular 11 β-HSD1 and β-actin protein expression analyzed by western blot and immunoreactive band intensity was quantified. Results are representative of two independent experiments. **(C)** RM were treated with vehicle or the PPARγ antagonist GW9662 (1μM) in the absence or presence of GW1929 (600nM) for 24h. **(D)** RM were transfected with scrambled or human PPARγ siRNA and subsequently treated with GW1929 (600nM) or DMSO during 24h. **(E)** RM were infected with Ad-GFP or Ad-PPARγ and subsequently stimulated for 24h with or without GW1929 (600nM). 11β-HSD1 mRNA levels were measured by Q-PCR and normalized to those of cyclophilin. Results are expressed as the mean value ± SD of triplicate determinations, representative of three independent experiments. Statistically significant differences are indicated (*p<0.05;**p<0.01;***p<0.001).

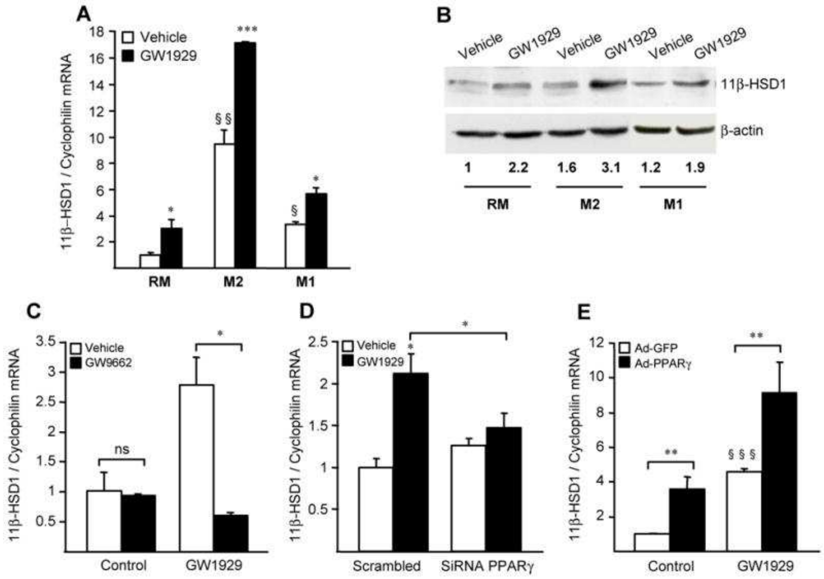

Figure 5

PPAR binds more avidly to the 11β-HSD1 PPRE in M2 macrophages

(A) EMSA was performed using the end-labeled DR1-consensus-PPRE or 11β-HSD1-PPRE oligonucleotide in the presence of unprogrammed reticulocyte lysate or *in vitro* translated hPPARγ and hRXRα. Supershift assays were performed using an anti-PPARγ antibody. **(B)** RM were transfected with the indicated reporter constructs $(DR1\text{-}11\beta\text{-}HSD1\ PPRE)_6$ or $(DR1\text{-}consensus\ PPRE)_6$ **(C)** , in the presence of pSG5-empty or pSG5-PPARγ vector, treated or not with GW1929 (600nM) and luciferase activity was measured. Statistically significant differences are indicated (*p<0.05;**p<0.01;***p<0.001). **(D)** EMSA was performed using *in vitro* produced RXR and PPARγ or nuclear extracts from RM, M1 and M2 in the absence or in the presence of exogenous hRXRα and supershift assays performed using an anti-PPARγ antibody. **(E)** ChIP assays were performed and quantified using chromatin from RM and M2 macrophages, immunoprecipitated with rabbit IgG or PPARγ-specific antibodies and then subjected to PCR using primer pairs covering either the 11β-HSD1 gene promoter or the β-actin gene. **(F)** H3K9ac ChIP-seq data for RM and M2 macrophages. The Y-axis shows the number of mapped tags sequenced on ChIP DNA from both RM and M2 macrophages. Promoters P1 and P2 as well as the identified PPRE are indicated. ChIP experiments were performed on H3K9-immunoprecipitated chromatin from two independent donors using primers covering the two identified 11β-HSD1 PPRE sites on the P1 and P2 promoters. Relative fold enrichments relative to a negative control region (set at 1) are shown. **(G)** RM and M2 macrophages were transfected with the $(DR1\text{-}11\beta\text{-}HSD1\ PPRE)_6$ construct, treated or not with GW1929 (600nM). Statistically significant differences are indicated (*p<0.05;**p<0.01).

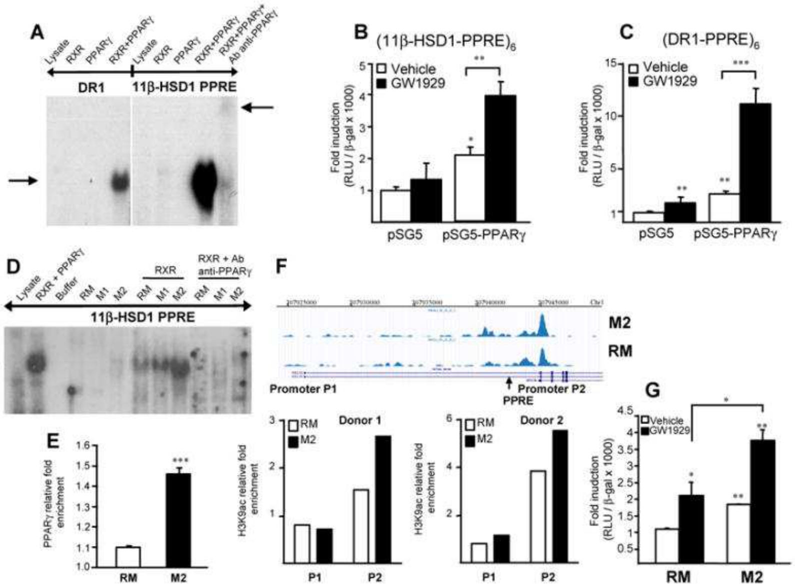

Figure 6

PPARɣ activation increases 11β-HSD1 activity in M2 macrophages leading to the induction of GR-target genes by cortisone

RM **(A)** , M1 **(B)** and M2 **(C)** macrophages were treated for 24h in the absence or in the presence of GW1929 (600nM) and subsequently incubated with radio-labelled cortisone (E) for the indicated time periods. Production of cortisol (F) was then measured. 11β-HSD1 reductase activity was determined as the percentage conversion of cortisone to cortisol. Results are representative of two independent experiments. Statistically significant differences between control and treated cells are indicated (*p<0.05). RM and M2 macrophages were activated or not with GW1929 (600nM) for 24h and subsequently treated for another 24h with cortisone (1μM). PDK4 **(D)** , GILZ **(E)** and ANGPTL4 **(F)** mRNA levels were measured by Q-PCR. Statistically significant differences between control and treated cells are indicated (*p<0.05;*p<0.01; ***p<0.001).

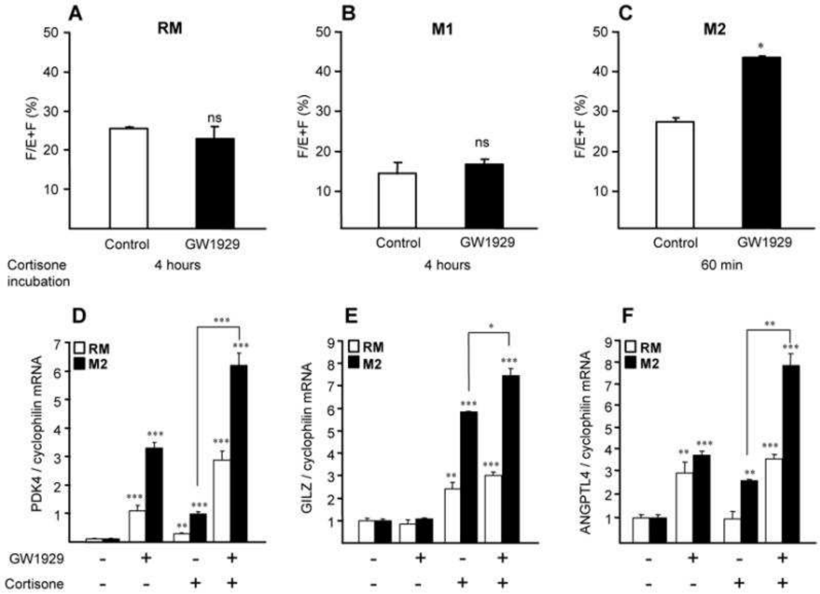

Table 1

Sequences of primers used

AMAC1	forward	*5′-AGC TCT GCT GCC TCG TCT AT-3′*
	reverse	*5′-CCC ACT TCT TAT TGG GGT CA-3′*
MR	forward	*5′-CGA GGA AGA GGT TCG GTT CAC C-3′*
	reverse	*5′-GCA ATC CCG GTT CTC ATG GC-3′*
IL-1Ra	forward	*5′-TTG AGC CTC ATG CTC TGT TC-3′*
	reverse	*5′-CAG TGA TGT TAA CTG CCT CCA G-3′*
IL-10	forward	*5′-GAT CCA GTT TTA CCT GGA GGA G-3′*
	reverse	*5′-CCT GAG GGT CTT CAG GTT CTC -3′*
***TGF*β	forward	*5′-CTC CGA GAA GCG GTA CCT GAA C-3′*
	reverse	*5′-CAC TTG CAG TGT GTT ATC CCT-3′*
Human 11*β*-HSD1	forward	*5′-CAT GTG GTG GTG ACA GCG AGG TC-3′*
	reverse	*5′-GGT TGA GAA TGA GCA TGT CTA GTC-3′*
Mouse 11*β*-HSD1	forward	*5′-AAC CAC ATC ACT CAG ACC-3′*
	reverse	*5′-GAG TTC TGT TCT AAT GGT G-3′*
Mouse CD36	forward	*5′-GCA CCA CTG TGT ACA GAC AG-3′*
	reverse	*5′-GTG CAG CTG CTA CAG CCA G-3′*
***IL-1*β	forward	*5′-AGC TCG CCA GTG AAA TGA TGG-3′*
	reverse	*5′-CAG GTC CTG GAA GGA GCA CTT C-3′*
GILZ	forward	*5′-GCA CAA TTT CTC CAT CTC CTT CTT-3′*
	reverse	*5′-TCA GAT GAT TCT TCA CCA GAT CCA-3′*
ANGPTL4	forward	*5′-GAT GGC TCA GTG GAC TTC AAC C-3′*
	reverse	*5′-TGA TGC TAT GCA CCT TCT CCA G-3′*
PDK4	forward	*5′-GGT TAC GGC TTG CCA ATT TCT CGT C-3′*
	reverse	*5′-TTG GGA TAC ACC AGT CAT CAG CCT C-3′*
cyclophilin	forward	*5′-GCA TAC GGG TCC TGG CAT CTT GTC C-3′*
	reverse	*5′-ATG GTG ATC TTC TTG CTG GTC TTG C-3′*