Thesis for the degree of Doctor of Philosophy (Ph.D.)

Role of nuclear receptors in macrophage development and function

By

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1. INTRODUCTION

1.1. Nuclear receptors

1.1.1. Nuclear receptors: Introduction

Nuclear hormone receptors are ligand activated transcriptional factors that play important roles in the differentiation of tissues and organs and regulate several key metabolic processes (1). These receptors are located in the cytoplasm or in the nucleus depending on the receptor and are activated by small lipophilic molecules that cross the plasma membrane or got generated inside the cell. After ligand binding receptors activate transcription of specific genes. Nuclear receptors make up a superfamily including classical steroid hormone, thyroid hormone (TR) and vitamin D receptor (VDR), retinoic acid receptors (RARs), receptors activated by intermediary metabolites: e.g. peroxisome proliferator-activated receptor (PPAR) by fatty acids, liver X receptor (LXR) by cholesterol metabolites, farnesoid X receptor (FXR) by bile acids, pregnane X receptor (PXR) by xenobiotics and several orphan receptors. The term orphan stems from the fact that ligands of these receptors were unknown when the receptors discovered were (4). In several cases metabolites have been identified, which could activate some of these receptors. Most of these molecules have low binding affinity to the receptors. It is likely that these receptors have more than one endogenous ligand, usually metabolites, which are highly abundant and therefore high affinity binding is not critical as for other hormone receptors. Nevertheless, identification of low affinity activators has led to the adoption of some orphan nuclear receptors (e.g. PPARγ, LXR, FXR).

Members of the superfamily share a conserved domain structure (Figure 1): 1. DNA binding domain (DBD) for anchoring the protein to specific DNA sequences, hormone response elements (HREs), 2. ligand-binding domain (LBD) for binding of small lipophilic molecules, 3. transactivation domain for activating the basal transcriptional machinery (5), (1).

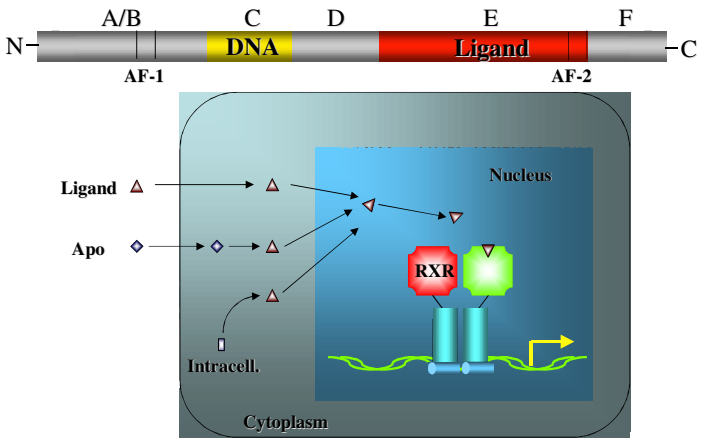


Figure 1: Domain structure of nuclear receptors.

Most nuclear receptors function as dimers. The dimerization occurs through both the DNA-binding and the ligand-binding domains and results in the formation of homo- or heterodimers depending on the receptor. Classical steroid hormone receptors like estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) form homodimers sitting in the cytoplasm in a complex with heat shock proteins and upon ligand activation they enter the nucleus and regulate transcription of their specific target genes. The non-classical nuclear receptors like RARs, VDR, TR are localized in the nucleus sitting on their hormone response elements in the enhancers of target genes and most receptors repress transcription. Upon ligand binding a conformational change occurs that results in the release of co-repressor complex and recruitment of co-activator complex leading to transcription of target genes. In a search for receptors mediating retinoid signaling first all-trans retinoic acid receptor (RAR) was identified. Later, another receptor termed retinoid X receptor (RXR) was discovered, which was capable of mediating retinoid-signaling pathways (6). RXR was also independently identified as a "co-regulator" necessary for efficient binding of RARs to their response elements (7), (8), (9). Most importantly RXR was shown to form heterodimers with many other nuclear receptors (10), (11), (12), (13) making it unique among the members of the nuclear receptor family and postulating a role for RXR as a master heterodimerization partner of many other nuclear receptors. The homo- or heterodimers recognize specific DNA sequences (hormone response elements): direct, indirect or inverted repeats. The consensus nucleotide sequence of the repeat is PuG(G/T)TCA$(X)_n$PuG(G/T)TCA (1).

The number of spaces between the two half sites of the repeat distinguishes the direct repeat of one dimer from the one of the other. When a heterodimer binds to the response element RXR is usually bound to 5' half of the repeat.

1.1.2. Nuclear receptors: Evolution

Since the initial cloning of the glucocorticoid receptor in 1984 (14), (15), (16) hundreds of nuclear receptors have been isolated, cloned, sequenced and studied from a variety of organisms. The completion of genome projects gave us more definitive answers regarding the number of nuclear receptors in the different species. For example in the worm *Caenorhabditis elegans* 270 putative nuclear receptor genes exist, the complete genome of *Drosophila melanogaster* contains 21 nuclear receptors while 48 and 49 receptors have been identified in the human and mouse genomes, respectively (17), (18). The nucleic acid and the deduced protein sequences derived from various species show high degree of conservation. The phylogeny of nuclear receptors has been studied by several authors and it has been established that nuclear receptors appeared very early during metazoan evolution and are present in all metazoan phyla (19), (20), (21). No nuclear receptors have been found in fungi, plants or unicellular eukaryotes so far. Sequence alignment and phylogenetic analysis led to a comprehensive classification of nuclear receptors. The majority of nuclear receptors can be grouped into six subfamilies (22):

1. A large subfamily, includes the well-characterized TR, RAR, VDR, PPARs, receptors that form heterodimers with RXR as well as orphan receptors like ROR, Rev-erb, CAR, etc.
2. This group contains RXRs and the related chicken ovalbumin upstream promoter-transcription factor (COUP-TF), hepatocyte nuclear factor 4 (HNF4). Several receptors function as homodimers, RXR forms heterodimers with Group 1 and 4 receptors.
3. This is the classical steroid receptor subfamily with estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR), etc.
4. A smaller group including NGFIB, Nurr1, Nor1.
5. Another small group has receptors related to the Drosophila Fushi Tarazu Factor-1 (FTZ-F1) and the mammalian steroidogenic factor (SF1) receptors.

6. This group contains germ cell nuclear factor (GCNF), which does not fit well into any other groups.

The conserved domain structures and the distribution in the various subfamilies suggest that nuclear receptors underwent a rapid evolution during early metazoan development. It is presumed that nuclear receptors emerged explosively after two waves of gene duplication events: the first wave during the emergence of metazoans, leading to the formation of members in the six subfamilies and the second wave later mainly in vertebrates leading to the divergence of the paralogue receptors (e.g. RARα, β, γ, RXRα, β, γ). Group 3 steroid receptors were reported to be absent in invertebrates and to be the most recently emerged family. However, recently an estrogen receptor orthologue was discovered in the mollusk, *Aplysia californica* (23). It was also suggested that steroid receptors were derived from an ancient gene before the appearance of the bilaterally symmetric animals and this gene was lost in arthropods and nematodes. Cnidarians (coelenterates) that includes jelly fish and sea anemones were reported as the earliest metazoans where nuclear receptors emerged (24). Interestingly, these organisms express Group 2 and 5 receptors: RXR, COUP-TF homologue and an FTF-F1 homologue. Very recently a nuclear receptor was identified in a sponge, *Suberites domuncula* that belongs to Group 2 indicating that nuclear receptors were present at the base of metazoan evolution and RXR homologues might be in deed the most ancient members of the family (25). From an evolutionary point of view RXR seems to be an ancestral nuclear receptor from which many of the other receptor families emerged. It is interesting to note that the ligand-binding domains of the RXRs share high degree of similarity from jellyfish to human, all show binding specificity for 9-*cis* RA as ligand (24). An intriguing observation is that the early RXR homologue in jellyfish was capable of monomeric DNA binding. Jellyfish RXR binds 9-*cis* RA and probably induces transcription. The more advanced arthropods including the insects have no RXR *per se* but express ultraspiracle (USP), a homologue of the RXR (26), (27). Although the amino acid sequence of USP is different from that of RXR and USP does not bind 9-*cis* RA, its crystal structure shows similar structural features in the two receptors (28), (29). This model suggests that RXR's ligand binding appeared very early in metazoan development and there was a secondary loss mainly in arthropods. RXR signaling appeared well before

RARs, PPARs and LXRs emerged suggesting that RXR signal pathway is independent from RAR and suggests that autonomous RXR signaling (independent of RAR and other partners) may still persist.

1.1.3. Nuclear receptors: Structure

Nuclear receptors have a common, conserved structure that will be discussed below using RXR as an example in more details. Receptors have a variable N-terminal domain (A/B domain), a highly conserved DNA-binding domain (DBD), a non-conserved hinge, and a moderately conserved C-terminus including the ligand-binding domain (LBD). The DBD consists of two cysteine-rich zinc finger motifs through which nuclear receptors bind to specific DNA sequences, hormone response elements PuG(G/T)TCA$(X)_n$PuG(G/T)TCA. These consensus nucleotide sequences form direct, indirect or inverted repeats that consist of two half sites separated by a short spacer (30), (31). A highly conserved part in the first zinc finger between the last two cysteines is the P-box, which determines the sequence specificity of the receptor-DNA binding (32), (33). Another conserved part in the second zinc finger is the D-box, which dictates the half site spacing. Transcriptional activation is mediated by the LBD, which contains four more-or-less overlapping surfaces: a ligand-binding pocket for binding of small, lipophilic molecules, a transactivation domain (AF2 or helix 12), a cofactor binding surface and a dimerization surface. The ligand-binding domain shares a common, overall structure among nuclear receptors: a three-layered alpha-helical sandwich as it is shown in Figure 2 in the case of RXR.

In the lower part of the structure a non-polar cavity, the ligand-binding pocket is located. This pocket is sealed by a two-stranded α-sheet and the C-terminal helix 12. In some nuclear receptors (e.g. PPAR, LXR, PXR) the ligand-binding pocket is relatively large and is not fully occupied by the known ligands. In contrast to these, nuclear receptors with high affinity ligands, like RXR have a tighter fit between ligand and receptor. The ligand-binding pocket is much smaller (generally 400-500 A^3) and 9-*cis* RA for example occupies most of the volume of the cavity. Binding of a ligand to the apoenzyme leads to conformational changes: it alters the ability of the receptor to multimerize, it also alters the surfaces that determine homo- or heterodimerization and it alters the receptor's

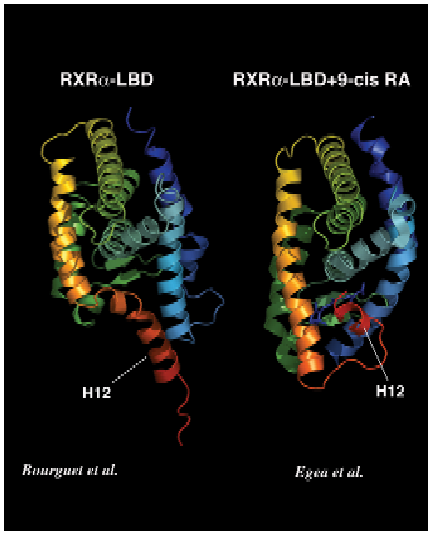


Figure 2: Structures of the unliganded and liganded RXRα's LBD. It shows a three-layered helical structure. Helix-12 (H12) is colored red. In the unliganded LBD (left) H12 stands away and upon ligand binding it is repositioned onto the body of the LBD (right). The structure of the human unliganded RXRα (PDB code: 1LBD) (2) and that bound 9-cis retinoic acid (RA) (PDB code: 1FBY) (3) are shown.

cofactor-binding surface. These changes (mainly due to the rearrangement of helices 10/11 and 12) cause a ligand-induced switch from the receptor's repressor activity to an activation state (Figure 2).

It is important to note that ligand binding in the ligand-binding cavity can also lead to conformational changes (mainly of helix 7) that affects the dimerization surface and the conformation of the partner's helix 12 that results in the activation of the partner. This phenomenon partially may explain the permissiveness or non-permissiveness of various RXR heterodimers. In the case of some receptors (e.g. PPARs, LXR, FXR) the length of helix 12 allows the positioning of these residues most likely making the heterodimer "permissive" for activation from the RXR side. In other receptors like RAR, TR, VDR this repositioning cannot occur and therefore these heterodimers might become "non-permissive" (i.e. cannot be activated from the RXR side) (34), (35). It is also notable that

other much weaker dimerization surfaces exist in the DBD establishing a weak response element specific interface.

Ligand binding is only a part of nuclear receptors' function. Several co-regulators have been identified that regulate unliganded and liganded nuclear receptor functions. A protein complex regulates nuclear receptors' actions that contain chromatin remodeling enzymes, co-repressors or co-activators depending on the state of the receptor. Both co-activators and co-repressors contain interaction domains with a motif of LxxLL in co-activators (36), (37) and LxxxIxxxI/L in co-repressors (38), (39), (40). From structural studies we know that the co-activator peptide binds to helices 3, 4 and 12 (37), (41), (42). Helix 12 position is critical in the interaction. In a liganded receptor it is placed in the active position (a charged clamp is formed on the surface) that favors co-activator binding and transactivation. In an unliganded receptor helix 12 is placed into an adjacent groove of the LBD forming an alternative binding-surface that favors co-repressor binding and repression (Figure 2). This phenomenon has been termed as ligand-induced switch and reviewed in (43).

Unliganded RXR monomer is likely to be rare because helix 12 extends away facilitating di- or tetramerization. Ligand binding induces conformational changes where helix 12 is repositioned onto the body of the LBD that generates an active conformation capable of co-activator binding. In the presence of a partial agonist ligand helix 12 is bound in an antagonist conformation (34) that allows co-repressor binding. Although, the structure of RXR with a full antagonist has not been solved, it is very likely that as in the case of other receptors RXR is also in an antagonist conformation because the ligand-binding pocket cannot be closed with helix 12. Similarly, deletion of helix 12 results in an antagonist conformation with increased co-repressor binding by both the RXR and its partner (44). In permissive heterodimers as PPAR binding of an activator results in conformational change, PPAR's helix 12 is repositioned and a strong co-activator binding site is generated (45). This leads to the binding of co-activators to the PPAR. Liganding of the RXR side is likely to be different. Conformational changes after binding of an RXR agonist may cause changes in the PPAR structure by docking its helix 12 to the co-activator binding site and contributing to the formation of an agonist conformation. In non-permissive heterodimers co-repressor binding is more complex. These receptors are

believed to unmask RXR's co-repressor binding surface leading to co-repressor binding that stabilizes this conformation. Binding of an RXR agonist alone is not sufficient for co-repressor release (46), (44). Several key mechanisms of the ligand-induced switch in heterodimers and the role of RXR in it need to be identified or further refined.

1.1.4. Nuclear receptors: Function

1.1.4.1. Retinoid receptors

Vitamin A and its derivatives, retinoids have profound effects in development, differentiation, homeostasis and various aspects of metabolism. The discovery of retinoid receptors substantially contributed to the understanding how these small, lipophilic molecules, most importantly retinoic acid (RA) exert their pleiotropic effects (47), (48). The two families of retinoid receptors (RARs and RXRs) now contain three isotypes, α, β and γ encoded by separate genes and giving rise to numerous alternatively spliced variants (30), (31). RARs can be activated by all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid, while RXRs can be activated only by 9-*cis* RA (49), (50). There are natural and synthetic compounds with selectivity for RXR (51), (52), (53) termed rexinoids (54). One of the most enigmatic and controversial areas of RXR research is the documentation of the presence of endogenous ligands activating RXR in vivo. Several molecules have been described as potential endogenous ligands for RXR: 9-*cis* RA (49), phytanic acid (55), docosahexaenoic acid (56) but none of these has been proved to be the bona fide endogenous ligand so far. Perlmann and colleagues created a ligand detector mouse line (57). Transgenic lines were constructed using Gal-DBD RXR-LBD fusion constructs and a reporter gene containing Gal binding sites and a β-galactosidase reporter gene. X-gal staining of mouse embryos revealed sites of ligand production. Specific regions of the spinal cord lit up suggesting that endogenous ligand production took place. Luria et al. using a similar system with green fluorescent protein as a reporter have confirmed these results recently (58). They also obtained high RXR activation in spinal cord and upon exogenous ligand treatment in the brain an in the olfactory epithelia.

RARα is expressed in a general fashion during murine embryonic development while RARβ and γ are more restricted: RARβ is more rostral and RARγ is expressed in the open neural tube. RARs have been implicated in embryonic (59), skeletal, (60) myeloid

development (61), (62), (63), wound healing, keratinization (64) and also in the developing nervous system (65). Homozygous RARα mutant mice exhibited early postnatal lethality and testis degeneration. RARγ null mice showed several malformations previously associated with vitamin A deficiency (VAD), including transformations of the cervical vertebra, skull, fusion of the first and second ribs. RAR double or compound mutants died either *in utero* or shortly after birth. Many defects found in these animals correspond to fetal VAD.

RXR is unique among nuclear receptors because in addition to forming homodimers it forms many heterodimers and therefore ligand activation has potentially pleiotropic effects on numerous biological pathways and has been implicated not only in retinoid responses but also in various metabolic pathways.

In 1990 it was discovered by Mangelsdorf et al. that one of the orphan receptors represented a novel retinoid responsive transcription factor (6), that was called retinoid X receptor (RXR). The fact that RAR is more similar to the thyroid hormone receptor (TR) than it is to RXR suggests that these two groups of retinoid receptors are implicated in the regulation of distinct pathways and do not represent redundant retinoid signaling pathways. The homology of the three RXR subtypes, termed RXRα, β and γ indicates that these receptors regulate common target sequences and respond to common ligands (66). The expression pattern of the subtypes is rather different. RXRα shows abundant expression in liver, kidney, spleen, placenta, epidermis and a variety of visceral tissues, RXRβ is expressed widely and can be found in almost every tissues (like RARα), RXRγ expression is mainly restricted to muscle and brain (66). These and other studies about differentiation and metabolic processes suggest that RXRs play critical roles in a wide range of developmental processes, from embryo implantation to organogenesis as well as in the regulation of adult physiology and metabolic processes.

Defect in the expression of RXRα has much more severe consequences than the loss of RXRβ and γ. Sucov et al. and Kastner et al. published a targeted loss-of-function mutation in the RXRα gene in the mouse germ line, which resulted in embryonic lethality between E13.5 and E16.5 in homozygous embryos (67), (68). The major defect led to lethality was the hypoplasia of the ventricular chambers of the heart manifested in extremely thin ventricular wall and defects in ventricular septation. They also found

ocular abnormalities and growth deficiency in the heterozygous mice. Deletion of one RXRα allele in mouse embryos revealed an intermediate phenotype with trabecular and papillary muscle defects, ventricular septal defects, conotruncal ridge defects and pulmonic stenosis, indicating a dosage effect for RXRα in maintaining normal cardiac morphogenesis (69). Hepatic differentiation was also transiently retarded. RXRα -/-, RXRβ -/-, RXRα -/- RXRβ -/-, RARα -/-, RARβ -/- and vitamin A deficient embryos exhibited a precocious differentiation of ventricular myocytes. A number of targeted mutations resulted in embryonic perturbation of cardiac development. This raised the possibility that this phenotype might represent a final common pathway of lethality occurring secondary to dilated cardiomyopathy. The abnormalities observed in RXR null mice were highly similar to the effects of embryonic VAD. Defects in retinoid signaling, achieved either by the generation of VAD or by deletion of individual retinoic acid receptors (particularly RARγ) resulted in ocular and skeletal developmental abnormalities (70). As in the case of the RXRs, there appeared to be functional redundancy among the RARs since animals with deletion of two RARs (particularly RARγ and α and to a lesser extent RARγ and β) showed a more extensive and severe phenotype than RARγ nulls. In each of these animal models, the function of the residual RARs was highly dependent on RXRα, since RXRα/RARγ double mutant animals displayed more severe ocular malformations and RXRα/RARα, RARγ double null mutants exhibited abnormalities not seen in the single mutants (60). Detailed analysis of the inactivation of one or both RXR alleles combined with that of one or both alleles of a given RAR subtype concluded that however RARs and RXRs could function independently on activating their own network of target genes they synergized in combined mutants, led to specific phenotypes could not be found in single mutants and sensitivity to the morphogenetic and teratogenic effects of retinoids depends critically on the levels of both RARs and RXRs in a particular tissue (71). During development, RARs appear to be the most important partners for the RXRs; most of the phenotypic features of the RXR null animals are attributable to perturbations in RAR dependent pathway. Furthermore, during development RXRα is the primary heterodimeric partner of RXR and it functions to support the activity of all three of the RARs (72).

Mouse fetuses with targeted disruption of the RXRα gene also developed defects in the chorioallantoic placenta (73). The labyrinthine zone of the placenta appeared histologically disorganized. Signs of necrosis, endothelial defects, blood stasis, abnormal thickening of the trophoblastic region were displayed with abnormal blood stasis leading to thrombosis, abnormal thickness and impaired transfer of nutrients and oxygen. It was suggested that the differentiation of the placental parenchymal cells into mature trophoblasts was facilitated by RXRα and this process was impaired in mutant mice. These abnormalities were also similar to those observed in mice with vitamin A deficiency suggesting a role of RAR/RXR heterodimer. Trophoblastic cells from mutant placentas were poorer in lipid droplets which phenomenon could reflect an abnormal PPARγ function (74).

RXRα -/- RXRβ-/- double mutant mice were also generated (75) to address the issue of redundancy. These animals died between 9.5 and 10.5 days of gestation, earlier than RXRα null mice and showed a wide range of abnormalities: truncation of caudal region, abnormal body turning, dilated heart cavities, wavy aspect of neural tube, shortened pharyngeal arches, hypoplasia of the frontonasal region, failure of neural tube closure. The cause of lethality was the lack of formation of the labyrinthine zone of the chorioallantoic placenta.

Approximately 50% of RXRβ null mutants died before or at birth (76). Those that survived appeared normal except that the males were sterile. The epididymis contained very few and abnormal spermatozoa. Sertoli cells showed progressive lipid accumulation.

The RXRγ null mice developed normally and were indistinguishable from the heterozygous and wild type animals (77). These mice had higher serum T4 levels and TSH (thyroid stimulating hormone) levels and increased metabolic rate than wild type animals (78). RXRα -/- RXRγ -/- double mutants were similar to the RXRα -/- mice. Viable RXRβ -/- RXRγ -/- double and RXRα +/- RXRβ -/- RXRγ -/- triple mutant mice could be obtained (77). They showed growth deficiency and, due to the loss of RXRβ, male sterility but reached adult age to date indicating that one single RXRα allele was sufficient for development and morphogenesis.

Retinoids were also implicated in the processes of learning and memory (79). RARβ deficiency eliminated hippocampal long term potentiation (LTP) and long term depression (LTD) the most widely studied forms of synaptic plasticity. RXRγ appeared to be required only for LTD. These findings showed that these two forms of plasticity were retinoid dependent and revealed different ways of contribution of retinoid receptors. RXRβ -/- RXRγ -/- double mutants exhibited locomotor deficiencies due to a dysfunction in dopamine signaling pathway (80).

To address the issue whether transactivation through RXR was essential for biological processes a mutant mouse line was generated that expresses truncated RXRα lacking helix 12 of the ligand binding domain (LBD) which also contained the activation function domain 2 (AF-2) (81). These animals exhibited a similar range of abnormalities than the RXRα null mice, often with incomplete penetrance. The mutation was lethal, the embryos died at E14.5 and E18.5 while others did so only at birth or shortly afterwards. They also displayed defects that were not seen in RXR null littermates: agenesis of esophageal septum, hypoplastic lungs, diaphragmatic hernia, ectopic opening of the ureter into the urogenital sinus. RXR AF-2 null/RXRγ -/- mice were indistinguishable from RXR AF-2 null mice but RXR AF-2 null/RXRβ -/- or RXR AF-2 null/RXRβ -/- /RXRγ -/- mice were more severely affected. In these double and triple mutants nearly the full spectrum of malformations could be observed that characterized fetal vitamin A deficiency syndrome (81). These data suggested that liganded RXRα and/or its transactivation function were required for the developmental functions of RAR/RXR heterodimers. Eliminating the other activation function (AF-1) domain resulted in similar but less severe abnormalities (82). AF-1 but not AF-2 domain was showed to be dispensable for placentation. This fact indicated that other RXR dimers (probably PPARγ/RXR) were responsible for placentagenesis (83), (84).

1.1.4.2. PPARs

PPARs are involved in the regulation of important metabolic pathways, most of them play critical role in fatty acid and cholesterol metabolism (85). The family contains three isoforms: PPARα, γ and δ. PPARα is mostly expressed in brown adipose tissue and liver,

then kidney, heart and skeletal muscle. PPARγ is mainly expressed in adipose tissue, macrophages and to a lesser extent in colon, in the immune system and the retina. PPARδ is found in many tissues, ubiquitously expressed but the highest expression is in the gut, kidney and heart. The function of the PPARs have been extrapolated from the identity of their target genes (Figure 3), which so far all belong to pathways of lipid transport and metabolism (86).

Receptor	Activator or ligand	Function	Knockout phenotype	Key target genes
PPAR-alpha	POVPC (1-palmitoyl-2-oxovalaroyl-sn-glycero-phosphocholine) PGPC (1-palmitoyl-2-glutaroyl-sn-glycero-phosphocholine) Clofibrate (synthetic)	Regulation of fatty acid catabolism Lipid lowering Endothelial cell activation Production of chemokines	Abnormalities in triglyceride and cholesterol metabolism Decreased atherosclerosis Obesity	IL-8 MCP-1 CPT-1
PPAR-gamma	9(S)-HODE (9(S)-hydroxy octadecadienic acid) 13(S)-HODE (13(S)-hydroxy octadecadienic acid) Rosiglitazone (synthetic)	Early embryonic development Adipogenesis Glucose metabolism Cholesterol influx Foam cell formation Regulation of cholesterol metabolism	Lethality Lack of adipose tissue Abnormalities in triglyceride and cholesterol metabolism Insuline resistance Increased atherosclerosis	CD36 FABP4 LXR-alpha
LXR-alpha	27OH-cholesterol 24(S)25 Epoxicholesterol	Cholesterol efflux Reverse cholesterol transport Regulation of fatty acid synthesis Regulation of cholesterol metabolism	Abnormalities in triglyceride and cholesterol metabolism Cholesterol accumulation Increased atherosclerosis	hLXR-alpha ABCA1 ApoE

Figure 3: Summary of PPARα, PPARγ and LXRα function, ligand and phenotype of the knockout mice.

Target genes of PPARα are a relatively homogenous group of genes that participate mainly in aspects of lipid catabolism through peroxisomal β-oxidation and immune processes. Fibrates are synthetic ligands of PPARα and in the past few years they have been used as potent hypolipidaemic drugs to treat cardiovascular disease. Fibrates lower plasma triglyceride levels markedly and increase high-density lipoprotein (HDL) levels. They may also have hypoglycaemic and thus anti-diabetic effect. Previous studies showed that oxidized phospholipids from minimally modified MM-LDL: oxPAPC (oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphocholine, POVPC (1-palmitoyl-2-oxovalaroyl-sn-glycero-phosphocholine) and PGPC (1-palmitoyl-2-glutaroyl-sn-glycero-phosphocholine) could activate PPARα leading to the production of interleukin-8

(IL-8) and monocyte chemotactic protein-1 (MCP-1) and concomitantly increased recruitment of monocytes/macrophages to endothelium (87). Mice homozygous for PPARα deficiency are viable and fertile. Pathological changes, like hepatomegaly, peroxisome proliferation followed by the administration of classical peroxisome proliferators, clofibrates could not be observed in mutant mice (88). Basal levels of serum total cholesterol and high density lipoprotein (HDL) cholesterol were elevated and levels after fibrate treatment were unaffected in PPARα null mice indicating important role of PPARα in the regulation of cholesterol level (89). Hepatic triglyceride secretion was higher in PPARα-deficient female mice than in controls, but there was no difference in male mice (90). After a high fat diet, mutant mice displayed elevated LDL cholesterol levels and triglyceride levels were not affected. Effects of receptor deficiency were also tested in a mouse atherosclerosis model: PPARα -/-, apoE -/- double knockout mice were generated. Under a high fat diet these mice showed severe atherosclerosis. Concentration of atherogenic lipoproteins was higher, however the area of the atherosclerotic lesion was reduced in PPARα-deficient mice than in control apoE-/- mice (91). This latter supports the observations above.

PPARγ controls a broad range of cellular responses: differentiation, proliferation, cell death and inflammation. It is essential for the development of adipose tissue (92), plays critical role in glucose homeostasis. In mice it is essential for placental development and vascularization (83), (84). PPARγ can be activated by natural ligands such as fatty acids, oxidized low density lipoprotein (oxLDL) component lipids, 11, 13-hydroxy octadecadienoic acid, 15-deoxy-d-(12,14)PGJ2 (15D-PGJ2) and the thiazolidinedione class of insulin-sensitizing drugs that have been developed and used in the treatment of type two diabetes mellitus (93), (94). PPARγ gene knockout resulted in early embryonic lethality. Deficiency interferes with terminal differentiation of the trophoblast and placental vascularization, leading to severe myocardial thinning and death by E10.0 (74). Tetraploid-rescued mutant exhibited another lethal combination of pathologies, including lipodystrophy, fatty liver and multiple hemorrhages (95), (96). Those any PPARγ -/- mice survived to term were deficient for all forms of fat providing evidience for the fundamental role of PPARγ in adipogenesis (74). Transplantation of PPARγ null bone

marrow into LDL receptor -/- mice results in a significant increase in atherosclerosis (97). It was reported by the Glass lab that TZDs greatly inhibited the development of atherosclerosis in LDL receptor deficient male mice (98). Similar results were shown by Z. Chen and colleagues in apoE-/- mice another murine atherosclerosis model (99). PPARγ has been implicated in the modulation of immune responses and has been identified as a key regulator of lipid metabolism in macrophages. Because of the early lethality of PPARγ -/- embryos conditional myeloid specific knockout mice were produced using the Cre/loxP system. The targeted disruption of the PPARγ gene resulted in reduced total plasma and HDL cholesterol levels and cholesterol efflux was significantly decreased from macrophages elicited by thioglycolate in mutant mice (100). Therefore, the lack of the PPARγ gene in macrophages is likely to be pro-atherogenic. Role of PPARγ in macrophage lipid metabolism was extensively analyzed and will be discussed in details below in macrophage lipidology chapter.

In contrast to PPARα and γ the function of PPARδ is relatively unknown. PPARδ is expressed in a wide range of tissues but the function of this transcription factor remained enigmatic, due to in part to the lack of selective ligands. It has been implicated in physiological and pathophysiological processes such as embryonic implantation, wound healing, inflammation, cancer and osteoporosis. It has been recently shown that PPARδ can be activated with very low-density lipoprotein (101) and also has been implicated in cardiac muscle function (102). It was also shown that a new, selective PPARδ agonist promoted reverse cholesterol transport in primates (103). These results and facts reveal the possibility that PPARδ has an unexpected and exciting role in fatty acid and cholesterol metabolism. It activates fat metabolism and prevents obesity (104). PPARδ has been implicated in colon carcinogenesis but the role is not clear: both inhibition and increase has been reported.

1.1.4.3. Liver X receptors

Two LXR proteins (α and β) are known to exist in mammals. LXR also function as heterodimer with RXR. Expression of LXRα is restricted, with highest levels in the liver and lower, but significant levels in macrophage, kidney, intestine, spleen and adrenals.

LXRβ expression is more ubiquitous and has been found in nearly every tissues examined (105). LXRs induce the transcription of the gene encoding cholesterol 7a-hydroxylase (Cyp7a) the rate-limiting enzyme in bile acid synthesis.

Several oxysterols, like 22(R)-OH cholesterol 25-OH and 27-OH cholesterol have been identified as ligands for LXR. LXRs have been implicated in the regulation of cholesterol metabolism (106) and clearance (107) and recently in inflammation (108), (109), (110). Mice lacking LXRα lose their ability to respond normally to dietary cholesterol and unable to tolerate any amount of cholesterol in excess of that they synthesize de novo (111), (112), (113). These mice develop severe atherosclerosis. Serum and hepatic cholesterol levels and lipoprotein profiles of cholesterol-fed animals showed no significant differences between LXRβ-/- and wild type mice. LXRβ-/- mice – in contrast to LXRα-/- mice maintain their resistance to dietary cholesterol. Synthetic LXR agonist was shown to reduce atherosclerosis in two mouse models, in apoE-/- and in LDL receptor -/- mice (114). Its control on macrophage lipid metabolism is tightly connected to PPARγ, which will be discussed below.

1.2. Monocytes-macrophages

1.2.1. Monocytes-macrophages: Introduction

Monocytes and polymorphonuclear phagocytes develop from pluripotent stem cells characterized by the expression of CD34. These cells give rise to granulocyte-monocyte, granulocyte- and monocyte-colony forming units (CFU-GM, CFU-G and CFU-M). Monocyte differentiation proceeds from CFU-M through monoblast to circulating monocytes, which mature to macrophage in various tissues leading to the formation of multiple types of tissue-specific macrophages (63). Markers for this lineage are M-CSF receptor, lysozyme, macrosialin and cell surface proteins (e.g. CD36, CD14, CD11b and CD18). The differentiation of myeloid cells is principally regulated by cytokines and orchestrated via cooperative gene regulation by various transcription factors: Maf-B, c-Jun, Egr-1, PU.1, Sp1. PU.1:GATA-1 interaction and CCAAT/enhancer binding protein α (C/EBPα) suppression of Friend of GATA (FOG) transcription inhibits erythroid and megakaryocyte gene expression. C-Jun:PU.1 complex induce monocytic genes. PU.1 and C/EBPα induce their own promoter, C/EBPα induce PU.1 and C/EBPε and RAR

activates its promoter. Higher level of PU.1 is required for monocytic development whilst higher C/EBP level favors granulopoiesis.

RARs are expressed in nearly all hematopoietic lineages (115) and play critical role in hematopoiesis (61), (62), (63). RARα and γ knockout mice display a block in granulocytic differentiation (116). Dominant negative RAR blocks neutrophil development at promyelocyte stage (117) and switches normal granulocyte/monocyte differentiation to basophil/mast cell development (115). RAR was reported to act as a differentiation checkpoint switch at the promyelocytic stage of granulopoiesis resulting in granulocytic differentiation (118). Following induction of myelomonocytic differentiation an induction of RARα was observed (119). Retinoic acid (RA) stimulates the maturation of myeloid precursors in cytokine-stimulated CD34 positive cells (120). The 15;17 chromosome translocation in acute promyelocytic leukemia (APL) generates a PML-RARα fusion protein that inhibits RARs, resulting in a block of terminal differentiation of granulocytes (121), (122), (120).

PPARγ was shown to influence myeloid development (93), (94). PPARγ has not appeared to regulate the formation of the monocytic lineage but modulates differentiation and metabolic functions of macrophages (123), (124). Recent observations suggest that although PPARγ is not necessary for monocyte differentiation (123), (124), modulation of the level and activity of PPARγ has consequences in the fate and metabolism of a macrophage. The mechanism by which PPARγ affects differentiation is still unknown and the possible interconnection between retinoid and PPARγ signaling in the maturation process has not been studied in detail yet.

1.2.2. Monocytes-macrophages: Lipid metabolism – Atherosclerosis

Atherosclerosis is a progressive, degenerative disease of blood vessels. Initially, it is characterized by lipid accumulation in the wall of arteries. The formation of the atherosclerotic lesion begins after birth and clinical manifestations can be observed usually from the sixth decade. Complications of atherosclerosis such as myocardial infarction and stroke are responsible for most of the deaths in western societies. This fact gives particular importance and relevance to studies on the formation of atherosclerotic lesions and to the efforts to identify molecular targets for potential anti-atherogenic

drugs. Handling of lipids by macrophages is an important metabolic process in the context of hypercholesterolemia and the development of atherosclerotic lesions (125), (126), (127). Atherosclerosis is the disease of vessel walls. Endothelium (a single cell layer lining the vessel wall) forms a barrier between the lumen and the wall of blood vessels and coordinates the migration of molecules and cells from circulating blood towards sub-endothelial structures. A multicasual disturbance in endothelium function is the key event of initiating lesion formation. Preferred sites of lesions are where endothelium is vulnerable, where blood flow is disturbed or not laminar. Endothelial dysfunction results in the accumulation of low-density lipoprotein (LDL) in the sub-endothelial matrix (125). It is not clear how LDL gets modified but this modification leads to the appearance of minimally oxidized/modified (mmLDL) and subsequently fully oxidized LDL (oxLDL) containing multiple oxidized lipid molecules (126). Oxidatively modified lipoproteins activate both endothelial cells and monocytes/macrophages resulting in monocyte migration into the sub-endothelial space (128). Uptake of the modified LDL and oxidized lipids is not complete so they are able to modulate the function of the neighboring cells, which lead to production of inflammatory cytokines by multiple cell types such as endothelial cells, lymphocytes and monocytes. The release of these inflammatory mediators evokes a characteristic inflammatory response around lipid accumulation by attracting additional monocytes to the lesion and their maturation into macrophages (129), (130). Macrophages accumulate lipids from oxLDL leading to lipid-loaded foam cell formation. Foam cells are the characteristic cells of the early, cellular phase of atherosclerotic lesions. Accumulated foam cells and lipids are visible as fatty streak in the intima of the vessels. Foam cells eliminate lipids from the sub-endothelium but unable to get rid of all of it if overwhelmed so they accumulate lipids continuously and this contributes to a sustained chronic inflammatory process mediated by cytokines and chemokines (126), (125). This chronic inflammation with the secreted mediators and growth factors make smooth muscle cells migrate from the vessel wall's media, proliferate and produce extracellular matrix. This leads in the formation of the late, fibrous atherosclerotic plaques. This late lesion is characterized by sclerosis, which makes the artery wall rigid and fragile. Finally, the lesion may rupture the endothelium covering it leading to the formation of thrombus and intravascular

coagulation. The complications (myocardial infarction and of these latter events) make atherosclerosis the number one enemy in our societies. It is critical to understand the regulatory processes associated with cholesterol and fatty acid uptake and release (efflux) of this cell type.

By 1999 it was shown that PPARγ is most prominently expressed in adipose tissue and myelomonocytic cells, monocytes and macrophages (131), (132). PPARγ promoted uptake of oxidized LDL and subsequent differentiation of the monocytes to foam cells (94). Synthetic PPARγ agonists caused similar differentiation both in myelomonocytic cell lines and in primary human monocytes, too. It was shown that PPARγ was expressed in foam cells of atherosclerotic lesion. Its expression could further increased with oxLDL. Thus, oxLDL but not native LDL promoted uptake of itself by scavenger receptor CD36. Characterizing this phenomenon it was proved that PPARγ had a key role in the regulation of lipid uptake into foam cells. Briefly, the scavenger receptor CD36 could be regulated by PPARγ as a direct target gene of the receptor. Two components from the lipids in oxLDL, 9-hydroxy octadecadienoic acid (9-HODE) and 13-HODE were identified as endogen activators and *bone fide* ligands of PPARγ (93). These results suggested a model of macrophage lipid metabolism. Macrophages internalize modified LDL via scavenger receptors (i.e.: CD36), which unlike LDL receptor are not downregulated by high cholesterol levels. On the contrary, oxLDL increase the expression of a scavenger receptor CD36 by PPARγ and potentiates its own uptake. Oxidative modification of the LDL particle endows it with the ability to bind scavenger receptors and at the same time converts a component of the particle, linoleic acid, into an effective activator of PPARγ, 9- and 13-HODE. Thus, the consequence of oxLDL internalization is the initiation of a positive feedback loop that enhances the expression of CD36.

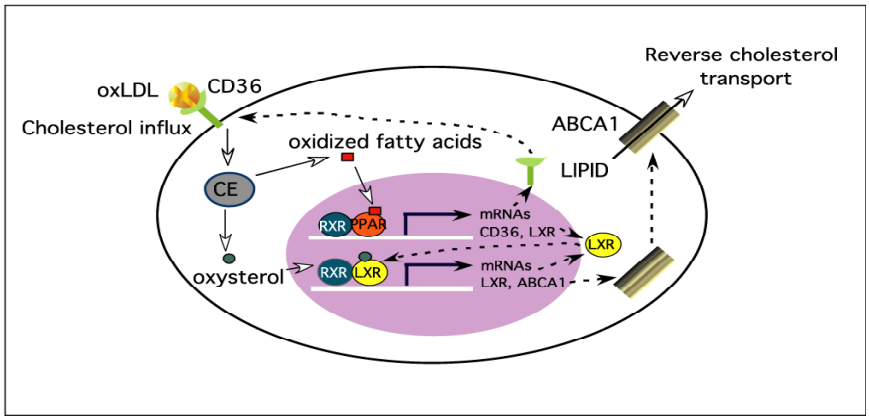


Figure 4: Role of PPARγ and LXR in the regulation of macrophage lipid transport.

OxLDL also induces scavenger receptor A (SR-A) expression via a PPARγ independent way. This regulatory circuit we called gamma-cycle (Figure 4) suggested novel mechanisms how oxLDL contributed to foam cell formation and atherosclerosis. These findings provided explanation for the formation of lipid-filled macrophages (foam cells) and suggested the existence of a vicious cycle leading to atherosclerosis.

The model for foam cell formation referred to as gamma cycle above put PPARγ on the dark side of atherogenesis, suggesting its activation may be proatherogenic. However, synthetic agonists of PPARγ, the thiazolidinediones (TZDs) are widely used in treatment of type II diabetes. TZDs and rexinoids (retinoid X receptor agonists) lower hyperglycemia and hyperinsulinemia. Studies in diabetic rodents and in man demonstrated that TZDs and rexinoids increase insulin sensitivity in adipose tissue and muscle (133). PPARγ has also been shown to reduce inflammatory cytokine production by macrophages and inhibit macrophage activation (134), (132), effects which should be anti-atherogenic. The question, how PPARγ regulates atherosclerosis remained unclear. Is its activation beneficial or harmful for the patients with atherosclerosis or diabetes? To address to these questions A. Chawla and colleagues used a genetic approach, PPARγ null mutant embryonic stem (ES) cells were created by homologous recombination. They were able to show that PPARγ was not essential for the development of the macrophage lineage in vitro and in vivo, but was an important regulator of macrophage gene expression. PPARγ deficient ES cells gave rise to macrophages in an in vitro differentiation assay and in chimeric mice generated with mutant ES cells PPARγ deficient cells were also able to contribute to the macrophage lineage in vivo (123). Retroviral transfer of the receptor facilitated the induction of CD36 and the uptake of oxLDL in response to PPARγ-specific agonists. No change in the CD36 mRNA level was observed when treated PPARγ-deficient macrophages with TZDs. This suggested that PPARγ was required for the induction of CD36. In these studies it was also shown that

PPARγ was not essential for PPARγ ligands to exert anti-inflammatory effects in macrophages. The expression of pro-inflammatory genes was also inhibited by natural and synthetic ligands in both wild-type and PPARγ deficient macrophages. At the same time K. J. Moore and colleagues reported a similar study with essentially the same results (124). These results made the interpretation of pharmacological studies on the role of PPARγ in atherogenesis even more complicated because both receptor dependent and independent effects had to be considered. Next PPARγ was shown to not only regulate lipid uptake, but had a central role in cholesterol efflux from cells. PPARγ induces ABCA1 expression and cholesterol removal from macrophages through a transcriptional cascade driven by PPARγ and LXRα. ABCA1 and ABCG1 are members of the ATP-binding cassette family of transporter proteins. It has been shown that these transporter proteins are highly expressed in lipid-loaded macrophages (135). Mutations in ABCA1 gene result in Tangier-disease, a disease characterized by marked cholesterol accumulation in macrophages and other reticuloendothelial cells (136), (137), (138). Cholesterol clearance is impaired from fibroblast isolated from patients with Tangier-disease which suggests that ABCA1 has a pivotal role in cholesterol efflux. Several studies reported that LXRs mediate cholesterol efflux by inducing cholesterol transporters ABCA1, ABCG1 and later ABCG5 and ABCG8 (139), (107), (140), (135). Cholesterol efflux from macrophages is likely to be anti-atherogenetic. The effect of oxLDL to induce cholesterol efflux from macrophages is also anti-atherogenic, but as described above oxLDL promotes a potential vicious circle activating PPARγ and enhancing its own uptake to the macrophages. This contradiction was resolved by showing that activation of PPARγ leaded to primary induction of LXRα (141). These data suggested that LXRα was a direct target gene of PPARγ. Since both PPARγ and LXR could be activated by lipid components of oxLDL, it was hypothesized that these nuclear receptors composed a transcriptional cascade that regulated macrophage response to oxLDL. Tontonoz and colleagues analyzed the promoter of the ABCA1 gene and showed that LXR:RXR could activate it but PPARγ:RXR heterodimer could not. They also identified the response elements and proved that these were functional. They compared PPARγ- and LXR-induced cholesterol efflux and found that agonists of both

nuclear receptors induced cholesterol efflux and the combination of the ligands was additive. To clarify the functional role of PPARγ in atherogenesis they in collaboration with the Evans lab performed transplantation with PPARγ null bone marrow into LDL receptor knockout mice, a murine model of atherosclerosis. They placed the transplanted mice on atherogenic diet for 8 weeks to induce atherosclerosis. Plasma total cholesterol levels were similar before and after the diet in control and PPARγ bone marrow transplanted mice. The degree of atherosclerosis was significantly increased in PPARγ-deficient transplanted mice (141). The pattern of PPARγ expression in atherogenic lesions was similar to that observed by others (142). G. Chinetti et al. reported similar results (143). The first model about the regulation of oxLDL uptake by PPARγ in macrophages was re-constructed to modified gamma cycle (Figure 4). This involves elements of the first cycle: oxLDL induces PPARγ and CD36 levels. Increased CD36 causes further oxLDL uptake. PPARγ also induces the expression of LXRα, which activates transcription of cholesterol transporters e.g. ABCA1 and these lead to increased cholesterol efflux to ApoAI from macrophages. The existence of this transcriptional cascade now predicts that modulation of one of the elements in the cascade will affect all the others and the net effect on cholesterol level in the cell depends on how the balance between the influx and efflux changes. The model also explains why TZDs are beneficial in atherosclerosis: by activating PPARγ they contribute to oxLDL scavenging and cholesterol efflux, and the net effect of these pathways is likely to be lipid removal from the artery wall.

A coordinated lipid transport is likely to be coordinated by these receptors that results in cholesterol efflux from the cells towards high-density lipoprotein (HDL). Linking of the two receptor systems (PPARγ and LXRα) provides an attractive but not well understood model to explain lipid/cholesterol uptake and efflux from macrophages. The issue how the activation of the two receptors may be coupled has not been addressed yet. It was assumed that lipid content of lipoproteins may act as activators or ligands for both PPARγ (93) and LXR (112). The fact that LXR signaling is activated in macrophages exposed to acetylated LDL (112), which does not contain oxidized cholesterol suggests that there must be other ways to activate/produce ligand for this receptor. PPAR-related

induction of LXRα is not enough for getting an activated LXR that induces cholesterol efflux. It needs to be activated by its endogenous ligand. Production of endogenous LXR activator that itself induces changes through LXR has not been reported yet. Furthermore, LXR:RXR heterodimers were originally identified as mediators of an alternative retinoid signaling pathway (144), (145) showing that LXR:RXR heterodimer is highly permissive and can be activated from either the RXR side by retinoids or the LXR side by oxysterols (107). The possibility of retinoids to activate PPARγ:RXR and LXR:RXR dimers from the RXR side has also not been well characterized. A number of oxysterols were identified as potential endogenous ligands for LXR (111), (146), (147), (148). One of these compounds, 27-hydroxycholesterol is produced by a p450 enzyme CYP27. CYP27 is a mitochondrial enzyme representing an alternative bile acid synthesis pathway (149), (150), (151), (152), (153) and was reported to be expressed besides the liver in the lung and also in macrophages (154), (155). It has been also associated with atherosclerotic lesions (156), (157). A mutation in this enzyme leads to a human disease cerebrotendinous xanthomatosis (CTX), a rare sterol storage disease characterized by xanthomas in tendons and also in the CNS leading to ataxia, spinal cord paresis, neurological dysfuntions, normolipidemic xanthomatosis and accelerated atherosclerosis (158), (159), (160). The enzyme's product 27-hydroxycholesterol has been shown to activate LXR (106), (146). These data raised the possibility that CYP27 might serve as a regulator of LXR activity by generating ligand to it.

In the context of atherosclerosis macrophages' cholesterol metabolism is only one key question, other main issues are: the control of the absorption, metabolism of cholesterol in the liver and its distribution to peripheral tissues. All of these processes are tightly controlled by two nuclear receptors, the LXRs and FXR (161). Both of these receptors form permissive heterodimers with the RXR so perturbations in the level of expression and the activity of the RXRs can have dramatic effects on cholesterol metabolism. Administration of rexinoids to mice and hamsters can cause a complete block in the absorption of dietary cholesterol (107). This effect is due in part to disruption of the production of bile acids essential for normal cholesterol absorption and also for alterations in the absorptive properties of the intestinal epithelium. The former effect is due to the activation of hepatic RXR/FXR heterodimers by the rexinoids and the

consequent repression of the CYP7a1 and 7b1 hydroxylases that are critical for normal bile acid production (162). The effect on the absorptive properties of the intestinal epithelium reflect the activation of RXR/LXR heterodimers in the enterocytes and the induction of ATP-binding cassette protein 1 (ABC-1), a transport protein that facilitates the efflux of free cholesterol from the interior of the enterocyte back in to the intestinal lumen (163). It is the combination of these distinct pharmacologic responses that leads to the functional block in cholesterol absorption. The effects of rexinoids on cholesterol absorption provide an excellent example of how rexinoids, by regulating the activity of multiple nuclear receptor heterodimers, can generate integrated control of complex metabolic pathways in ways that are not replicated by individual nuclear receptor ligands. The ability of rexinoids to provide concerted induction and activation of both the PPAR and LXR signal transduction pathways was exploited by Auwerx and associates to demonstrate that an RXR agonist could be used to drastically reduce the development of atherosclerosis in an experimental mouse model (apoE null animals) (113). Both RXR and LXR agonists induced ABCA1 expression and stimulated ABCA1-mediated cholesterol efflux from macrophages from wild type, but not from LXRα and β double -/- mice suggesting that activation of RXR/LXR heterodimers contributes to the beneficial effects of rexinoids on atherosclerosis (113).

From these results it is clear know that the control of macrophage cholesterol influx and efflux are the major sites where regulation may occur. In the context of macrophages an alteration in the balance of the two processes and the subsequent overloading of the macrophages with lipids results in foam cell formation as an early step in atherogenesis. Analyzing nuclear receptors in macrophages revealed that PPARγ and LXRα have key functions in the regulation. Nevertheless, many questions remain to be answered about nuclear receptor activation. Our aim was to define some of these open issues and try to find answers to them.

2. SUMMARY AND QUESTIONS REMAIN TO BE ANSWERED

Many aspects of nuclear receptors' function in macrophages have been studied. As a conclusion one can see that there are predominant receptors in macrophages: RAR (mainly α and γ), RXR (mainly α and β), PPAR (mainly γ) and LXR (mainly α). The major processes regulated by RARs are likely to be related to differentiation. Little is known about RXR's role, though. It can be linked to RARs, PPARs and LXRs but there are pieces of evidence that indicate the existence of separate RXR activity. This obviously needs to be further characterized. Most data from recent years show that PPARγ is a key nuclear receptor in macrophages. PPARγ is the regulator of another nuclear receptor, LXR in these cells and besides the anti-inflammatory effects of its ligands PPARγ was also tied to an important macrophage function, the clearance of oxidized cholesterol. A cycle has been described with PPARγ in the center coordinating lipid traffic into and from the cells. Once lipids from oxLDL have activated PPARγ it leads to a feed-forward regulation and increased expression of scavenger receptors that leads to further oxLDL uptake. Second, it induces LXRα which if becomes activated induces reverse cholesterol transport, cholesterol efflux from the cells.
However, several questions remain to be answered. It was shown that activation of PPARγ leads to the induction of maturation markers but PPARγ *per se* is not necessary for macrophage differentiation. It is clear from other studies that RARs regulate myelopoiesis. How are these two receptors interconnected, if at all?
PPARγ was shown to be activated by oxidized fatty acid coming from oxLDL. What about LXR? How is it activated? Where does the ligand come from? LXR:RXR is also a highly permissive heterodimer. What is the role of retinoids in LXR activation?
From a functional point of view macrophages are heterogeneous. Does the activation state affect nuclear receptor regulated processes? If yes, how?
These are the questions we set out to answer.

3. AIMS

We wanted to analyze three potential interactions in details: 1. RAR-PPAR crosstalk in myeloid differentiation, 2. PPARγ-LXR crosstalk in macrophage lipid metabolism and 3. relationship between macrophage activation and PPARγ response.

1. Crosstalk between RARs and PPARs

RAR was reported to act as a differentiation checkpoint switch at the promyelocytic stage of granulopoiesis resulting in granulocytic differentiation as detailed above but recently it was more generally connected to myelopoiesis suggesting a role in the early common steps of granulopoiesis and monopoiesis and has been also implicated in later stages of monocytic development. We wanted to characterize the expression of PPARγ in various stages of development and analyze if retinoids modulate the maturation process and/or PPAR activity, PPAR-mediated maturation.

2. Crosstalk between PPARγ and LXR

PPARγ induces transcription of LXRα. But how is LXR activity regulated? Is it constitutively active by a huge excess of endogenous activators or is there a need for the synthesis of endogenous agonist? Our preliminary data suggested that endogenous ligand synthesis was required and we wanted to find how this happens and if it is regulated by PPARs or other nuclear receptors. First we identified similarly expressed genes in macrophages and compared these to the ones regulated by PPARγ. By global gene expression profiling we were able to find potentially interesting genes in ligand production that were further characterized.

3. Role of macrophage activation state in gene expression

Last but not least we were interested in the type of macrophages where PPARγ and/or LXR are present and active. Several studies have been reported about nuclear receptor-regulated processes so far but none of these analyzed nuclear receptor activity from the macrophage point of view. Therefore we differentiated monocytes to macrophages and activated these cells with various agents (cytokines or pathogen derivatives) to compare

the expression and function of nuclear receptors implicated in lipid metabolism to determine if the activation state of the cells can add a third dimension to the regulation of receptor activity besides the presence of receptors and the presence/production of their ligands.

4. MATERIALS AND METHODS

4.1. Materials

Cells were treated with the following ligands: AM580 (Biomol), LG268, a gift from R. Heyman (Ligand Pharmaceuticals), Wy14643, Rosiglitazone, T0901317 (Alexis Biochemicals), GW501516 and GW9662 were gifts from T. M. Willson (GlaxoSmithKline), 27-hydroxycholesterol, 25-hydroxycholesterol, 24S,25-epoxycholesterol, 22R-hydroxycholesterol (Steraloids Inc.), 9-hydroxyoctadecadienoic acid (Cayman Chemicals), LDL, oxidized LDL (Intracel), AGN193109, a gift from Roshantha A. S. Chandraratna (Allergan Inc.) or vehicle (ethanol-dimethyl-sulfoxide). All other reagents were obtained from Sigma or as indicated.

4.2. Plasmids

Mammalian expression vectors expressing CMX-hRARα, CMX-hRXRα, CMX-mPPARγ, β-galactosidase, TK-Luc were described previously (164). hCYP27-853-pCAT, hCYP27-649-pCAT, hCYP27-217-pCAT plasmids were constructed earlier (165). pcDNA3.1-hCYP27 was a gift from E. G. Lund (Merck Research Laboratories, Rahway, NJ).

4.3. Cell culture

KG-1, HL-60, THP-1 cells were obtained from ATCC (Manassas, VA). MonoMac-6 cells were kind gift of E. Duda (Biological Research Center, Szeged, Hungary). The cells were grown in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) or 10% charcoal-stripped serum (Invitrogen) or 10% lipoprotein-deficient serum (Intracel), 2mM glutamine, penicillin and streptomycin (Sigma) or without serum in the presence of insulin-transferrin-sodium selenite medium supplement (Sigma) or in AIMV (Invitrogen). Primary human fibroblast were isolated from healthy adults and patients with cerebrotendinous xanthomatosis as described earlier (166) and cultured in DMEM (Sigma) supplemented with 10% FBS, 2mM glutamine, penicillin and streptomycin.

4.4. Isolation and culture of human primary monocytes and CD34 positive cells

Human monocytes were isolated from healthy volunteer's buffy coat obtained from the Regional Blood Bank. Monocyte separation was carried out according to the manufacturer's instructions using CD14 MicroBeads (Miltenyi Biotec). Monocytes were differentiated for 2-4 days. Primary human cells were cultured in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, penicillin and streptomycin or as indicated.

Human CD34 positive stem cells were isolated with CliniMax (AmCell GmbH) from peripherial blood of granulocyte colony stimulating factor treated patients according to the protocol. Stem cells were expanded with recombinant human Flt-3L (25 ng/ml), stem cell factor (20 ng/ml), interleukin-6 (20 ng/ml) and interleukin-3 (20 ng/ml) for 10 days and then differentiated to macrophages with recombinant human M-CSF (10 ng/ml) for 8 days. All cytokines were purchased from Peprotech Inc.-Rocky Hill, NJ.

4.5. Flow cytometry

Analysis of cell surface expression of proteins was performed on a Coulter Flow Cytometer (Beckman Coulter Inc.-Fullerton, CA). Briefly, cells were washed in PBS (phosphate buffered saline) pH7.4 supplemented with 0.5% (BSA) bovine serum albumin then were incubated with antiCD14-RPE (Dako-Glostrup, Denmark), antiCD36-FITC (Beckton-Dickinson-Franklin Lakes, NJ) antibody, respectively for 1 hour at 4^{o}C, finally cells were washed in PBS-BSA and 10000 cells were counted on the cytometer.

4.6. OxLDL uptake

For oxLDL uptake experiments we used diI-labeled oxidized human LDL (Frederick, MD). Following two days of treatment with the indicated ligands cells were treated with 5µg protein/ml diI-oxLDL for 6 hours at 37^{o}C. Cells were washed and 10000 cells were counted by flow cytometer. Median values of the fluorescence intensities are shown. The experiment was repeated three times with similar results.

4.7. RNA extraction and real-time quantitative PCR

Total RNA was isolated from cells using Trizol Reagent (Invitrogen) according to the instructions. Atherosclerotic lesions were dissected off from underlying tissues and were homogenized. Control samples were obtained from the neighboring non-atherosclerotic part of the vessel wall. In case of the human samples lesion areas were dissected off abnormal, fatty not ulcering lesions of the femoral a., common and int. carotid a. After homogenization total RNA was extracted with Trizol Reagent. Transcript quantitation was performed by quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using Taqman probes. Transcript levels were normalized to the level of cyclophilin D and 36B4. Sequences of primers and Taqman probes used in transcript quantitation are listed in Supplemental Table at www.biochem.dote.hu/nagylab.

4.8. Microarray analysis

Total RNA was isolated using Trizol Reagent (Invitrogen) and further purified by using the RNeasy kit (Quiagen). cRNA was generated from 5 μg of total RNA by using the SuperScript Choice kit (Invitrogen) and the High Yield RNA transcription labeling kit (Enzo Diagnostics). Fragmented cRNA was hybridized to Affymetrix (Santa Clara, CA) arrays (HU95A) according to Affymetrix standard protocols. Data analysis was performed using GeneSpring 6.0 (Silicon Genetics, Redwood City, CA). These analyses provided a signal for each specific transcript that was subsequently normalized by comparing to the median signal (arbitrary value of 1.0) obtained from the whole array.

4.9. Northern blot analysis

Total RNA (10-15 μg) was separated on 1% agarose/formaldehyde gel electrophoresis and transferred to nylon membrane that was hybridized with radioactive [α-^{32}P]-dATP labeled DNA probes using Random Hexamer DNA Labeling Kit (Fermentas) and QuickHyb Hybridization Solution (Stratagene). Transcript levels were determined after overnight exposure to Kodak X-OMAT films and were normalized for 36B4 expression levels.

4.10. Western blot analysis

Cells were treated for two days as indicated and were washed in PBS then lysed in buffer A (Tris-HCl pH7.5, 1mM EDTA, 15mM β-mercaptoetanol, 0.1% Triton X 100, 0.5mM PMSF (phenyl-methyl-sulfonyl fluoride). 25µg total protein was separated on 10% SDS-PAGE (polyacrylamid gel electrophoresis) and transferred to PVDF membrane (Bio-Rad Laboratories). After blocking with 5% dry milk the membrane was probed with antiCYP27 antibody, a kind gift from Dr. D. W. Russell (University of Texas, Southwestern Medical Center, Dallas) or with anti-PPARγ antibody (Biomol) and subsequently with peroxidase-conjugated secondary antibody. ECL detection kit (Pierce) was used for signal detection.

4.11. Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out as described (167) with modifications. Briefly, cells were fixed with 1% formaldehyde for 10 min at room temperature. Fixation was stopped by adding chilled glycine to a final concentration of 150 mM. Cells were scraped and washed twice with ice-cold PBS that contained proteinase inhibitors (1mM PMSF, 1µg/µl aprotinin and 1µg/µl pepstatin A). Nuclei were prepared by incubation for 10 min on ice in a buffer containing 5mM Pipes pH8, 85mM KCl, NP40 0.5% and proteinase inhibitors. After centrifugation with 3000g for 10 minutes at 4 °C, nuclei were resuspended in sonication buffer (1%SDS, 0.1M $NaHCO_3$ and proteinase inhibitors), lysed on ice for 10 minutes and sonicated on ice to an average fragment size of 300 base pairs. Cell debris was pelleted twice by centrifugation with 10000g for 30min at 4°C in a bench-top centrifuge. Soluble chromatin was aliquoted and frozen in liquid nitrogen and stored on –70°C. For immunoprecipitation chromatin was diluted, 10-fold in an IP buffer (0.01%SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris pH8.1, 16.7mM NaCl and proteinase inhibitors). 1 ml of diluted chromatin was precleared twice with 40µl, blocked protein A/G-sepharose beads. Immunoprecipitation was carried out with specific antibodies purchased from Upstate Biotech (Anti Acetyl H4 2) and Santa Cruz (Anti PPARγ, Anti RXRα). Anti RARα was a kind gift of L. Tora (CNRS/INSERM/ULP Strasburg, France). Immunoprecipitated nucleosomes were eluted twice from beads with elution buffer (1% SDS, 0.1M $NaHCO_3$) and eluates were combined. Crosslinks were

reversed by incubating for 6 hours at 65°C after adding 20µl 5M NaCl. Eluate was combined with 10 µl of 0.5M EDTA, 20µl 1M Tris pH6.5 and 2µg Proteinase K and incubated for 1 hour at 45°C. DNA was recovered after phenol: chloroform extraction and ethanol precipitation using 20µg of glycogen as a carrier. DNA was resuspended in 50µl of 50ng/µl yeast tRNA (Invitrogen). 2µl of this solution was used for real-time Q-PCR in a 25µl reaction. All measurements were done in triplicates. All chromatin results were verified from independent chromatin preps.

4.12. Transient transfections and reporter gene assays

Human CYP27-CAT (chloramphenicol acetyl-transferase) promoter plasmids were constructed as described earlier (165). The following deletion constructs were used and co-transfected with the indicated full-length receptors: hCYP27-853-pCAT, hCYP27-649-pCAT, hCYP27-217-pCAT relative to the translational start site, +1. The response elements were cloned into TK-Luc to obtain Enhancer trap vectors that were transfected along with the indicated receptors. All transfection experiments were performed in CV1 cells (Green monkey kidney fibroblasts) as described earlier (94).

4.13. Electromobility shift assays

Electromobility shift assays were performed as described earlier (168). Full-length hRARα, hRXRα and mPPARγ receptors were produced using the T7 Quick TNT *in vitro* Transcription/Translation Kit (Promega). For supershift experiments the receptors were preincubated with the indicated antibody prior to binding reaction: anti-RAR (Santa Cruz), anti-RXR (a kind gift from L. Tora (CNRS/INSERM/ULP Strasburg, France), anti-PPARγ (Biomol). The oligonucleotides that were used are listed in Supplemental Table at www.biochem.dote.hu/nagylab.

4.14. Determination of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid

Levels of 27-oxygenated sterols were determined as described previously (154). Briefly, $1x10^6$ cells were treated as indicated and 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid content was measured by quantitative mass spectrometry with use of

deuterium-labeled 27-hydroxycholesterol and 3β-hydroxy-5-norcholestenoic acid as internal standards, respectively.

4.15. Determination of retinoid levels

Retinoid levels were determined by high-performance liquid chromatography (HPLC) as described previously (169).

4.16. Statistical analysis

All data are presented as means ±SD. In real-time quantitative PCR and transient transfection experiments the mean and standard deviation were calculated for both the normalized and the normalizer values. To incorporate the random errors of the measurements we used the propagation of errors to determine the standard deviation of the normalized values. For all experiments we made at least four biological replicates and on the fold changes we performed an F test followed by an unpaired (two tail) t test and results were considered significant with $p<0.01$.

5. RESULTS

5.1. Retinoids potentiate PPARγ response in differentiation, gene expression and lipid metabolic processes in developing myeloid cells

Here we show that PPARγ is the most highly induced nuclear receptor in differentiating myeloid cells and its expression increases parallel with the degree of maturation. Moreover, activation of PPARγ contributes to subsequent differentiation in the monocytic pathway. We have also found a crosstalk between retinoid and PPARγ signaling: retinoids potentiate developing cells' response to PPARγ activators. This crosstalk represents a novel convergence of the two signal pathways important in the maturation of myeloid precursors and suggests new opportunity for regulating PPARγ-related metabolic processes.

5.1.1. PPARγ is the most highly induced nuclear receptor during macrophage development

We compared the expression of PPARα, γ and δ with real time quantitative PCR in primary human CD34 positive cells after isolation and following differentiation with M-CSF to primary human macrophages for eight days. Surprisingly, we found a significant difference in the mRNA expression profile in the various conditions (Figure 5A).

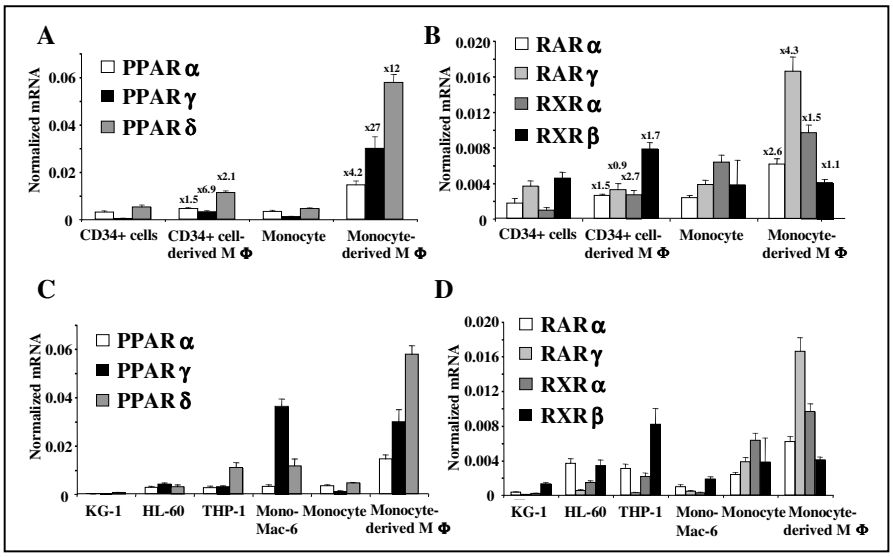


Figure 5: PPARγ is the most highly induced nuclear receptor in macrophage development. Normalized transcript levels are shown.

During the maturation of stem cells we could detect a 7-fold induction of PPARγ with minor increase in PPARα and δ levels. Similarly, in the monocyte/macrophage transition PPARγ induction was the highest (27-fold) and PPARα and δ were induced to a lesser degree however these changes were greater than those observed in stem cells. We also measured the expression levels of RARs and RXRs (Figure 5B). All of these receptors were expressed at levels lower than PPARs. RXRα showed the biggest (almost 3-fold) change in the CD34 positive cell-derived macrophages compared to the CD34 positive cells while we could detect a 2.6-fold and a 4.3-fold induction of RARα and RARγ, respectively during monocyte/macrophage transition. The other retinoid receptor levels were not changed or the changes were below 2-fold. RARβ and RXRγ could not be detected in these cells.

As a model of the different stages of myeloid differentiation we chose myeloid leukemia cell lines: KG-1 representing acute myelogenous leukemia, HL-60 representing acute promyelocytic leukemia (FAB M3), THP-1 (FAB M5) and MonoMac-6 (FAB M5) representing two monocytic leukemia cell lines. MonoMac-6 proved to be most committed to the monocytic lineage characterized by its increased phagocytic capacity, chemotactic potential, cytokine production and cell surface expression of monocytic markers (170), (171). We determined the absolute mRNA copy numbers of PPARs, RARs and RXRs expressed in these cells and compared to the levels of monocytes and macrophages (Figure 5C and D). PPARs are expressed at high levels in these cell types except the least matured KG-1 cells. PPARα level is similar in the other three cell lines, PPARδ is higher in the two monocytic leukemia cell lines and PPARγ mRNA is the most abundant in the most matured MonoMac-6 cells reaching the level of that of the macrophage's (Figure 5C). RARα, RXRα and β are expressed in the two differentiated cell lines (HL-60 and THP-1) and in monocytes-macrophages at the highest level and interestingly, only at low levels in the least differentiated KG-1 cells. RARγ level in the cell lines is lower than that in monocytes. These data suggested that monocyte/macrophage differentiation was accompanied by an induction in PPARγ and at a lesser degree of PPARδ levels. The significant induction in PPARγ levels raised the possibility that activation of the receptor may be part or contribute to the maturation

process. Expression of retinoid receptors did not show such a change during maturation but their presence indicated possible roles for these receptors in macrophage development. These data also suggested that the leukemic cell lines are likely to serve as an appropriate and representative model for studying the contribution of varying PPARγ levels to myeloid cell differentiation and function.

5.1.2. Activation of PPARγ induces expression of macrophage markers

To characterize the role of PPARγ in these cells we activated them with a synthetic agonist, Rosiglitazone and analyzed the effects on monocytic differentiation and on activation of target gene transcription. In the least differentiated cell line (KG-1) activation resulted in no significant changes in cell surface markers (data not shown) which was probably due to the very low level of the receptor expressed in these cells. In the most matured MonoMac-6 cells activation of the PPARγ:RXR heterodimer with synthetic agonists, Rosiglitazone for PPARγ and LG268 for RXR, induced the expression of CD14, CD36 differentiation markers on the cell surface (Figure 6A, B). In Figure 6C and D we show that the induction was caused by changes in mRNA levels and a synergy between Rosiglitazone and LG268 could also be observed at the mRNA expression level. These results are in agreement with our previously reported findings on THP-1 cells (93), (94) with one important difference:

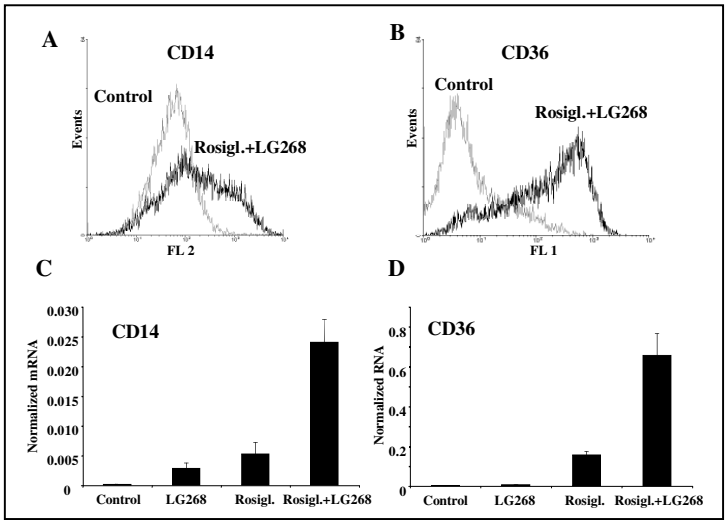


Figure 6: PPARγ activation induces expression of macrophage differentiation markers. Cell surface expression of proteins and normalized transcript levels are shown.

in the case of MonoMac-6 cells phorbol-12-myristate 13-acetate (PMA) pretreatment was not necessary to obtain PPARγ responses unlike in the case of THP-1 cells. This phenomenon can be explained by the fact that PPARγ level, which is induced upon PMA treatment (data not shown) (93), (94), was high enough in the MonoMac-6 cells (Figure 5C), higher than in THP-1, to ensure a sufficiently high level of the receptor.

5.1.3. Retinoid pretreatment enhances PPARγ agonist induced PPAR response

These data suggested that activation of PPARγ:RXR heterodimer enhanced differentiation of cells in the monocytic pathway and also supported that this process could occur only if the cells were in a permissive stage of their maturation (cells need to be committed in the monocytic pathway but not fully differentiated yet) and expressed sufficient amount of PPARγ. Our next question was how one could prime the cells to enter this final differentiation stage and regulate PPARγ expression levels in order to become matured cells and acquire a full capacity to induce transcription of PPARγ target genes that regulate lipid homeostasis. There is extensive evidence suggesting that retinoids play important roles in the early developmental processes of hematopoietic cells (61), (63). We tested whether they induced myeloid differentiation. On one hand we found that RAR agonists caused maturation only in the least differentiated cells (KG-1, HL-60) examined directing them towards the granulocytic lineage but we have found no evidence that retinoids could induce monocyte/macrophage differentiation in these cell lines (data not shown). On the other hand, RXR specific agonists caused similar effects than PPARγ activators (Figure 6C), suggesting that RXR specific ligands might activate and function through PPARγ:RXR heterodimers. Very surprisingly, combination of RAR and PPARγ activators resulted in an unexpected synergy on PPARγ induced gene expression. In the experiments we used sequential treatment (retinoids followed by PPARγ activators), because we reasoned that it probably represented a more physiological setting (i.e. previous reports propose a role for retinoids in the earlier steps of myeloid cell differentiation (61), (63)). When we treated the MonoMac-6 cells sequentially first with RAR agonists and then with PPARγ agonists we observed an

increased effect of PPARγ specific ligands on gene expression of CD14 and CD36 (Figure 7A and B), the latter one is a direct target gene of PPARγ:RXR heterodimers.

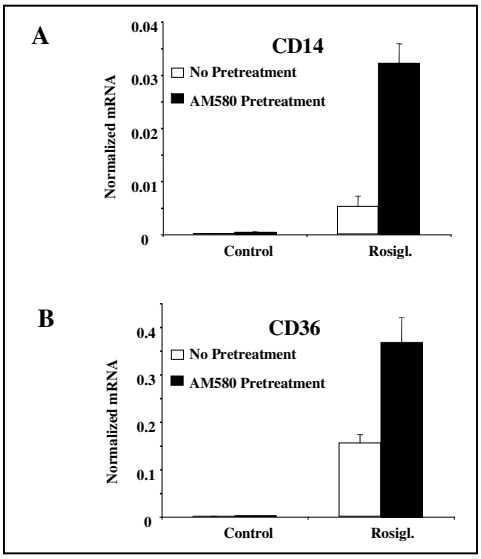


Figure 7: Retinoid pretreatment enhances PPARγ agonist induced PPAR responses. Normalized transcript levels are shown.

We could show that this phenomenon was present in both THP-1 and MonoMac-6 cells (Figure 8) but not in the least matured KG-1 and HL-60 cells (data not shown). The cells were treated first with receptor selective retinoids (RAR□ or RXR specific agonists), the combination of both or natural retinoids (ATRA or 9-*cis* RA) for 48 hours followed by a vehicle or Rosiglitazone treatment for an additional 48 hours. We characterized this retinoid-evoked potentiation of PPARγ response by measuring the induction of various PPARγ target genes: fatty acid-binding protein 4 (FABP4) (172) (Figure 8A, B), CD36 (93), (94) (Figure 8C, D), adipose differentiation-related protein (ADRP) (173), (174) (Figure 8E, F) and PPARγ angiopoietin-related protein (PGAR) (175) (Figure 8G, H). Rosiglitazone readily induced (see bars C/Rosigl. in the figure) target gene expression in MonoMac-6 cells in the cases of FABP4 (x42), CD36 (x5) and PGAR (x150) and in THP-1 cells in the case of FABP4 (x43) and PGAR (x3). We also noted that all retinoids induced CD36 expression in both cell lines (Figure 8C and D). ATRA caused potentiation in both cell lines of all the measured target genes: the fold inductions are x213, x19, x3, x150 compared to x42, x5, x0.7, x150 respectively in MonoMac-6 cells in

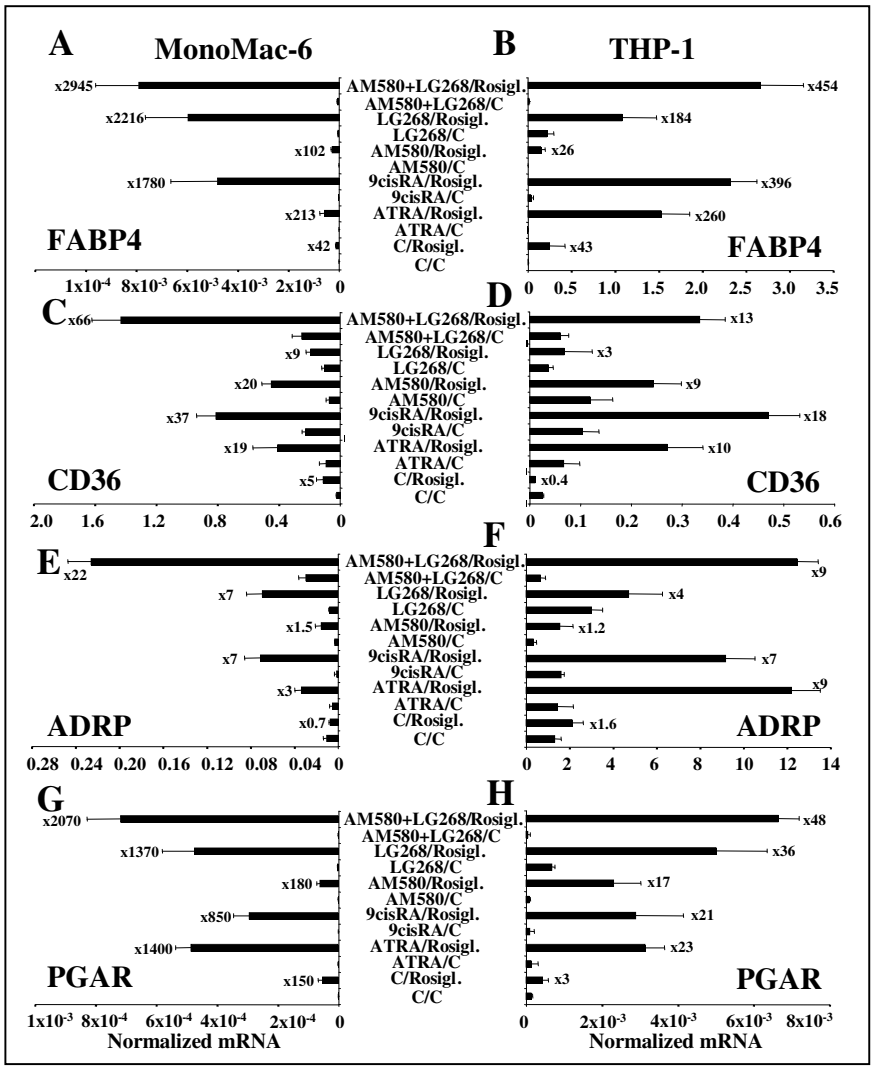


Figure 8: Retinoids potentiate PPARγ responses as shown by mRNA levels of four target genes in two cell lines.

the case of FABP4 (A), CD36 (C), ADRP (E) and PGAR (G). THP-1 cells behaved similarly (B, D, F, H). The effect of 9-*cis* RA was similar to ATRA with even higher inductions. AM580 was less effective than ATRA but also showed enhanced induction with Rosiglitazone (e.g. see (A) x102 compared to x42, (C) x20 compared to x5, (D) x9 compared to x0.4, (H) x17 compared to x3). LG268 pretreatment potentiated FABP4, ADRP, PGAR and CD36 expression induced by Rosiglitazone. Synergy was particularly striking in MonoMac-6 cells for FABP4 and PGAR probably due to the fact that these genes are not induced by LG268 alone. Some of the effects may be explained by remnant

LG268 binding to RXR in heterodimers and synergy with Rosiglitazone. The highest inductions could be observed in the case of AM580+LG268 pretreatments.
Probably the most striking and unexpected effect was that AM580, an RARα selective compound could readily potentiate Rosiglitazone-induced CD36 expression in both cell lines (C and D) (x20 compared to x5 and x9 compared to x0.4), FABP4 (A) (x102 compared to x42) induction in MonoMac-6 and PGAR (H) (x17 compared to x3) induction in THP-1 cells. Significantly, pretreatment of cells with either the pan-retinoid receptor agonist 9-*cis* RA or a combination of AM580 and LG268 followed by Rosiglitazone produced the highest level of target gene expression in both cell types for all four target genes. These results clearly indicated that retinoid pretreatment although differentially affected the two cell types and the four target genes as a general rule potentiated PPARγ responses. A significant component of this potentiation is induced by RARα selective retinoids.
Next we wanted to know how long the pretreatment was necessary for the potentiation to develop. We found that 6 hours of pretreatment was sufficient for the enhancement of PPARγ response (data not shown).

5.1.4. Retinoids induce transcription of PPARγ mRNA

Obviously, there are many potential mechanisms to account for this effect ranging from epigenetic changes to changes in cofactor and receptor levels. We tested the most obvious, the levels of PPARs. We measured PPAR mRNA levels and found that both natural and synthetic RAR and RXR agonists induced PPARγ mRNA levels while PPARα and δ mRNA levels were unaffected in both THP-1 and MonoMac-6 cells (Figure 9A and B). We also found that this transcriptional activation of PPARγ resulted in an elevation in the protein level (Figure 9C). Interestingly, retinoids proved to be as effective as PMA in the induction of PPARγ. We note here that unlike PMA retinoids do not induce cellular differentiation *per se* in these cells therefore this effect is unlikely to be differentiation related. In order to obtain data from cells of *ex vivo* origin we examined monocyte-derived macrophages. We found a similar induction of PPARγ transcription in

primary human monocyte-derived macrophages when treated with 9-*cis* RA, while Rosiglitazone had

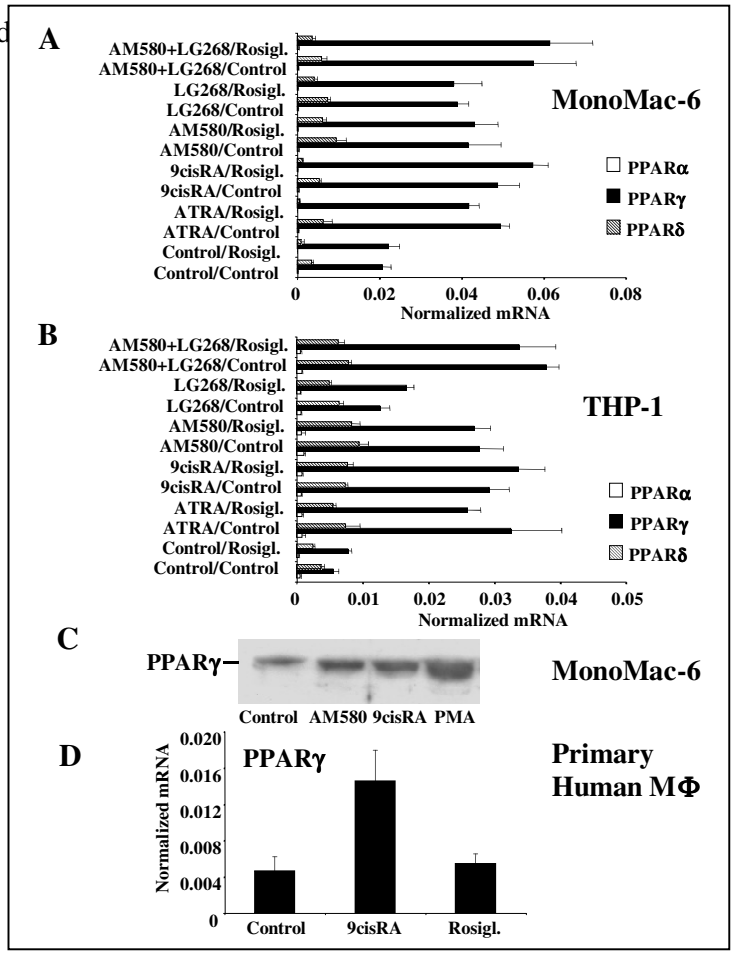


Figure 9: Retinoids induce PPARγ mRNA (A, B, D) and protein (C).

5.1.5. Retinoid-induced PPARγ response potentiation enhances oxLDL uptake

Finally we sought to obtain evidence for the biological consequence of retinoid enhanced PPARγ response. Therefore, we measured the uptake of diI-labeled oxLDL in retinoid-pretreated MonoMac-6 cells after activation with PPARγ agonists (Figure 10).

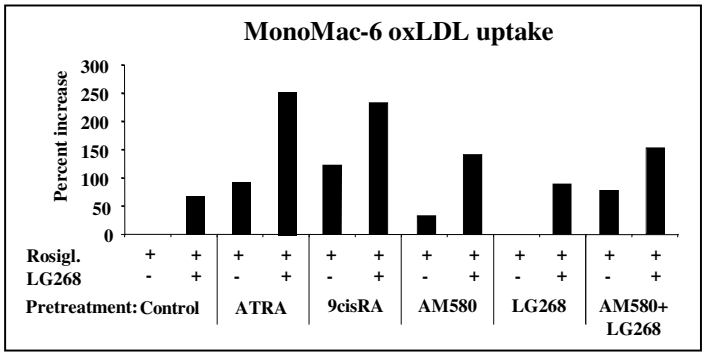


Figure 10: Retinoids enhance Rosiglitazone-induced oxLDL uptake. Percent increase is shown measured with flow cytometer using diI-labeled oxLDL.

Inter :one-induced oxLDL uptake only in the presence of an RXR agonist in control cells while retinoid-treated cells and only those that were treated with RAR agonists became capable of taking up oxLDL after PPARγ agonist treatment alone. These data suggest that suboptimal class of PPARγ activators could be brought up to effective levels with retinoid pretreatment. The PPARγ and RXR agonists induced level of uptake was also increased in retinoid-pretreated cells. These results showed that retinoid pretreatment not only induced increased transcription of PPARγ target genes but also facilitated oxLDL uptake into the cells providing a potentially new target for the modulation of cholesterol-eliminating machinery.

5.2. Transcriptional regulation of human CYP27 integrates retinoid, PPAR and LXR signaling in macrophages

5.2.1. Human CYP27 is acutely regulated by retinoids and PPARγ agonists

The other question we wanted to answer was how LXR got activated in macrophages and what the contribution of PPARγ to LXR activity and LXR-induced cholesterol efflux is. As we show above PPARγ is highly induced in monocyte/macrophage transition. In order to identify the common elements of the transcriptional changes during monocyte-macrophage transition and that of PPAR:RXR heterodimer activation we have carried out DNA microarray analysis on monocytic leukemia cells (MonoMac-6) treated with PPARγ (Rosiglitazone) and the RXR selective retinoid LG268 and also on primary human monocytes induced to differentiate to. As part of these global gene expression profiling efforts we found that transcript levels of the p450 enzyme CYP27 increased in monocyte-derived macrophages and also in PPARγ and RXR ligand treated myeloid cells along with several other genes associated with lipid metabolism (data not shown) suggesting that these genes may be under PPARγ and/or retinoid regulation. We found CYP27 the most interesting because its product 27-hydroxycholesterol was previously

shown to activate LXR and CYP27 had been connected to atherosclerosis on multiple levels before. In order to provide a baseline for further studies we determined the transcript levels of various nuclear receptors (PPARs, retinoid receptors and LXR) during monocyte-macrophage transition. Comparing the relative abundance of nuclear receptor transcript levels by quantitative PCR we found an increased PPARγ and LXRα level (Figure 11A, B). Strikingly, it was paralleled by an increase in CYP27 transcript levels (Figure 11C), the enzyme with the potential to produce a soluble cholesterol metabolite and a ligand for LXRα.

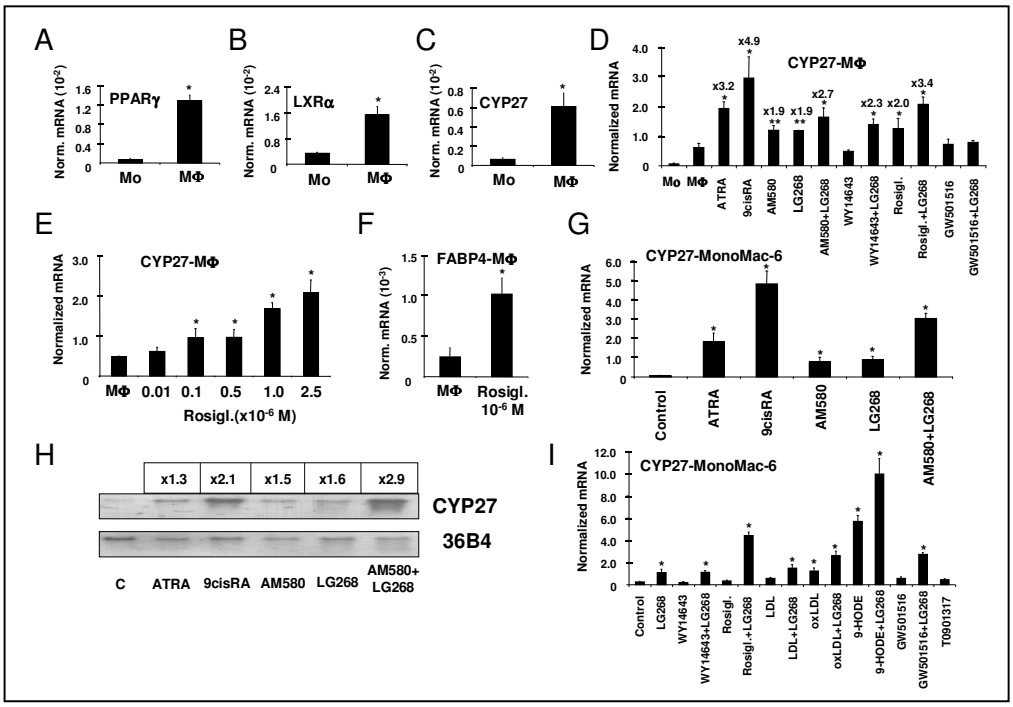


Figure 11: Retinoids and PPARγ induce CYP27 expression as shown by normalized mRNA levels and Northern blot.

It was intriguing to speculate that there was a link between the well-established PPARγ-LXR signaling and induction of CYP27 in macrophages. Therefore, we decided to determine if ligand activation of nuclear receptor pathways could activate CYP27 gene transcription. Using monocyte-derived macrophages and the monocytic leukemia cell line (MonoMac-6) we tested the effect of RAR, RXR, PPAR and LXR specific ligands on the expression of CYP27. The results of Northern blot and RT Q-PCR analyses are presented in figures 11D-I. We found that retinoids and activators of PPARγ could induce the

transcript levels of CYP27 to high levels in human macrophages (Figure 11D, E). To further substantiate the Rosiglitazone induction a dose-response experiment was carried out (Figure 11E) showing that CYP27 is induced by Rosiglitazone in a dose dependent manner similarly to the bone fide target gene fatty acid binding protein (FABP4/aP2) (172) (Figure 11F). In MonoMac-6 cells this represents the induction of a single transcript (~2 kb) (Figure 11H and Figure 12A). Compounds with RAR selectivity (such as all trans retinoic acid (ATRA), AM580) and also one with RXR selectivity (LG268) proved to be effective inducers and we could detect an increased induction upon treatment with the PPARγ specific Rosiglitazone in combination with the RXR specific LG268 (Figure 11G, H, I). In MonoMac-6 cells the retinoid induction profile of CYP27 was very similar to that of macrophages (Figure 11G). Among all the PPARγ activators tested interestingly, only oxidized LDL and its lipid component 9-hydroxyoctadecadienoic acid proved to be active on there own in MonoMac-6 cells (Figure 11I). Rosiglitazone induced CYP27 mRNA expression only in combination with the RXR selective LG268. This profile is very similar to the one reported by us on the regulation of CD36 in another myeloid leukemia cell line THP-1 (93). Significantly, neither PPARδ, nor LXR selective ligands induced CYP27 in this cell type. PPARδ agonist showed an at least additive or synergistic response when combined with LG268 (Figure 11I). In order to gain insight into the mechanism of induction, we next wanted to test if this induction with PPARγ agonists and retinoids was acute or required prolonged exposure to the compounds. As shown in figure 12A Northern blot analysis revealed that both retinoids (AM580 in this case) and combination of PPARγ and RXR agonist induced an acute (after 2-6 hours) induction of CYP27 transcription. These experiments strongly suggested that RAR:RXR and PPARγ:RXR heterodimers can transcriptionally regulate the human CYP27 gene. These observations were further substantiated by carrying out time-course experiments using RAR and RXR selective compounds. As shown in figure 12B both the RAR□ selective AM580 and the RXR selective LG268 induced CYP27 gene expression, whilst the combination of the two or the pan-agonist 9-*cis* RA proved to be more effective inducers of gene expression. The efficacy of ATRA induced expression has fallen in between the receptor selective compounds and the pan-agonists indicating

that perhaps some of ATRA is converted into pan or RXR agonist compounds during the course of the experiment.

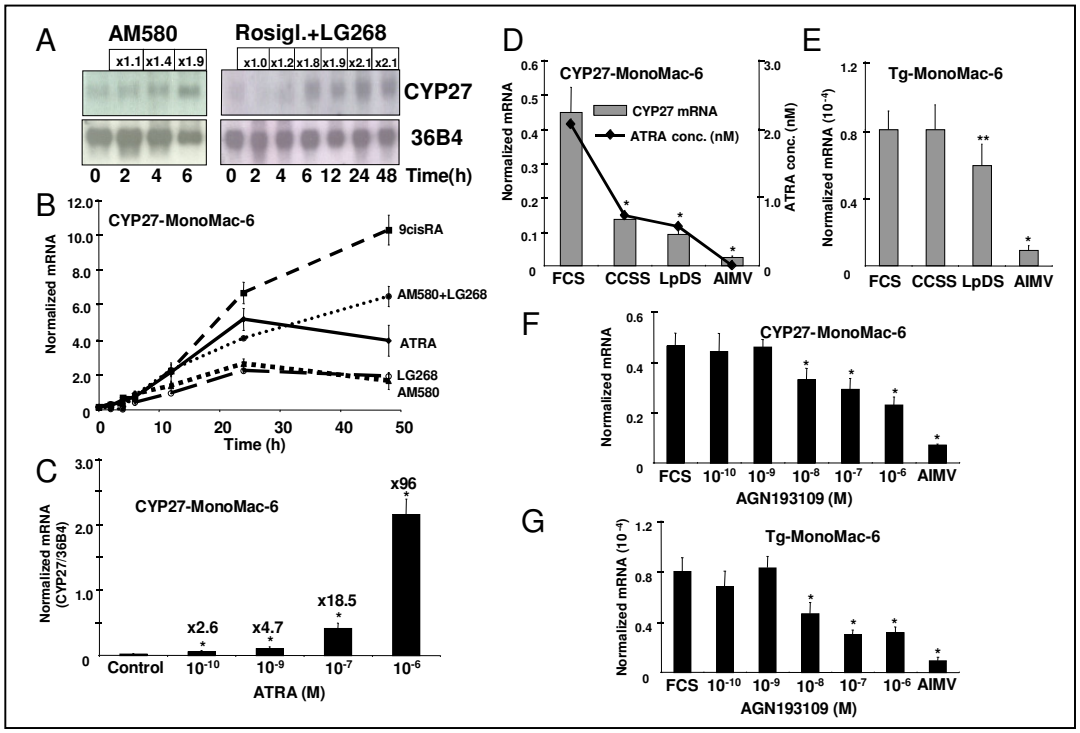


Figure 12: Analysis of CYP27 mRNA induction. Normalized transcript values and Northern blot (A) are shown.

In order to establish that the induction is dose dependent and induced with doses biologically relevant for receptor activation dose responses experiments were carried out. As it is shown in figure 12C ATRA induced CYP27 expression in a dose dependent manner in the range of 0.1 nM to 1 μM. These experiments established that CYP27 is acutely regulated by retinoids in a dose and time dependent manner and the doses required for activation were in the same range as the compounds K_ds for the receptors. This raised the possibility that endogenous retinoids might regulate CYP27 expression also. We compared the expression levels of human CYP27 and a known retinoid receptor target gene tissue transglutaminase (Tg) (176) in MonoMac-6 cells cultured in four different media (FBS, Charcoal stripped, lipoprotein deficient or serum free) in which we determined endogenous ATRA concentrations. We plotted then mRNA levels of CYP27 along with ATRA levels (Figure 12D), which showed a remarkable correlation. The more

ATRA detectable in the serum, the more transcripts were detectable. A similar profile was obtained for tissue transglutaminase expression (Figure 12E). Retinoic acid receptor levels did not change under the conditions used (data not shown). Obviously other differences may exist between the different sera therefore in order to obtain further evidence on the role of endogenous retinoids in the induction of CYP27 we used a well-characterized RAR antagonist/inverse agonist (AGN193109) to block activation through endogenous retinoic acid receptors. Increasing amount of antagonist decreased the expression level of CYP27 in serum (FBS) containing medium by 50-60% (Figure 12F). Similarly, tissue transglutaminase expression was also reduced (Figure 12G). These two pieces of evidence although did not prove but strongly suggested that normal serum levels of endogenous retinoids are likely to contribute to the regulation of CYP27 in myeloid cells and established human CYP27 as a gene regulated by endogenous levels of retinoids.

5.2.2. RAR:RXR and PPARγ:RXR heterodimers bind to and activate the human CYP27 promoter

Our findings prompted us to look at the previously defined promoter region of human CYP27 (165). Using an 853 bp fragment we carried out transient transfection experiments asking if receptor heterodimers can activate this promoter. Co-transfection of RAR and RXR receptors and addition of 9-*cis* RA induced a more than 5-fold increase in promoter activity (Figure 13A). Similarly, co-transfection of PPARγ and RXR in the presence of both PPARγ and RXR specific ligands increased transcriptional activity (Figure 13A and data not shown). These results indicated that the 853 bp fragment contained all the necessary information to mediate both retinoid and PPARγ-dependent regulation. To define the response elements we looked at various deletion mutants of the 853 bp fragment (a 649 bp and a 217 bp fragment) and found that the detected induction was approximately half of that of the 853 bp promoter fragment (Figure 13B) when a shorter fragment was used (649 bp). An additional deletion (217 bp fragment) led to retained core promoter activity and to the complete loss of inducibility.

These experiments suggested that transcriptional regulation takes place on the human CYP27 promoter by RAR:RXR and PPARγ:RXR heterodimers and raised the possibility

that more than one response element may be localized between -853 and -217 bp. In silico analysis of the promoter identified two regions with potential binding sites for RAR:RXR and PPARγ:RXR heterodimers. We termed these PRRE (Peroxisome Proliferator Activat B.

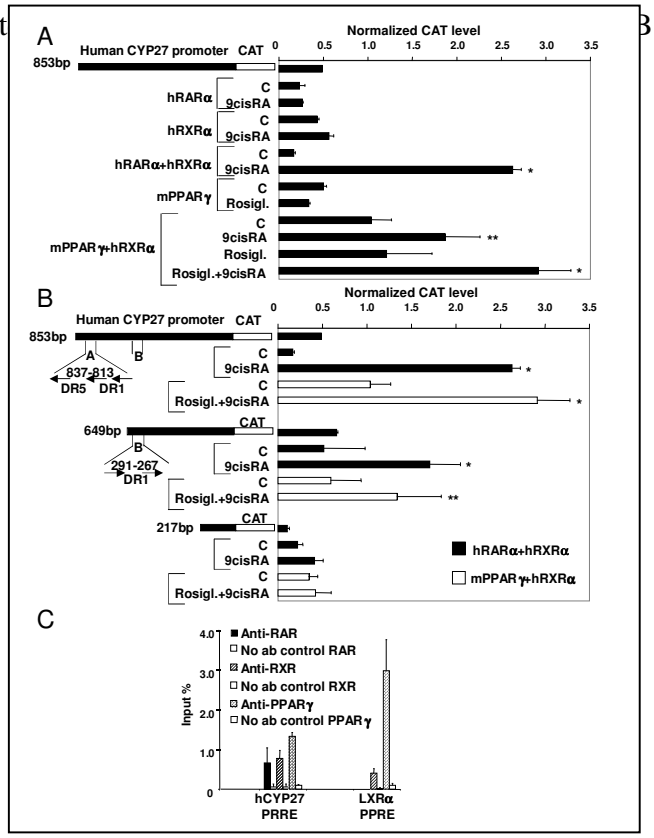


Figure 13: Promoter analysis of human CYP27 gene. Retinoids and PPARγ activates CYP27 promoter measured by CAT-reporter activity (A, B). RAR, RXR and PPARγ bind to hCYP27 PRRE as shown by chromatin immunoprecipitation (C).

The A element contained an unusual arrangement of binding sites, three binding sites arranged in an overlapping DR1-DR5 configuration (i.e. two direct repeats sharing a half site). The B element looked simpler, it was a direct repeat separated by one nucleotide (DR-1).

Before embarking on a detailed promoter analysis we wanted to see if the promoter was transcriptionally active in myeloid cells and if endogenous levels of receptors were bound to the identified elements. We have carried out chromatin immunprecipitation experiments first to assess the acetylation status of histone 4 lysines. This would provide direct evidence for promoter activation. Both 9-*cis* RA and combination LG268 and

Rosiglitazone induced significant acetylation of the promoter (data not shown). Next we used receptor specific antibodies to immunoprecipitate chromatin with RARα, RXRα and PPARγ from ligand treated MonoMac-6 cells. TaqMan probes were designed for PRRE-A and PRRE-B and the LXRαPPRE as a positive control to quantitate reliably immunoprecipitated genomic DNA. Both elements could be precipitated with the PPARγ antibody, whilst the RARα and RXR antibody readily immunoprecipitated the B element and to a much lesser degree the A element. The LXR-PRRE was precipitated with the anti RXR and PPARγ antibody. Collectively the transfection and immunoprecipitation data established that the identified region of the promoter was transcriptionally active and the B element could be important in the regulation of the gene, whilst the A element might have an accessory role. Therefore we continued with the characterization of the B element.

5.2.3. Characterization of a response element mediating retinoid and PPARγ signaling

In order to test if the identified element could indeed bind receptor heterodimers *in vitro* we carried out Electrophoretic Mobility Shift Assays (EMSA). It is shown in figure 14A that the major element (hCYP27-PRRE-B) has a DR-1 arrangement. EMSA analysis revealed that this DR-1 is able to mediate both PPARγ:RXR and RAR:RXR binding (Figure 14A). As shown in figure 14A neither PPARγ nor RXR alone was able to bind to the labeled oligonucleotide, the two receptors together show strong specific binding, which can be effectively competed by either unlabeled self (S) or a canonical DR1 oligonucleotide (Figure 14A, left panel). The specificity of binding was demonstrated by the use of antibodies against RXR and PPARγ inducing supershifts or reduced binding, respectively (Figure 14A). A similar set of experiments were carried out using RAR:RXR heterodimers. As shown in figure 14A, right panel RXR:RAR heterodimers can bind to hCYP27 PRRE and this binding can be competed by self and canonical DR5 elements. RAR:RXR and PPARγ:RXR can bind to the same element utilizing a DR1. We have established a transfection-based assay for the determination of receptor binding independent of transactivation in CV-1 cells in which the transfection experiments were carried out. By using VP16 fusion receptors (ligand-mimic receptors) and transient

transfection in figure 14B we could show a strong and robust binding of the PRRE-B with VP-RXR and VP-RXR and PPARγ. To prove that the identified response element is a functional enhancer, enhancer trap vectors were constructed fusing two copies of the element to a minimal TK promoter and a luciferase reporter gene.

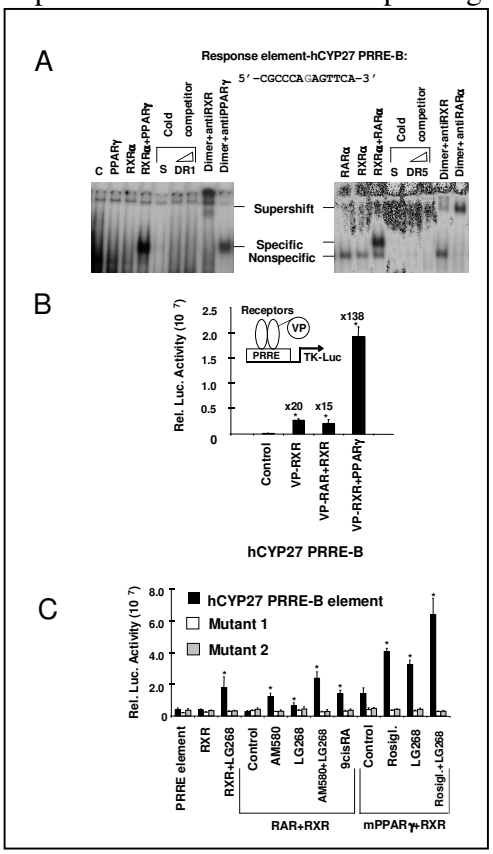


Figure 14: hCYP27 PRRE-B binds strongly RAR:RXR and PPARγ:RXR heterodimers. Electromobility shift assays are shown in (A). Enhancer trap reporter construct was used to show activation (B) and binding (C) in transient transfection experiments.

Co-transfection analysis revealed that the identified element conferred both RAR:RXR and PPARγ:RXR heterodimer responsiveness (Figure 14C). Similar experiments carried out with mutations in either of the two halfsites resulted in a complete loss of induced transcription.

We have also analyzed the other *in silico* found response element (hCYP27-PRRE-A). There appears to be a discrepancy between the *in vitro* binding and the apparent enhancer activity of this element because it shows strong *in vitro* binding, but only weak enhancer activity (data not shown). Therefore the role of the A element may be cell type specific or more likely part of a more complex enhancer interaction in the context of the full

promoter. Based on these data the B element (hCYP27-PRRE-B) appeared to be critical in mediating transcriptional activation with a potentially minor contribution of a weak, accessory element (hCYP27-PRRE-A). Identification and analysis of this element provided evidence for direct regulation and a potentially complex interplay between retinoid and PPARγ signaling on the human CYP27 promoter.

5.2.4. Retinoid- and PPARγ-induced CYP27 expression results in 27-hydroxycholesterol formation and efflux

Having established that human CYP27 gene is transcriptionally regulated by two nuclear receptor heterodimers (RAR:RXR and PPARγ:RXR) next we wanted to see what the biological consequences of this regulation were. We carried out Western blot analysis in MonoMac-6 cells treated with natural retinoids (ATRA or 9-*cis* RA). Protein expression is increased as demonstrated by the appearance of the expected single 59 kDa band (Figure 15A insert). In order to establish that the protein is functional we carried out mass spectrometric determination of the metabolic products of CYP27 from cell pellets and media supernatants. The determined compounds were 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid.

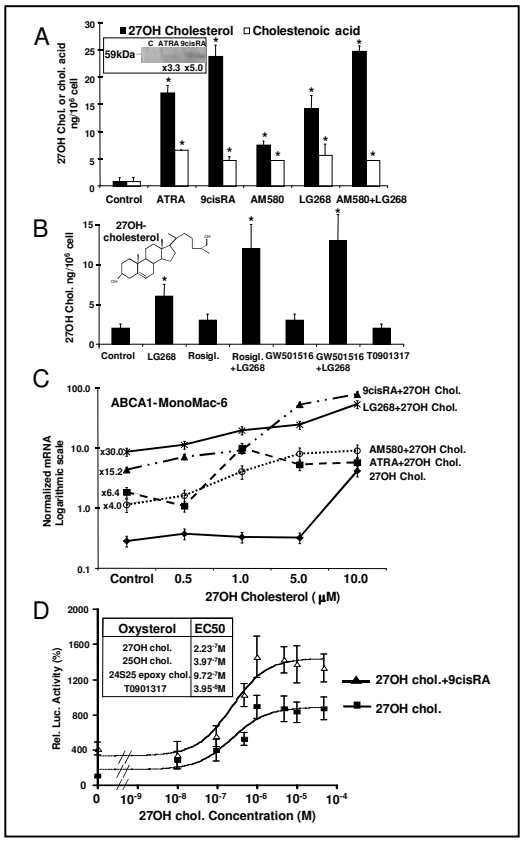


Figure 15: Induction of CYP27 results in increased protein level and activity. 27-hydroxycholesterol levels were measured by mass spectrometry (A, B). 27-hydroxycholesterol activates LXR as shown by induction of LXR target gene, ABCA1 mRNA levels (C) and by transfection-based reporter assay.

Mon

simultaneously loaded with cholesterol. After two days 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid levels were determined in the supernatant and cell pellet. As it is shown in figure 15A and B treatment with naturally occurring retinoids ATRA and 9-*cis*RA as well as synthetic ligand for RAR (AM580) or RXR (LG268) induced 27-hydroxycholesterol formation and release to the medium (data not shown). Combination of RAR and RXR ligands, PPARγ and RXR or PPARδ and RXR ligands also induced this effect. Neither Rosiglitazone nor a synthetic PPARδ ligand (GW501516) was able to induce 27-hydroxycholesterol formation on its own. Ligands for LXR alone or in combination with RXR ligands failed to induce it at all. In molar terms the supernatant contained 20-50 nM 27-hydroxycholesterol whilst the highest intracellular levels deduced from the pellets were approximately 12-20 μM. It is unsettled if high levels of 27-hydroxycholesterol is able to activate LXR:RXR heterodimers to sufficiently high levels. The fact that retinoids were able to induce the enzyme's expression and also to synergize with the enzyme's product offered a testable hypothesis. It is possible that under some conditions both retinoids and 27-hydroxycholesterol are required for full activation of LXR:RXR heterodimers. We tested this hypothesis by looking at LXR-dependent gene expression induced by retinoids and 27-hydroxycholesterol. As it is shown in figure 15C LG268 and 9-*cis* RA synergizes with 27-hydroxycholesterol to induce gene expression to a very significant degree. Note that 27-hydroxycholesterol at 10 M concentration was able to induce a 14-fold induction whilst if combined with 9-*cis* RA the induction was further induced with an additional 19-fold resulting in a nearly 300-fold induction altogether. These data suggest that retinoids via the induction of a partial agonist of LXR gain competence to induce robust LXR-dependent transcription. This notion was further substantiated by determining the EC_{50} values for 27-hydroxycholesterol alone and in combination with 9-*cis* RA in better defined transient transfection experiments. Figure 15D shows that 27-hydroxycholesterol is capable to induce LXR dependent gene

expression with an EC_{50} of $2x10^{-7}$ M and addition of 9-*cis* RA increased the efficacy of the response. EC_{50}'s of other oxysterols were also determined (Figure 15D insert) for reference in the same assay. These fall in the same range as that of 27-hydroxycholesterol.

5.2.5. Retinoids induce LXR-mediated gene expression which involves activation of CYP27

After having established that retinoids and PPARγ ligands induce CYP27 expression and this leads to the formation of 27-hydroxycholesterol in biologically relevant quantities we sought to understand the biological consequence of this induction. One of the key biological consequences of the pathway described, one may predict, is the induction of LXR responses. To address this issue we treated human monocyte-derived macrophages with various RAR, RXR and PPARγ ligands and determined the expression level of key LXR target genes, ABCA1 and ABCG1. As shown in figure 16A and B retinoids and LXR ligand induced ABCA1 and ABCG1 to varying degrees. The induction of ABCA1 and ABCG1 by RXR or pan-retinoid agonist ligands is not unexpected since they are believed to regulate LXR:RXR heterodimers from the RXR side, but the RAR selective ligand AM580 could also induce ABCA1 and ABCG1 (Figure 16A and B). Combination of RAR and RXR ligands were slightly better than the RXR ligand alone. Our findings therefore suggest that retinoid induction of LXR target genes could involve activation of CYP27 and production of 27-hydroxycholesterol. To obtain genetic evidence for the role of CYP27 in the described regulatory network and its biological relevance we used primary cell lines derived from patients with CYP27 deficiency (CTX).

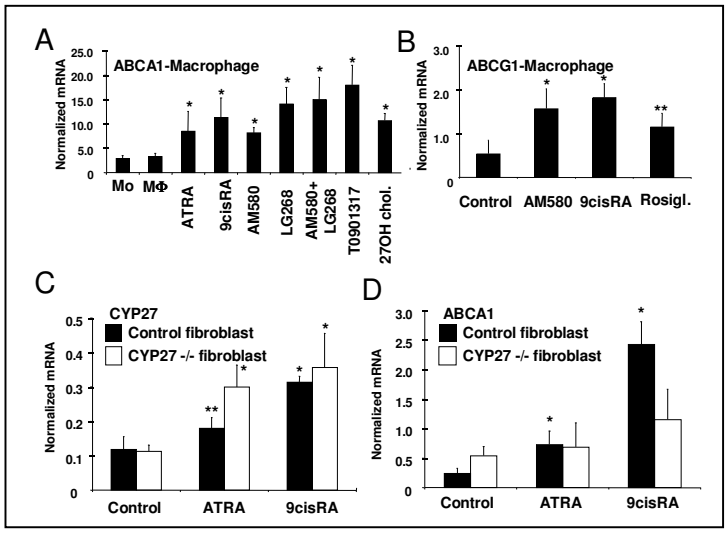


Figure 16: Retinoids induce LXR activity through CYP27. Normalized mRNA levels of LXR target genes are shown. This induction is missing in CYP27 -/- cells.

We could demonstrate that retinoids induced CYP27 expression not only in myeloid cells, but also in fibroblasts (Figure 16C) and that retinoid induced ABCA1 expression is strongly attenuated in the absence of CYP27 (in CTX fibroblasts) (Figure 16D).

5.2.6. Evidence for retinoid- and PPARγ-regulated gene expression in human atherosclerotic lesions

In order to obtain evidence for the physiological relevance/disease relatedness of the described pathway in atherosclerosis, a disease linked to macrophage cholesterol metabolism, we analyzed the gene expression profile of human macrophage rich atherosclerotic lesions.

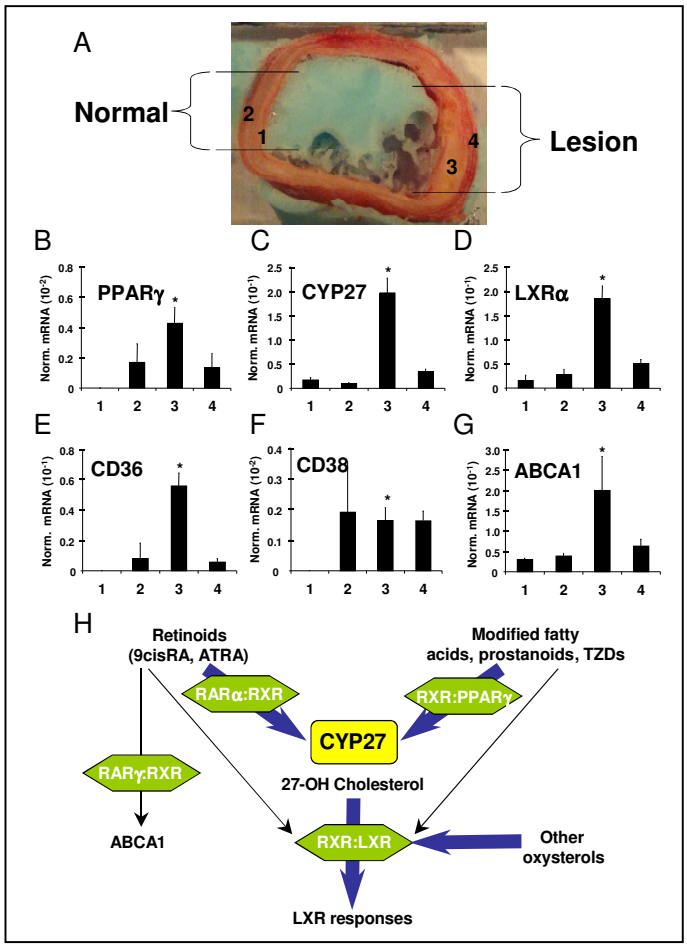


Figure 17: Members of the RAR-PPARγ-CYP27-LXR pathway are present and functional in human atherosclerotic lesion. Normalized transcript levels are shown.

mRNA levels of LXRα, PPARγ and their relevant target genes ABCA1, CD36 as well as CYP27 were determined from lesions and lesion-free intima and vessel wall tissue along with transcript levels of enzymes responsible for the production of ATRA retinaldehyde-dehydrogenase (RALDH2) and markergene for retinoid action CD38. Lesions were defined as abnormal fatty tissue on the luminar side of the internal elastic membrane of arteries. As shown in figure 17, in human lesions a remarkable increase of PPARγ (Figure 17B), LXRα (Figure 17D), their target genes ABCA1 (Figure 17G), CD36 (Figure 17E) and CYP27 (Figure 17C) was detected if compared to normal vessel wall, along with high expression of RALDH2, CD38 (Figure 17F and data not shown).
These data suggest that lesion tissue (mostly macrophages) have an expression profile similar to that of *in vitro* differentiated macrophages showing retinoid, PPARγ and LXR regulated transcription including increased CYP27 expression. These collectively provide a very strong link and correlation between retinoid, PPARγ and LXR signaling in the context of human macrophage rich atherosclerotic lesions and also establish CYP27 as a potentially key mediator of this interrelated signaling network.

5.3. PPARγ responsiveness in differently activated macrophages

PPARs are expressed at the high levels in macrophages and PPARγ showed a significant induction during monocyte/macrophage transition.
Once monocytes enter the subendothelial tissues they are transformed to macrophages and become activated by extracellular signals to fulfill their task. Basically, there are two forms of macrophage activation: the classical one which is induced by inflammatory agents and cytokines and results in an inflammatory macrophage and an alternative activation pathway is also exists that was found to be an inhibitory, anti-inflammatory process induced by mainly interleukin-4 (IL-4) but recently many positive consequences of alternative macrophage activation have been described e.g. roles in antigen presentation, resolving the inflammation, clearance of tissue debris, phagocytosis and

repair (177), (178). We wanted to test if the state of macrophage activation affects/correlates with the expression and function of PPARγ. Therefore, we activated monocyte-derived macrophages in vitro by using INFγ+TNFα for the classical and IL-4 for the alternative activation. As it is shown in figure18A the activation state of the macrophage influences the level of PPARγ whilst other PPARs does not show any differences. PPARγ is highly induced upon IL-4 treatment we identified the specific cell type, which express (much) PPARγ and separated this cell type, namely the alternatively activated macrophages from others that contain less PPARγ or do not express any. AMAC1 (alternatively activated macrophage chemokine 1, also named DC-CK, CCL18, PARC or MIP4) expression is shown in figure 18B as a marker for alternative activation.

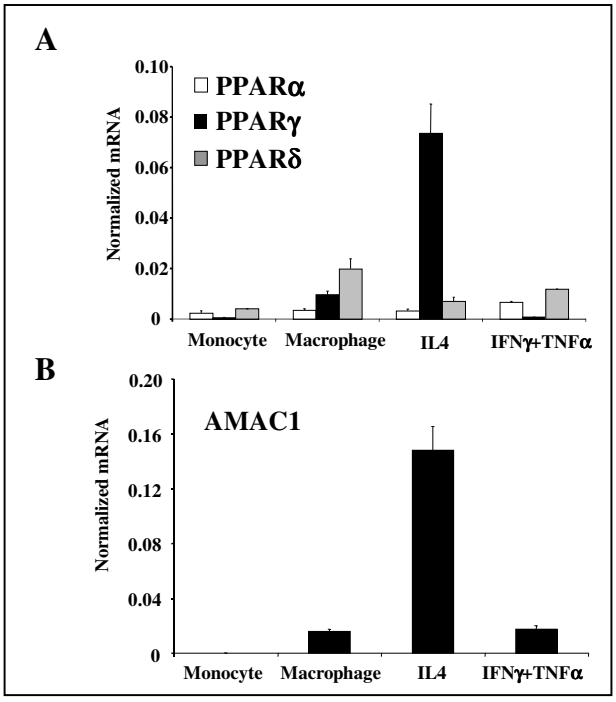


Figure 18: PPARγ is predominantly expressed in alternatively activated macrophages. Normalized transcript levels are shown.

Next we wanted to determine the time course of the inductions. By this we dissected the processes that affects PPARγ expression (Figure 19). First we identified an immediate-early induction of PPARγ and termed this "out-of-vessel induction". Another early induction is driven by IL4, "IL4-induction". Both inductions reach their maximum level

after 4-6 hours and then a slow down-regulation begins, “in-time-down-regulation” and finally classical activation results in the total downregulation of PPARγ, “classical-downregulation”.

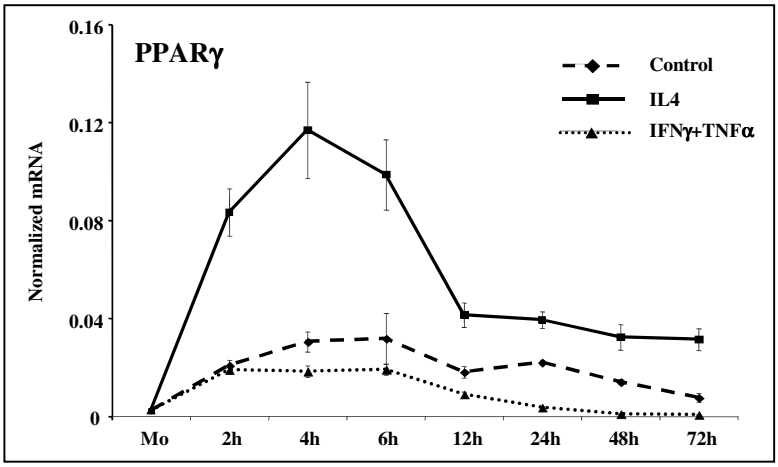


Figure 19: Time course of PPARγ expression during macrophage activation. Normalized mRNA levels are shown.

The regulation of PPARγ transcription raises two possibilities: on one hand PPARγ itself can be privy to alternative activation and in the function of an alternatively activated macrophage and/or on the other hand the induction can be a consequence of activation.. So far, we have performed in vitro studies; we treated human cells with synthetic PPARγ agonist, Rosiglitazone and used global gene expression profiling combined with gene-specific real-time Q-PCR to identify the regulated genes/processes. Our preliminary data show that there is a major difference in the PPARγ responses in the differently activated cells. As in figure 20A, B and C shown, PPARγ target genes can be differently induced in the differently activated cells. mRNA levels of three PPARγ target genes were measured after 6 hours, when PPARγ levels were at their maximum level in all cases of activation. Nevertheless, target genes were inducible only in the alternatively activated macrophages and to a minor degree in the non-activated cells. The different responsiveness cannot be explained with the different PPARγ levels, further characterization is needed to find the underlying mechanism(s).

We have also analyzed PPARγ expression and responses in murine macrophages. We used resting peritoneal macrophages, thioglycolate-elicited macrophages and bone

marrow-derived macrophages. These cells are different from the human ones, but they resemble the human macrophages at least in the pattern of PPAR expression. PPARγ is hardly detectable in resting peritoneal macrophages and is not induced during culturing (data not show

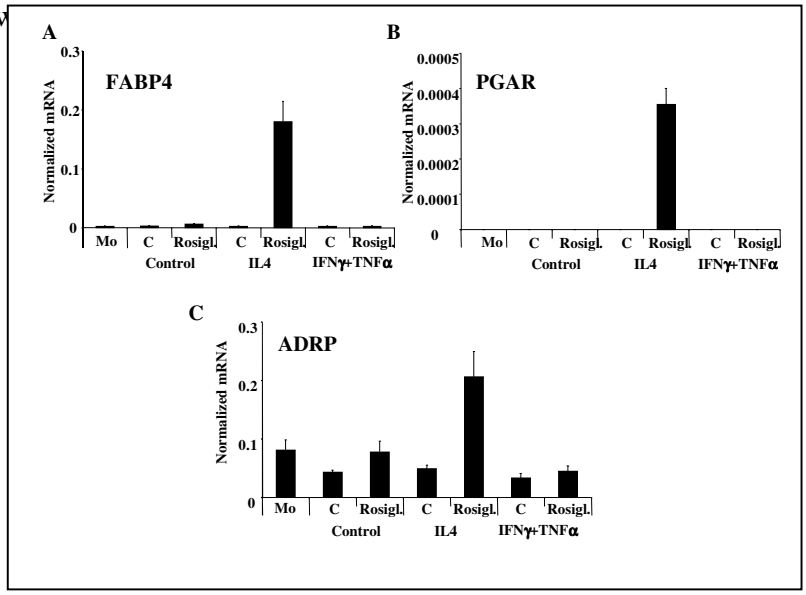


Figure 20: PPARγ target genes are predominantly induced in alternatively activated macrophages. Normalized transcript levels are shown.

In spite of this, thioglycolate-elicited cells express plenty of PPARγ, which is slightly downregulated during culturing but alternative activation results in the induction and classical activation (cytokines or lipopolysaccharide (LPS)) results in the downregulation of PPARγ (Figure 21A).

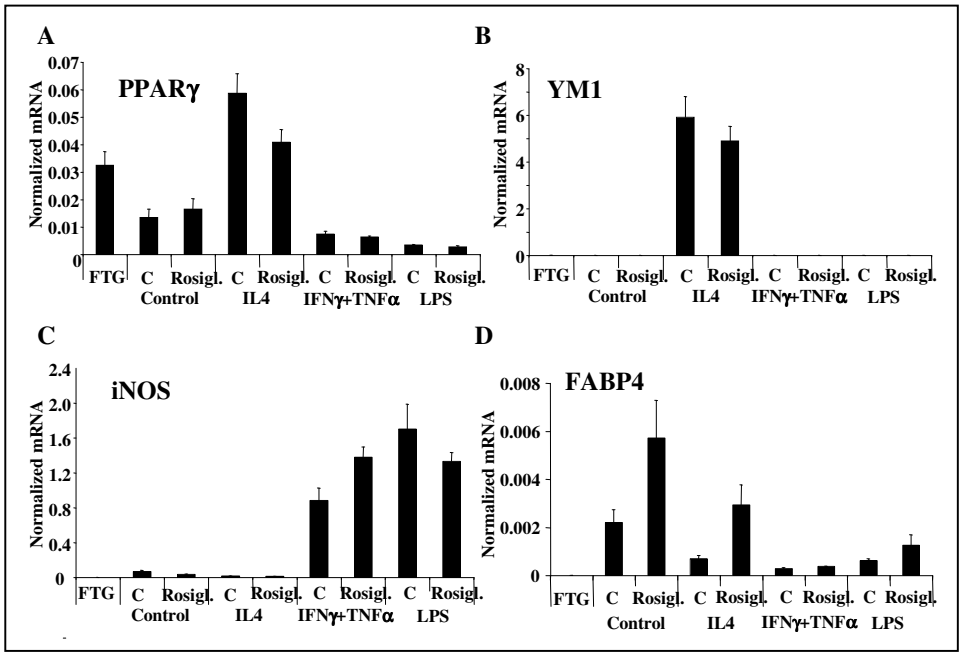


Figure 21: PPARγ expression and response in mouse peritoneal macrophages. Normalized mRNA levels are shown.

PP 1

during macrophage differentiation (Figure 22A).

Activation results in similar changes like in peritoneal cells: PPARγ level is increased after alternative and decreased upon classical activation. YM1 serves as a marker for alternative and iNOS for classical activation (Figure 21 and 22B, C). PPARγ target gene FABP4 cannot be induced in the classically activated cell in contrast to the alternatively activated ones both in peritoneal and bone marrow derived macrophages (Figure 21D and 22D).

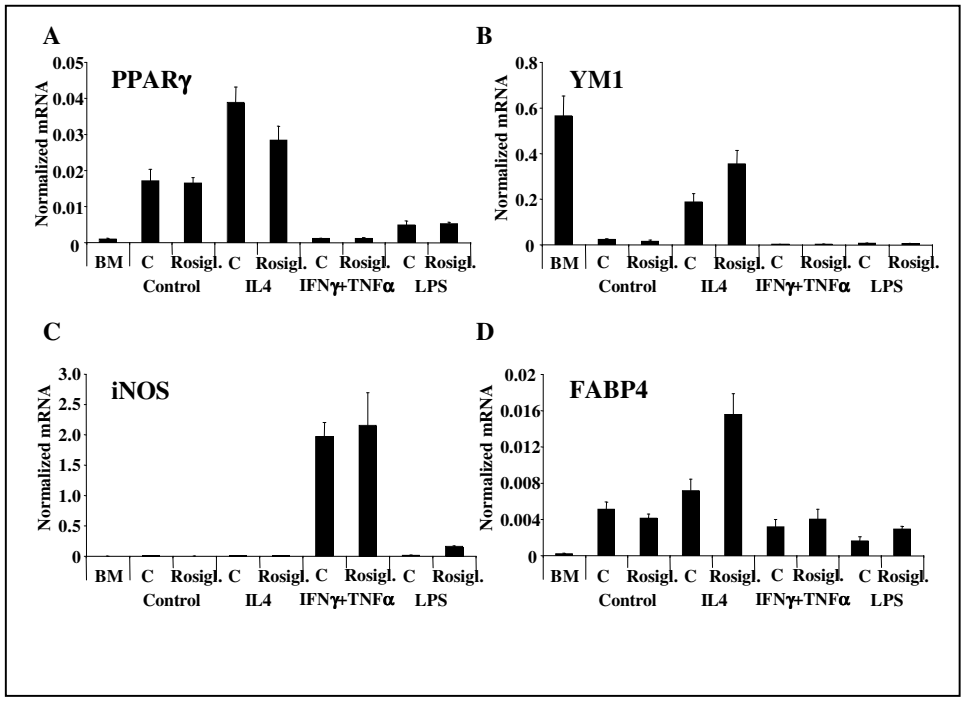
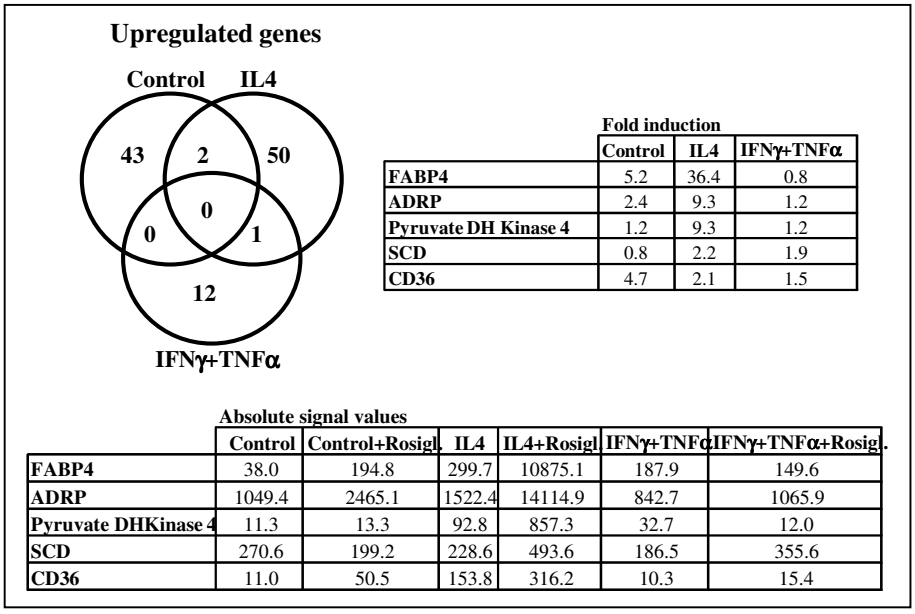


Figure 22: PPARγ expression in mouse bone marrow-derived macrophages. Normalized mRNA levels are shown.

We performed microarray experiments from the human macrophages to see if the enhanced PPARγ responsiveness a general phenomenon is or is limited for a subset of target genes. Interestingly, we found that more genes were upregulated in the

alternatively activated macrophages than in the control or classically activated cells (Figure 23).



Fold induction

	Control	IL4	IFNγ+TNFα
FABP4	5.2	36.4	0.8
ADRP	2.4	9.3	1.2
Pyruvate DH Kinase 4	1.2	9.3	1.2
SCD	0.8	2.2	1.9
CD36	4.7	2.1	1.5

Absolute signal values

	Control	Control+Rosigl.	IL4	IL4+Rosigl.	IFNγ+TNFα	IFNγ+TNFα+Rosigl.
FABP4	38.0	194.8	299.7	10875.1	187.9	149.6
ADRP	1049.4	2465.1	1522.4	14114.9	842.7	1065.9
Pyruvate DHKinase 4	11.3	13.3	92.8	857.3	32.7	12.0
SCD	270.6	199.2	228.6	493.6	186.5	355.6
CD36	11.0	50.5	153.8	316.2	10.3	15.4

Figure 23: Distribution of PPARγ-induced genes in human macrophages.

Furthermore, not only the number of genes induced were higher but those genes that were induced in at least two conditions (the known PPARγ target genes are shown in Figure 23 B and C) were expressed at higher levels shown by the absolute signal values. The most exciting observation was that only a few genes were regulated in at least two cell types and none in all the three conditions. These results clearly show that alternatively activated macrophages are a cell type where PPARγ is expressed andactive among all cell types studies.

6. DISCUSSION

6.1. Retinoid-PPAR crosstalk

After the initial discovery of PPARγ in myeloid cells (179), (180) there have been several studies trying to define its biological role. Many studies including some of our own has established PPARγ as a differentiation related transcription factor in myeloid cells (93), (134). However, no systematic analysis of its expression and the regulation of the PPARγ response have been carried out on myeloid cells of human origin.

In this work we have attempted to systematically characterize the level of PPARγ in human normal and leukemic myeloid cell. We have established a role for nuclear receptors in myelogenous differentiation, by showing that PPARγ expression is tightly linked to the differentiation stage of myeloid cells in the monocytic lineage and that promotion of differentiation induces the expression level of this gene. Moreover, activation of this nuclear receptor results in the induction of differentiation markers of macrophages. We also showed that retinoids potentiate the effects of PPARγ activators by inducing the transcription of PPARγ itself. We provided evidence for that retinoids may contribute to the physiological/pathophysiological function of PPARγ by increasing the uptake of oxLDL particles. These findings establish a link between retinoid receptors and PPARγ in myeloid differentiation and implicate RAR as a potential "jump-starter" of the non-established PPARγ signal pathway in macrophages.

PPARγ has been implicated in numerous developmental processes. Disruption of PPARγ gene in mice is lethal during early development: it is required for differentiation of the trophoblast and placental vascularization and homozygous PPARγ-deficient embryos die at day 10 of embryonic development (74), (95), (96). The PPARγ-null mice that survived to term were deficient in all forms of fat, substantiating the fundamental role for PPARγ in adipogensis, i.e. fat storage (74). Although PPARγ-null embryonic stem cells were capable to in vitro differentiate into macrophages (123), it was shown that PPARγ activators enhance monocytic development (93), (94) and PPARγ specific roles have been identified in macrophage lipid metabolism (93), (94), (141). These were the regulation of oxidized LDL uptake and the transcriptional activation of LXRα̃ Via this

latter pathway PPARγ can also contribute to cholesterol efflux by indirectly inducing ABCA1 transcription. Myeloid specific disruption of PPARγ gene in mice resulted in reduced total plasma and HDL cholesterol levels. In addition, cholesterol efflux was significantly decreased from macrophages elicited by thioglycolate in mutant mice (100). The lack of the PPARγ gene in macrophages is therefore likely to be proatherogenic. Based on this evidence it is not difficult to see that understanding the regulation of PPARγresponsiveness in myeloid cells and macrophages is important and may prove to be therapeutically relevant. We have used myeloid leukemia cell lines blocked at different stages of differentiation and also normal human myeloid cells to study the regulation of PPARγ responsiveness during myeloid maturation. The expression level of PPARγshowed remarkable correlation with the stage of differentiation. Previously, retinoids have been implicated in myeloid differentiation on multiple levels but mainly in the granulocytic pathway (115), (117), (116), (118). Recently, it was reported that retinoids have a role earlier in myelogenesis affecting the common granulocyte/monocyte precursors (120). This obsevation is consistent with our findings and suggests that it is likely that retinoid action precedes the appearance of PPARγ responsiveness and may contribute to its development during macrophage differentiation.

This is not the first example of a crosstalk between retinoid and PPARγ signaling during a differentiation process. A crosstalk between retinoid and PPARγ signaling during fat cell differentiation has been extensively characterized by the Lazar group. They found that retinoic acid blocks adipogenesis by inhibiting C/EBPβ-mediated transcription (181), (182). In myeloid cells the situation is clearly different. Retinoids promote PPARγ expression and responsiveness indicating that the pathways are interrelated but the consequence of the crosstalk is cell-type specific. We have identified a link between RAR, PPARγ and LXR signaling in macrophages, where PPARγ and/or RAR mediated activation of a p450 enzyme, CYP27 leads to LXR activation. Our data suggest that the interrelatedness of the three nuclear receptor mediated pathways are more complex than previously believed and that retinoid signaling may have a larger role in macrophage gene expression and metabolism as previously suspected.

One may speculate that sequential effects of retinoids followed by PPARγ activators are coordinately regulating myeloid maturation and gene expression, a proposition only *in vivo* experiments can test. Needless to say that a pharmaceutically amenable pathway to modulate PPARγresponsiveness can be utilized to boost the anti-atherogenic effects of PPARγ-regulated gene expression. It clearly requires further studies before retinoid regulated PPARγ responsiveness becomes a valid clinical target.

6.2. Retinoid-PPARγ-LXR crosstalk

We have identified a p450 enzyme CYP27 as a gene commonly induced during monocyte-macrophage transition and as a PPARγ:RXR and RAR:RXR regulated gene in myeloid cells. Promoter analysis revealed complex regulation by retinoid receptors and PPARs via a response element on the promoter of human CYP27 further underscoring the interrelatedness of these pathways. These findings tie retinoid, PPAR and LXR signaling into one regulatory network requiring natural ligands: retinoids and modified fatty acids or prostanoids to activate an entire metabolic pathway and leading to coordinate regulation of lipid/cholesterol uptake, metabolism and efflux (Figure 17H). Furthermore, we provided evidence that all components of the described pathways exist in human atherosclerotic lesions.

Applying synthetic ligands to biological systems allows identification of pathways ascribable to the receptors. This proved to be a very fruitful approach to identify biological processes activated by the receptors but it also overwrites the need for the identification of natural ligands and more importantly sources and regulation of natural ligand production. This approach may also represent superphysiological and ectopic activation of receptors. This is a particular concern in cases of the metabolite receptors (PPARs, LXRs, FXR, PXR) for which only low affinity natural ligands have been identified so far, because activation with low affinity (partial agonist) ligands may lead to a different biological outcome than activating with a synthetic full agonist. Therefore the processes/enzymes leading to endogenous ligand production and their regulation should be also considered when assigning biological functions to receptors. This line of arguments led us to search for mechanisms linking PPARγ:RXR and LXR:RXR signaling pathways. In the case of LXR several low affinity oxysterols have been identified (22(R)-

cholesterol, 20(S)-cholesterol, 24-, 25- and 27-hydoxycholesterol as endogenous ligands (106), (111), (146), (147), (148) but none of them has higher affinity than 10 μM. It is important to note that among these oxysterols 27-hydroxycholesterol is present at the highest concentration in the circulation (183) suggesting an *in vivo* relevance in activation of LXR. We identified CYP27 as a nuclear receptor regulated enzyme and capable of producing endogenous ligands for LXR. Moreover, panagonists or RXR activating retinoids can contribute to receptor activation resulting in a robust synergistic response between retinoids and oxysterols. The tying of regulated enzyme expression and heterodimer activation suggests that partial agonists such as 27-hydroxycholesterol can contribute to full activation in the presence of the appropriate retinoid. This adds an additional layer of control to the receptor's activity by the regulation of the production of two endogenous lipid molecules.

CYP27 is an attractive target for regulated transcription because its product is an alternative bile acid synthesis precursor in the liver. Identification of a complex PPAR-RAR regulation and the fact that retinoids present in the serum are regulating the basal expression level of the enzyme in myeloid cells are significant novel aspects of the enzyme's regulation. The product generated, 27-hydroxycholesterol, is a polar compound capable of transversing membranes and therefore provides an alternative cholesterol efflux mechanism (155), (184), (185). The fact that 27-hydroxycholesterol is an endogenous ligand of LXR has been noted previously (106), (146). It was even suggested that 27-hydroxycholesterol is a partial agonist of LXR:RXR heterodimers (106). Our results are in agreement with this assessment, but go further and show that ligand production can be regulated and that, in combination with retinoids 27-hydroxycholesterol becomes a full agonist on LXR regulated target genes. The fact that retinoids (RAR and RXR selective compounds) and combination of PPAR and RXR selective compounds were able to induce CYP27 expression suggested that there is a cross talk between retinoid and PPAR signaling. It became apparent when the promoter analysis revealed the two enhancers mediating the effects. Further studies identified PRRE-B as the major element and regulator of human CYP27 gene's transcription, whilst PRRE-A might serve as an accessory site required for full activation. Further studies are needed to define the relationship between the two elements and the two heterodimers

(RAR:RXR and PPARγ:RXR) binding to them. The ChIP assays suggest two possibilities one is that both heterodimers could bind in one complex, the other is that there is heterogeneity between the cells and some bind one some the other heterodimer. It may also be possible that the two elements and two heterodimers are part of a larger complex (i.e. an enhancesome). Traditional promoter/enhancer analysis is not sufficient to dissect such complex relationships. Another interesting aspect of the cross talk between retinoid and PPAR signaling is the fact that PPAR ligands are only active in regulating CYP27 expression if rexinoids are present in some of the cell lines used. We termed this retinoid enabled PPAR response. This phenomenon can be observed in the monocytic leukemia cell line, MonoMac-6 and a similar observation was made during the transient transfection based analysis of the promoter also. This is inline with our initial observations on oxidized LDL uptake regulated by PPARγ□RXR heterodimers (93). At this point it is not clear if the identified elements are solely responsible for this effect or other factors contribute to it.

Regulated expression of CYP27 is also of interest because it has a key metabolic function converting cholesterol into a more polar compound, 27-hydroxycholesterol. Consequently it has two major effects on cholesterol efflux. It induces ABC transporter expression and subsequent HDL-dependent efflux via the activation of LXR:RXR. 27-hydroxycholesterol also represents an alternative cholesterol efflux pathway from macrophages that is independent of known transporters including ABCs and HDL and allows converted cholesterol to leave the cells and cleared by the liver as bile acids. It is estimated that under steady state conditions it may represent as much as 10-20 % of total cholesterol efflux as it was shown by Babiker et al. (154). Our data suggest that this efflux in macrophages may be regulated by retinoid and PPARγ-mediated induction of CYP27. It is also apparent that besides cholesterol efflux LXR:RXR heterodimers are capable to induce multiple other pathways involved in lipid metabolism such as SREBP1c induction (186), (187) or the induction of phospholipid transport protein (188) and have a more global effect on macrophage lipid homeostasis. As far as the physiological relevance of the pathway is concerned, due to the species specificity (exists in human but not in mouse) of this regulation and the lack of suitable mouse models we had to rely on approaches of gathering data from CTX (CYP27-/-) human fibroblasts and

atherosclerotic lesions of humans. The evidence from these approaches clearly demonstrates that CYP27 is required, at least in part, for retinoid and PPARγ ligand induced LXR mediated gene expression and potentially to cholesterol efflux. The striking similarity between gene expression patterns, including retinoid, PPARγ and LXR regulated gene expression as well as high levels of CYP27, of in *in vitro* differentiated macrophages and in tissue samples of macrophage-rich atherosclerotic lesions also underscores that this regulatory network is likely to have physiological and disease relevance. A recent study by Costet et al. (189) suggested that ABCA1 and cholesterol efflux can be regulated directly by retinoids via a RARγ mediated pathway. Our data presented here show that RARα activation has a broader effect on human macrophage cholesterol metabolism as those authors suggested. We suggest that retinoid regulated CYP27 is likely to act as a modulator of robust LXR response. In our view the identified pathway also represents a potential new target for the regulation of macrophage cholesterol efflux and for the management of diseases with increased foam cell formation and cholesterol overload. It also suggests, that retinoids may have more profound effect on lipid metabolism than previously suspected. This notion is further underscored by the observation that RXR agonists have significant atheroprotective effect in apoE-/- mice (113). Clearly more work needs to be done before regulation of CYP27 can be considered a valid target for pharmacological intervention in these conditions.

6.3. PPARγ - macrophage activation

Macrophages are formed in the tissues from monocytes where they become activated upon various stimuli (177). Classical activation occurs for example after stimulation of Toll-like receptors and results in the induction of IFNγ, TNFα dependent signal pathway leading to inflammation, induction of anti-proliferative, anti-microbial functions and Th1 dominant immune response. On the contrary, alternative activation is induced by IL-4, IL-13, results primarily in suppressive macrophage functions, elimination of tissue debris, wound healing, Th2 dominant response.

Several nuclear receptors have been implicated in various metabolic and immunological functions of the macrophage. Previously, we and others showed that PPARγ, LXRα, RXRα and RARα were highly expressed in macrophages.

We sought to characterize these two distinct activation forms of macrophages and find the role for PPARγ in the activated cells. We used human monocytes, mouse peritoneal and bone marrow-derived cells to generate macrophages.

We found that PPARγ showed a rapid induction during monocyte-macrophage transition and it was further induced upon alternative activation while was downregulated upon classical activation. We studied the activity of these receptors by determining target gene expression levels. Surprisingly, we could show that PPARγ could only be fully activated in alternatively activated and not in classically activated cells. Next, we extended our studies using global gene expression profiling and identified the genes induced in the classically and alternatively activated cells and also the PPARγ-regulated genes. More genes are induced in the alternatively activated cells and the expression levels of the regulated genes are much higher in these cells than in the classically activated ones. These data support the notion that PPARγ is significantly more active in alternatively activated macrophages suggesting roles in this newly defined cell type rather than in classically activated ones. It is very likely that the activation state of macrophages determines the activity of some metabolite activated nuclear receptor pathways. These data also suggest that increased PPARγ is a novel marker of alternative macrophage activation and that this cell type is the target of PPARγ ligands in vivo.

7. SUMMARY

Nuclear receptors are ligand-activated transcription factors that regulate many aspects of metazoan life. In vivo, a biologically active, functional nuclear receptor requires obviously the protein of the transcription factor, an endogenous ligand that activates it and appropriate conditions in the cell. This latter mainly depends on the presence of elements of co-activator complexes and the permissive, epigenetic status of the regulated DNA. Nuclear receptors have been shown to be important in regulating lipid metabolism in myeloid cells and were also implicated in differentiation processes of the myeloid lineage. Peroxisome Proliferator Activated Receptor γ (PPARγ) appears to be a key component of lipid uptake by inducing the scavenger receptor CD36 that mediates oxidized low-density lipoprotein (oxLDL) uptake to macrophages. Retinoic Acid Receptor (RAR) on the other hand was also shown to play important roles in myeloid cell differentiation.

- We present evidence for a crosstalk between these two nuclear receptor pathways in myeloid cells and show that expression level of PPARγ increases with the degree of monocyte/macrophage commitment. Activation of PPARγ leads to the increased expression of maturation markers (e.g. CD14, CD36). Interestingly, retinoid treatment potentiates PPARγ ability to induce transcription of its target genes.

Cholesterol uptake and efflux are key metabolic processes associated with macrophage physiology and atherosclerosis. PPARγ and Liver X Receptor α (LXRα) have been linked to the regulation of these processes.

- We identified CYP27, a p450 enzyme as a link between retinoid, PPARγ and LXR signaling. We show that the human CYP27 gene is under coupled regulation by retinoids and ligands of PPARs. Induction of the enzyme results in an increased level of 27-hydroxycholesterol that activates LXR and LXR-regulated processes, which leads to an alternative means of cholesterol efflux. Human macrophage rich atherosclerotic lesions have an increased level of retinoid, PPARγ and LXR-regulated gene expression and also enhanced CYP27 levels. Our

findings suggest that nuclear receptor regulated CYP27 expression is a key integrator of RAR-PPARγ-LXR signaling.

- We also analyzed the activation state of macrophages and found that PPARγ is dominantly expressed in alternatively activated macrophages and induces target genes' expression mostly in this specialized cell type.

New discoveries:

We found a correlation between the maturation stage and the level of PPARγ expressed in myeloid cells.

We identified PPARγ as a highly induced and expressed nuclear receptor in macrophage differentiation.

We showed that retinoids induce expression of PPARγ and potentiates PPARγ responses.

We identified an enzyme, CYP27 to be induced during monocyte/macrophage transition and be regulated by RAR, RXR and PPARγ activators.

We characterized the promoter of the human CYP27 gene and identified the response elements mediating the regulation by nuclear receptors.

We showed that induction of CYP27 by RAR, RXR or PPARγ agonists resulted in an increase in CYP27 protein level that was active showed by the elevated levels of oxidation products.

We found that 27-hydroxycholesterol not only activated LXR:RXR but showed a high level of synergy with retinoids.

We found elements of CYP27 regulation in human atherosclerotic lesion being active.

We characterized differently activated macrophages and found that PPARγ is predominantly expressed in alternatively activated macrophages and PPARγ response is a marker of this activation state.

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