THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Lipid Signaling and Inflammation in Macrophages and Dendritic Cells

Role of Liver X Receptor in dendritic cell biology

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Supplement 1.

Activation of LXR sensitizes human dendritic cells to inflammatory stimuli.

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Supplement 2.

Oxysterol signaling links cholesterol metabolism and inflammation via the liver X receptor in macrophages.

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Supplement 3.

Mycobacterium bovis bacillus Calmette-Guérin infection induces TLR2-dependent peroxisome proliferator-activated receptor gamma expression and activation: functions in inflammation, lipid metabolism, and pathogenesis.

Almeida PE, Silva AR, Maya-Monteiro CM, Töröcsik D, D'Avila H, Dezsö B, Magalhães KG, Castro-Faria-Neto HC, Nagy L, Bozza PT.

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LIST OF ABBREVIATIONS

9-HODE, 9-hydroxyoctadecadienoic acid;

13-HODE, 13-hydroxyoctadecadienoic acid;

22(R)-HC, 22(R)-hydroxycholesterol;

24(S),25-EC, 24(S),25-epoxycholesterol;

24(S)-HC, 24(S)-hydroxycholesterol;

25-HC, 25-hydroxycholesterol;

27-HC, 27-hydroxycholesterol;

ABC, ATP-binding cassette;

AF-1, activation function domain 1;

AF-2, activation function domain 2;

apo, apolipoprotein;

ATI-829, 3,6,24-Trihydroxy-24,24-di(trifluoromethyl)-5b-cholane;

BCG, Bacillus Calmette-Guérin

CFSE, 5-6-carboxyfluorescein diacetate succinimidyl ester;

COX-2, cyclooxygenase-2;

CTX, cerebrotendinous xanthomatosis;

DAB, 3,3'diaminobenzidine;

DBD, DNA binding domain;

DC, dendritic cell;

DMHCA, N,N-dimethyl-3b-hydroxycholenamide;

ER, estrogen receptor;

FXR, farnesoid X receptor;

GM-CSF, Granulocyte macrophage colony-stimulating factor;

GW, GW3962;

HDL, high density lipoprotein;

HRP, horse raddish peroxidase;

iDC, immature DC;

IL, interleukin;

iNOS, inducible nitric oxide synthase;

IRF3, interferon regulatory factor 3-dependent;

LAM, lipoarabinomannan;

LBD, ligand binding domain;

LDL, low density lipoprotein; LDLR, low density lipoprotein receptor;

LPL, lipoprotein lipase;

LPS, lipopolysaccharide;

LXR, liver X receptor;

LXREs, LXRresponsive elements;

MCP-1, monocyte chemoattractant protein-1;

MCP-3, monocyte chemoattractant protein-3;

mDC, mature DC;

NCoR, nuclear receptor corepressor;

NF-κB, nuclear factor κB;

oxLDL, oxidized low density lipoprotein;

PGJ2, 15-deoxy-D12,14-PGJ2;

PMSF, phenylmethylsulfonyl fluoride (PMSF);

PLTP, phospholipid transfer protein;

PPARγ, peroxisome proliferator-activated receptor;

RAR, retinoic acid receptor; RXR, retinoid X receptor;

SERM, selective estrogen receptor modulator;

SMRT, silencing mediator of retinoic acid and thyroid hormone receptors;

SR, scavenger receptor;

SREBP-1c, sterol regulatory element binding protein-1c;

SULT, sulfotransferase;

SUMO, small ubiquitin-related modifier;

TLR, Toll-like receptor;

TNF, tumor necrosis factor;

TZD, thiazolidinedione;

YT-32, (22E)-ergost-22-ene-1,3-diol;

1. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

A dendritikus sejtek immunológiai tulajdonságait a környezetben jelenlevő zsírmolekulák befolyásolni képesek. Ezen zsírmolekulák által kiváltott változások egy részét a sejtmagban található ún. magreceptorok irányítják, az általuk szabályozott géneken keresztül. Korábbi munkák igazolták, hogy a magreceptor család számos tagja, úgymint a PPAR-ok, a retinsav receptor vagy a D-vitamin receptor, melyek a dendritikus sejtekben magas szinteken fejeződnek ki, ezen jelátviteli útvonalak közvetítésében játszanak kulcsszerepet. A megfelelő aktiváló molekulák hatására (zsírsavak, retinoidok, aktív D-vitamin), a receptorok befolyásolni képesek, többek között a Toll-like-receptor 2, 3, 4 és 7 által bekapcsolt jelátviteli útvonalra hatva, a gyulladásos sejtfelszíni molekulák kifejeződését és a válaszként adott citokinek termelődését, a dendritikus sejtek tolerogén sajátságainak kedvezve. A dendritikus sejtek tompított aktivációja, a velük kapcsolatba kerülő T sejtek csökkent aktiválódásához vezet.

A Liver X Receptor (LXR) szintén a zsírok aktiválta magreceptorok család tagja. Bár makrofágokban betöltött szerepéről ismereteink jelentősek, a dendritikus sejtekben való kifejeződésükről és az általuk szabályozott útvonalakról keveset tudunk. Kísérleteinkben arra kerestük a választ, hogy az LXR-t aktiválni képes, szintetikusan előállított, valamint fiziológiásan is előforduló anyagok milyen változásokhoz vezetnek a vérben keringő monocitákból differenciáltatott dendritikus sejtekben. Meghatároztuk, hogy mind a vérben található, mind pedig az általunk a keringésben levő monocitákból in vitro differenciáltatott dendritikus sejtekben az LXR kifejeződik. A receptor aktiválása az differenciáltatott, majd a TLR3 illetve 4 útvonalakon keresztül aktivált sejtekben, a receptor által szabályozott gének fokozott mértékű kifejeződéséhez vezetett. A receptor aktiválását, az érési markerek, a CD80 és a CD86 emelkedett szintje kísérte, a gyulladásos citokinek, úgy mint az IL-12, TNFα, IL-6 és IL-8 megnövekedett termelődésével társultan, mely a $CD4^+$ T sejtek fokozott aktivációjához vezetett. A jelenség hátterében a TLR aktivátorok által bekapcsolt NFκB útvonal aktivitásának időtartam és intenzitásbeli növekedését találtuk. Alátámasztva az LXR pozitív dendritikus sejtek szerepét (pato)fiziológiás körülmények között, immunkémiai

módszerrel igazoltuk a receptor jelenlétét tuberkulózisos, sarkoidosisos illetve daganat asszociálta nyirokcsomókban jelenlevő dendritikus sejtekben.

Vizsgálatainkban, melyben a PPARγ receptort azonosítottuk szintén immunhisztokémiai módszerrel mycobacteriummal fertőzött makrofágokban, bemutattuk, hogy a PPARγ receptor központi szerepet tölt be a sejtek fertőzésre adott válaszában, a zsíranyagcsere és a baktérium sejten belüli túlélésének összekapcsolásán keresztül.

Eredményeink alátámasztják az LXR útvonal jelentős szerepét dendritikus sejtekben, valamint a magreceptorok szerepét a sejtek immunológiai sajátságai és a környezetükben levő zsírmolekulák közötti kapcsolat közvetítésében.

2. INTRODUCTION

2.1. Nuclear receptors

LXR and PPARγ are both members of the family of steroid hormone receptors that includes 48 members in the human genome. Programming growth, development and homeostasis, steroid receptors deliver hormonal or metabolic signals directly at a transcriptional level, by activating or repressing gene expression. First members, the classical hormone receptors for some steroids, like the estrogen receptor and the androgen receptor, were identified nearly 40 years ago. Over the last 20 years with the discovery of the so-called 'metabolic receptors' like LXR and PPARγ, the group was extended and widened our view and concepts on hormone and metabolic signaling. These discoveries put these receptors in the focus of research, because these are able to translate the signals of the intra- and extracellular lipid environment into physiological and patho-physiological processes, as oxysterols and intermediate products of the cholesterol biosynthetic pathway activate LXRs, various fatty acids, eicosanoids and prostanoids activate PPARs, or metabolites of vitamin A activate the retinoic acid receptors. A set of receptors, such as LXR and PPARγ form heterodimers with retinoid X receptor, allowing a more complex and combinatorial regulation integrating different signaling network.

The steroid receptors all share a common domain structure consisting of a DNA binding domain, and most of the receptors also have a ligand binding domain, an amino terminal activation function domain (AF-1), and a second carboxy terminal activation domain (AF-2) (1). While DBDs are very highly conserved and these are responsible for the nuclear localization, with two zinc-binding motifs, and bind to DNA enabling nuclear receptors to act on gene regulation, the LBDs are more diverse with different and specialized ligand binding pockets for each receptor. The ligands, either natural ligands or synthetic agonists and antagonists, unlike water-soluble peptide hormones, have a non-polar character enabling them to pass freely through the lipid bilayer of the cell membrane. The ability that different lipids such as cholesterol metabolites activate with different affinity could be best explained by looking at the structure of the LBD. Unlike hormone receptors such as ER and RAR, having so-called classical tight-fitting LBDs

(2), LXRs and also PPARs have large hydrophobic cavities that enable receptors to bind several different kinds of ligands but with a considerably lower affinity (3) (4).

The specific ligand–receptor interaction leads to allosteric changes, resulting in activation, inactivation or repression of the receptor's activity that is mediated by other transcription factors where the activation function domain is important in mediating the displacement of corepressors and the recruitment of coactivators to the receptor depending on the ligand-bound state. In a simplified way, corepressors bind to ligand-free, inactive nuclear receptors that, in the presence of activating ligands, go through conformational changes and the inhibitory transcriptional factors (corepressors) are displaced by coactivators that turn on the signaling cascade (5).

Some nuclear receptors can also act in 'trans' that occurs in a gene and signal-specific manner where the ligand-bound receptor is not the activator of transcription but a promoter specific repressor. The small ubiquitin-related modifier (SUMO)ylated forms of the ligand-activated both LXRs and PPARγ may be recruited to the promoters of inflammatory genes and inhibit the LPS induced corepressor (NCoR) clearance (6) (Figure 1).

In the classical way of gene expression regulation the interaction with other transcription factors allows transcriptional regulation of the target genes in a great variety of genetic programs putting nuclear receptors also in the focus of drug discovery. For example PPARs with metabolic diseases such as diabetes and hyperlipidaemia (7), RARs are associated with certain types of leukaemia (8), ER with breast cancer (9) and glucocorticoid receptors with inflammation control (10).

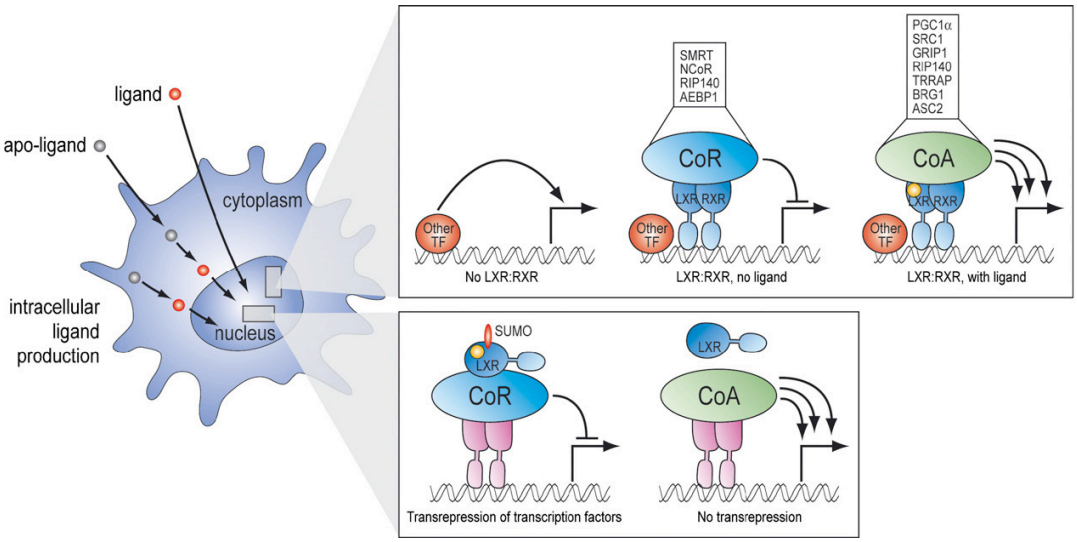

Figure 1. Mechanisms of target gene repression and activation by nuclear receptors through the possible interactions of LXR. In the upper part the three possible states of an LXR target gene is shown. First, in the absence of LXR the effects of other unrelated transcription factors result in a basal transcription level. In the presence of the LXR:RXR heterodimer this basal transcription is inhibited without ligands. And third this repression is relieved upon ligand activation of the heterodimer. This process is accompanied by the switch from corepressors to coactivators recruited to the LXR:RXR. In the lower panel the mechanism of transrepression is shown. This happens on the promoter of genes, which are directly not regulated by LXR. In this case upon ligand binding LXR is SUMOylated and inhibits the proteosomal degradation of the corepressor complex on the transcription factors, thereby inhibiting gene expression from these promoters.

2.2. Liver X Receptor

Expression and regulation of the receptor

Liver X receptors a and b (also known as NR1H3 and NR1H2) were cloned in 1994 based on sequence homology with other nuclear receptors from a liver-derived cDNA library, and named because for its high expression found in liver (11) (12). Further studies demonstrated that LXRs are expressed in different tissues also and while LXRα is

highly expressed in the liver and at lower levels in adipose, intestine, lung, kidney, the adrenal glands and macrophages, LXRβ is ubiquitously expressed (12).

Although LXRs have been studied intensely, very little is known about the expression regulation of the receptors. Generally, LXRβ is considered to be constitutively expressed, while LXRα levels can be modulated. LXRα is induced during monocyte–macrophage transition (13) (14) where PPARγ can induce its expression (15). In human but not in mouse cells another autoregulatory loop exists: LXR can induce its own transcription (16) (17) (18). Studying the promoter of LXRα CCAAT/enhancer-binding proteins were reported to isotype and cell-type specifically regulate LXRα expression (19).

The naturally occurring polyphenol resveratrol has been associated with the beneficial effects of red wine consumption on cardiovascular disease and shown to inhibit atherosclerosis in animal models. Resveratrol was shown to regulate the expression of LXRα in human macrophages, which could be a possible molecular explanation for the beneficial effects of polyphenols (20).

Activation of the receptor

Like many other nuclear receptors LXRα forms permissive heterodimers with the nuclear receptor RXR, where the complex can be activated through either partner. Like other nuclear receptors LXR, forming obligate heterodimers with RXR, bind DNA to specific nucleotide sequences, the LXR-responsive elements (LXREs) consisting of direct repeats (DRs) of the core sequence AGGTCA separated by 4 nucleotides (DR-4) (11) (12) (21). As at that time LXRs were considered ‘‘orphan” nuclear receptors, with no known natural ligands, but the heterodimer was readily activated by RXR ligands it was believed that LXRs are part of a new retinoid response pathway (12).

The breakthrough came in 1996 when LXRs became ‘‘adopted” via the discovery, that although not cholesterol itself, but its metabolites the naturally occurring oxysterols, such as 24(S)-hydroxycholesterol (24(S)-HC), 22(R)-hydroxycholesterol (22(R)-HC), and 24(S),25-epoxycholesterol (24(S),25-EC) can bind to and activate LXRs at physiologic concentrations pointing to its role as a lipid/cholesterol sensor (22) (23) (24). In the absence of ligand, LXRs reside in the nucleus, bound to DNA in a non-activating state, forming a complex with corepressors, such as the nuclear receptor corepressor (NCoR)

and the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), keeping its target genes repressed. Ligand binding of LXRs results in a conformational change of the receptor enabling the exchange of corepressors to coactivators turning on the transcription of the regulated target genes (25) (26) (27). As the ligand binding pockets of LXRα and LXRβ differ (the level of identity is estimated to be 77%) a different binding specificity of the ligands for each isotype can also be predicted. Therefore the aim to design LXR subtype selective ligands for pharmacologic application is also relevant but yet has to be delivered (28).

Ligands - Oxidized lipids as signaling molecules

LXR was originally described as an alternative retinoic acid response pathway due to the permissiveness of the LXR:RXR heterodimer (12) (29). That means that either LXR or RXR activators can activate the dimer. Later, a number of oxysterols have been identified as potential endogenous ligands for LXR (22) (28) (23) (24).

There are two synthetic LXR agonists existing and both of them have been widely used during the last several years. T0901317 is a non-steroid LXR activator with EC50 of 50 nM and Kd of 50 nM measured in a cell-based reporter assay and coactivator interaction studies, respectively (30). T0901317 also activates farnesoid X receptor (FXR) (31) (32). GW3965 is another potent non-steroid LXR activator with EC50 of 125 nM in a cell-free ligand-sensing assay and EC50 of 190 and 30 nM for LXRα and LXRβ in cell-based reporter assay, respectively (33).

The group of Mangelsdorf showed that both LXRα and LXRβ bind 24(S),25-EC with a Kd of approximately 200 nM in an in vitro ligand binding assay, corresponding well to the concentrations in the liver, while 24(S)-HC and 22(R)-HC, which are also present in vivo, bind to both isotypes with a similar Kd (28). They also presented data on that in tissues (e.g., liver, brain and placenta) where both cholesterol metabolism and LXR expressions are at high levels, these oxysterols exist at concentrations that can activate LXR.

Screening different oxysterols after mono- or multiple oxidation steps and introduction of amide, imino or ester moieties they found that poly-oxidation (at positions 22, 24, or 25 on the side chain of cholesterol) decreases binding and activation of LXR, whereas single

hydrogen bond acceptors on the cholesterol side chain make the compounds potent ligands. Switching the stereochemistry of 24(S),25-EC and 24(S)-HC from the S to R configuration they found that the transcriptional activation resulted in a 50% reduction and a 6-fold and 4-fold decrease in affinity of 24(R),25-EC to LXRα and LXRβ, respectively (28). When switching the naturally occurring R to synthetic S configuration in 22(R)-HC, they found a complete loss in the transactivation of LXRα and LXRβ, although 22(S)-HC was found to bind both LXR subtypes competitively and with high affinity. As a possible explanation the authors proposed that 22(S)-HC might not reach the nucleus based on its affinity to different cellular proteins or alternatively it could be a better substrate for cholesterol-acyltransferases that could inactivate them (28).

CYP27 is a p450 enzyme that generates 27-hydroxycholesterol (27-HC). It is a mitochondrial enzyme representing an alternative bile acid synthesis pathway (34) (35) (36) (37) (38) and was reported to be expressed besides the liver in the lung and also in macrophages (39) (40). It has been also associated with atherosclerotic lesions (41) (42). 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 central nervous system leading to ataxia, spinal cord paresis, neurological dysfunctions, normolipidemic xanthomatosis and accelerated atherosclerosis (43) (44) (45). The enzyme's product 27-HC has been shown to activate LXR (22) (46). Our group found that CYP27 is induced during macrophage development and is a direct target for RAR, RXR and PPARγ receptors (47). The overall effect of 27-HC became controversial by reports claiming that it is a competitive antagonist of estrogen receptor and increasing 27-HC levels in mice decreased estrogen-dependent expression of vascular nitric oxide synthase and repressed carotid artery re-endothelialization. Based on these studies 27-HC works as a selective estrogen receptor modulator (SERM), depending on the cell-type and promoters it can exert pro- or anti-estrogenic effects (48) (49).

3b-Hydroxy-5-cholesten-25(R),26-carboxylic (cholestenoic) acid, a metabolite of 27-HC is also a naturally occurring ligand for the LXR (50).

Although 25-hydroxycholesterol (25-HC) can be produced by testicular macrophages (51) and was found in lesion macrophages (52) (53), its synthesis during foam cell formation has not been studied in sufficient details.

By converting cholesterol to 24(S)-HC, cytochrome P450 46A1 (CYP46A1) initiates the production of another oxysterol that can activate LXR. Based on its expression level and function it is responsible for the major pathway of cholesterol removal from the brain (54) (55) (56) (57).

The oxysterol 24(S),25-EC is made in a shunt in the cholesterol biosynthetic pathway in all cholesterogenic cells and is an activator for the LXR (58) (23) (28). This compound may function as an endogenous inhibitor of newly synthesized cholesterol accumulation in cells (59). Inhibition of the enzyme 2,3-oxidosqualene:lanosterol cyclase elevates intracellular levels of 24(S),25-EC and activates LXR signaling in cultured macrophages (60) (61). Overexpression of an enzyme 2,3-oxidosqualene cyclase in Chinese hamster ovary cells selectively inhibits the synthesis of 24(S),25-EC. That results in decreased expression of LXR target gene, ABCA1 and increased acute cholesterol synthesis by an increase in HMG-CoA reductase gene expression (62). A recent observation that 24(S),25-EC impairs cholesteryl ester hydrolysis in macrophages and reduces the availability of cholesterol for efflux to cholesterol acceptors indicates that the in vivo role of oxysterols might be even more complex (63).

22(R)-HC is also a naturally occurring oxysterol that can activate LXR (28) and has been widely used as a positive control agonist for the receptor.

Recently, oxysterols have been proven to be LXR ligands in vitro and in vivo. Evidence came from the overexpression of an oxysterol catabolic enzyme, cholesterol sulfotransferase that inactivated LXR signaling in cell lines and in mice without altering the responsiveness to a non-sterol synthetic LXR agonist T0901317 (64). Song et al. reported that sulfated forms of 7-ketocholesterol and 5a,6a-epoxycholesterol, which are present in blood and have been found in atherosclerotic plaques inhibit LXR activity. If sulfated forms of these oxidized sterols are also present, they may have an important role in foam cell formation by inhibiting LXR function (65). Chen et al. also generated triple-knockout mice deficient in the biosynthesis of three oxysterol ligands of LXR, 24(S)-HC, 25-HC and 27-HC. These animals respond to dietary T0901317 by inducing LXR target genes in liver but show impaired responses to dietary cholesterol (64).

Another piece of evidence supporting the in vivo role of oxysterols in LXR activation came from the effect of statins, inhibitors of cholesterol synthesis, on LXR signaling.

Statins decrease expression of LXR target genes by inhibiting the synthesis of an oxysterol ligand for LXR, 24(S),25-EC (66) (67). Nevertheless, the influence of statins on LXR activity is controversial, because atorvastatin treatment was reported to activate PPARγ and increase LXR-mediated gene expression and cholesterol efflux through inhibition of RhoA signaling, leading to increased PPARγ activity, enhanced LXR activation, increased ABCA1 expression, and cholesterol efflux (68) while others showed the opposite that it inhibits LXR function in macrophages (69).

Certain natural 6a-hydroxylated bile acids are also receptor-specific activators of LXRα (50) and acetylpodocarpic dimer was shown to be a potent, selective agonist for both LXRα and LXRβ (70) (Figure 2).

The first nonoxysterol natural product that functions as a ligand for the LXR was paxilline, a fungal metabolite. Paxilline binds directly to both receptors and is an activator of LXR-dependent transcription in cell-based reporter assays (71). Guttiferone I, a new prenylated benzophenone from Garcinia humilis acts as an LXR ligand (72).

Screening of a natural product library of microbial extracts using a LXR-scintillation proximity binding assay and bioassay-guided fractionation led to the discovery of potential LXR ligands (73) (74) (75) (76). A novel LXRα activator identified from the natural product Gynostemma pentaphyllum (77).

Substituted 3-(phenylamino)-1H-pyrrole-2,5-dionesmaleimides have been identified in high throughput screens as LXR agonists and ligand-bound crystal structure has been determined (78). Liverwort-derived riccardin C and F were identified as an LXRα agonist/LXRβ antagonist and an LXRα antagonist, respectively (79). 15-Ketosterol was shown to be a partial agonist for LXRα and LXRβ (80).

A few gene-selective LXR modulators have been reported so far. Derivatives of phytosterols can act as LXR agonists. YT-32 ((22E)-ergost-22-ene-1,3-diol), which is related to ergosterol and brassicasterol, is the most potent LXR agonist. YT-32 directly binds to the receptor, induces interaction with cofactors and while general LXR activators simultaneously act on ABC transporters and lipogenic genes this compound was reported to selectively act only on ABC transporters without increasing plasma triglyceride levels (81).

A synthetic oxysterol, N,N-dimethyl-3b-hydroxycholenamide (DMHCA), represents a gene-selective LXR modulator that mediates potent transcriptional activation of ABCA1 gene expression while exhibiting minimal effects on SREBP-1c both in vitro and in vivo in mice. DMHCA has the potential to stimulate cholesterol transport through the upregulation of LXR target genes, including ABCA1, in liver, small intestine and macrophages. However, DMHCA exhibits only limited activity for increasing hepatic SREBP-1c mRNA and does not alter circulating plasma triglycerides (82).

The most unexpected molecule has been associated with LXR activity recently. Mitro et al. demonstrated that glucose could bind to and stimulate LXR activity assigning the receptor a potential glucose sensor function (83). However, others reported that glucose is required for the transcription factor carbohydrate-responsive element binding protein and that LXRs are not necessary for the induction of glucose-regulated genes in liver (84). Therefore the contribution of glucose to LXR activity requires further studies.

N-Acylthiadiazolines, a new class of LXR agonists have been characterized. These molecules show increased selectivity for LXRβ (85). Despite the selectivity and modest potency, the compound still induce cholesterol efflux from macrophages with full efficacy. These data show that LXRs are likely to act as a sensor of various and chemically diverse group of compounds, and these also show that the constrains on ligand binding are not particularly stringent.

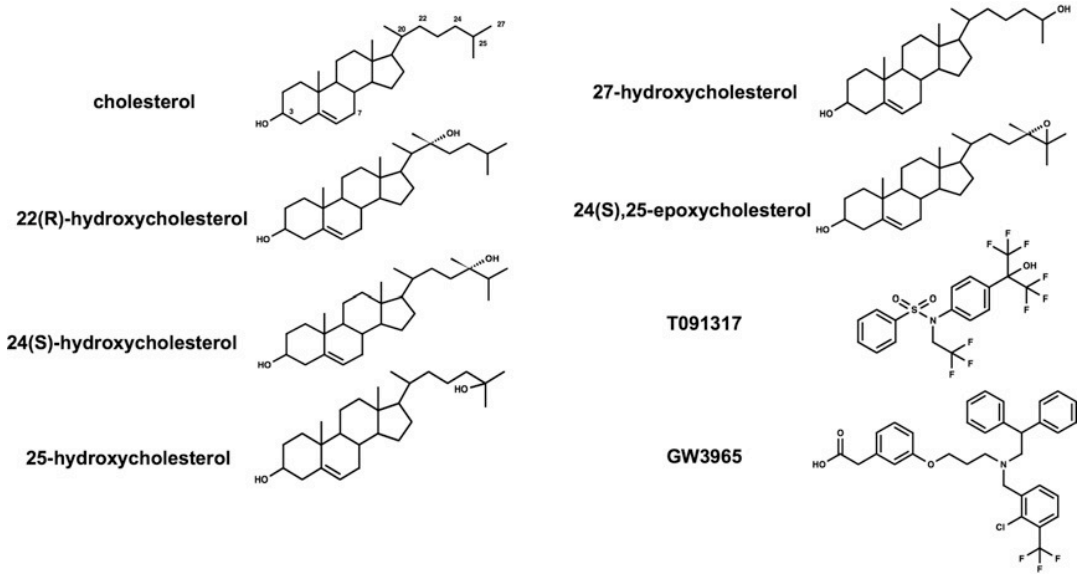

Figure 2. Natural and synthetic LXR activators. A list of the most common endogenous oxysterols, which can activate LXR and the two synthetic agonists, most often used for LXR activation.

Biological role of LXRα

The biological role of the receptor was revealed by using knockouts in mice and by the identification of the directly regulated genes in various cellular contexts.

The studies on LXRα-/- mice, but not the LXRβ-/- mice, showed a marked cholesteryl ester accumulation in their liver when fed with diets containing cholesterol. This led to the identification of CYP7A1, the rate-limiting cytochrome p450 enzyme in bile acid synthesis as the first known direct target of LXR (86). The different phenotype of the two knockout mice strains also indicated that the two LXR isoforms although share considerable sequence homology and with different activity but respond to the same ligands, might have distinct roles.

Further studies showed that LXR also regulates lipid metabolism at various levels. Activation of the receptor induces expression of sterol regulatory element binding protein-1c (SREBP-1c) a central regulator of fatty acid synthesis (30) (87) (66) (88). Induction of SREBP-1c and further identification of other target genes driving lipogenesis, such as, fatty acid synthase (89), acyl-CoA carboxylase (90), and stearoyl-CoA desaturase 1 (91) linked dietary cholesterol intake to triacylglycerol synthesis and explained the phenotype of mice treated with LXR ligands, displaying increased hepatic and plasma triglycerides. This effect poses a serious limitation on the efforts to introduce LXR ligands as therapeutics (87) (92) (93).

The spectrum of biological activities has been widened with the identification of other target genes involved in the regulation of lipid transport like lipoprotein lipase (LPL) (94), cholesteryl ester transfer protein (95), phospholipid transfer protein (PLTP) (the lipoprotein-remodeling enzyme) (96) (97) (98), apolipoprotein E (apoE) (99) (Laffitte et al., 2001a) and the apoCI/CIV/CII gene cluster (100) that control the secretion and metabolism of lipoproteins rich in triglycerides and has a key role in the reverse cholesterol transport in various cell-types by increasing high density lipoprotein (HDL) levels and net cholesterol secretion.

Furthermore, LXR induces genes involved in uptake of lipoprotein particles, like low density lipoprotein receptor (LDLR) (101) and scavenger receptor (SR) BI (102) (103). LXR also regulates lipid transport at the cellular level. Studies on LXRs indicate that they control the expression of members of the ATP-binding cassette (ABC) superfamily of membrane transporters such as ABCG5 and ABCG8 (104) (105) in the intestine (regulators of bile acid excretion and intestinal cholesterol absorption) or ABCA1 and ABCG1 in macrophages (involved in cholesterol efflux) (106) (107) (108) (109) (110).

By the discovery that LXRα is regulated by PPARγ, LXRs became integrated into an anti-atherosclerosis transcriptional cascade, the PPARγ–LXRα–ABCA1 axis that involves particle uptake, processing and cholesterol removal upon oxLDL stimulus during the process of atherogenesis as discussed later (15).

In vivo consequences of LXR activation have been studied via two approaches. First, LXR activators, generally highly potent synthetic agonist were administered to mice to activate LXR throughout the body. Second, LXR genes were knocked out from mice and the animals were challenged by various stimuli. Administration of a synthetic LXR agonist T0901317 induces expression of LXR target genes, like ABC transporters and lipogenic genes and results in an increase in triglyceride and phospholipid levels and in hepatic steatosis in mice (87) (92) (93). T0901317 decreased atherosclerotic lesion development in LDLR knockout mice (111).

Another LXR agonist, GW3965 induced expression of LXR target genes in the small intestine and also in macrophages and increased reverse cholesterol transport by increasing high density lipoprotein cholesterol concentration (33).

GW3965 was used to treat mice in two models of atherosclerosis, LDLR knockout and apoE knockout animals (112). Ligand treatment induced the expression of ABC transporters in the vessel walls and reduced atherosclerotic lesion size in males and females in both models.

By regulating the expression of ABCA1 and the rate-limiting enzyme in bile acid synthesis, CYP7A1, rexinoids (synthetic RXR activators) were shown to inhibit cholesterol absorption from the intestines and repress bile acid synthesis in the liver. This was due to the activation of FXR, another nuclear receptor by the rexinoid, which in turn inhibited bile acid synthesis through CYP7A1 and LXR was also activated, which led to

increased reverse cholesterol transport and blocked cholesterol absorption through ABCA1. LXR is required for the cholesterol absorption as shown with the lack of absorption inhibition in LXRα/β double knockout mice (107).

Similarly, induction of ABCA1 and enhanced cholesterol efflux were observed in mice after rexinoid treatment, which leads to decreased atherosclerosis (113). These were due to the activation of LXR:RXR heterodimer by the rexinoid.

Recently, T0901317 was reported to act at multiple levels to inhibit atherosclerosis. It suppresses endothelial monocyte adhesion and suppresses NF-κB activity and consequently expression of adhesion molecules (114).

A novel, steroidal synthetic LXR agonist N,N-dimethyl-3b-hydroxy-cholenamide was shown to attenuate plaque formation in apoE-deficient mice without inducing liver steatosis and hypertriglyceridemia (115).

Indazole-based LXR modulators are synthetic partial agonists for the receptor with slightly weaker potency and efficacy on LXRα than on LXRβ. These compounds reduced lesion size when tested in LDLR knockout mice but did not stimulate hepatic triglyceride synthesis (116).

3,6,24-Trihydroxy-24,24-di(trifluoromethyl)-5b-cholane (ATI-829) is a novel potent synthetic steroidal LXR agonist. It has poor effects on SREBP-1c expression while efficiently induce ABCA1. In male LDLR-deficient mice, ATI-829 selectively activated LXR target gene expression in mouse intestines and macrophages but not in the liver. Consequently, no significant increase in triglyceride levels was observed while atherosclerosis development was inhibited in certain vessels (117).

Mice lacking LXRα lose their ability to respond normally to dietary cholesterol and are unable to tolerate any amount of cholesterol in excess of what they synthesize de novo (24) (113) (99). These mice develop severe atherosclerosis.

While LXRα knockout mice accumulate cholesterol in the liver but not in the periphery LXRα apoE double knockout animals exhibit cholesterol accumulation in the peripheral tissues with accelerated atherosclerosis. However, the synthetic agonist GW3965 can compensate the lack of LXRα by activating LXRβ (118).

Serum and hepatic cholesterol levels and lipoprotein profiles of cholesterol-fed animals revealed no significant differences between LXRβ knockout and wild-type mice. On

normal chow increased expression of cholesterol biosynthetic enzymes were observed (119). These suggest a difference between LXRα and LXRβ in controlling cholesterol metabolism and a resistance to dietary cholesterol in LXRβ deficient mice.

By using bone marrow transplantation LXR was eliminated from bone marrow-derived cells. This condition resembled Tangier disease, a human disease caused by ABCA1-deficiency and subsequent decrease in HDL level. This provided evidence that macrophage LXR is required for the LXR agonist to exhibit its anti-atherosclerotic effects (120). Similar results were reported by Levin et al. (121).

Macrophage-specific overexpression of LXRα in LDLR-deficient mice results in normal plasma lipid and lipoprotein profile and significantly reduced lesion size with elevated expression of target genes and increased cholesterol efflux (122).

With the identification of its target genes LXR became a major player in our understanding of its role in the mechanisms of (i) bile acid metabolism in liver, through metabolism, storage and efflux (ii) enterohepatic cholesterol circulation, by limiting the extent of cholesterol absorption, and (iii) regulation of cholesterol metabolism in macrophages of atherosclerotic plaques. As a result of the work of the Tontonoz laboratory another important aspect of LXR in macrophage biology was uncovered by showing that in macrophages LXRs are not just in the centre of the pathogenesis of atherosclerosis but also control inflammation and immunity. This started a new era in LXR research by integrating lipid metabolism and immune functions in macrophages (123) (124) (Figure 3).

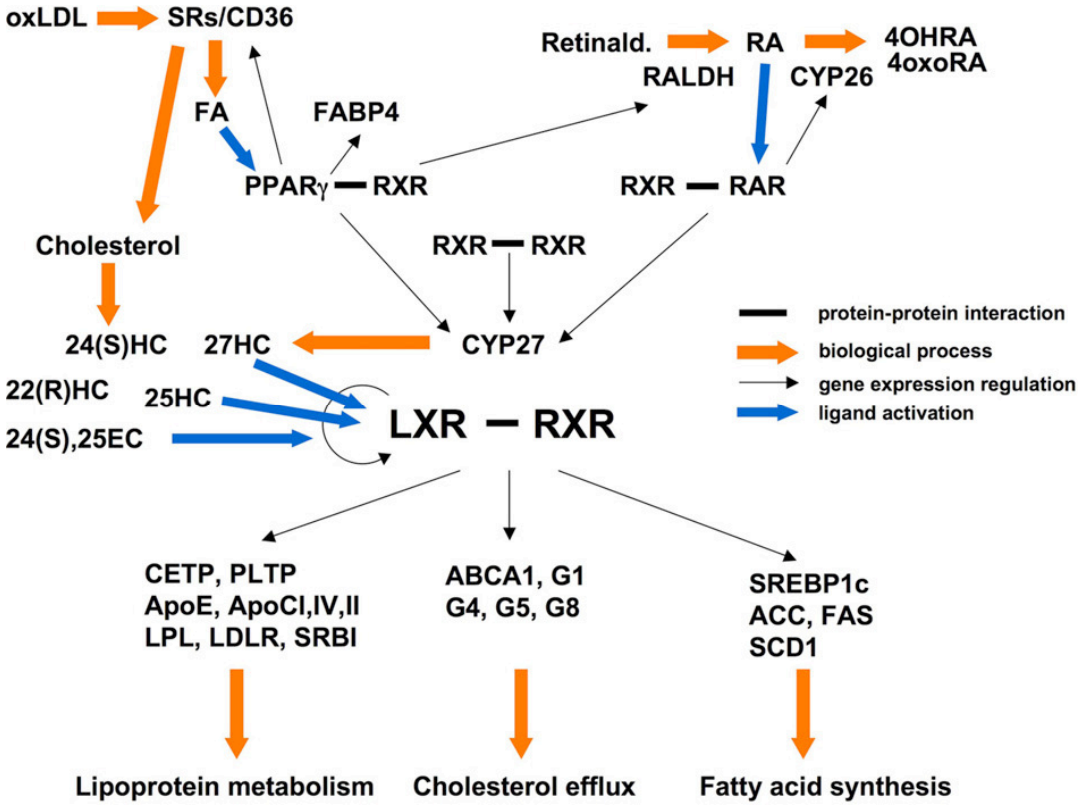

Figure 3. The LXR-centered regulatory network. There is a network of transcription factors, metabolic enzymes and target genes with several feed-forward and feed-back loops that regulate the activity of the PPARγ–LXR axis. The four types of interactions: (i) protein–protein interaction, (ii) activation of a biological process, (iii) activation of transcription and (iv) endogenous ligand production that results in the activation of a nuclear receptor.

2.3. Peroxisome Proliferator-Activated Receptor gamma

PPARγ controls a broad range of cellular responses: differentiation, proliferation, cell death and inflammation. It was originally shown to be required for adipocyte differentiation (125) (126). In mice it is essential for placental development and vascularization (127, 128) (129). PPARγ can be activated by natural ligands such as oxidized fatty acids (130), oxLDL components, 11,13-hydroxyoctadecadienoic acid (131, 132), 15D-PGJ2 (133-135) and the thiazolidinedione (TZD) class of insulin-sensitizing

drugs (136) that have been developed and used in the treatment of type II diabetes mellitus. The fact that the receptor has a relatively large ligand binding pocket results in the possibility that two ligands can simultaneously bind to the receptor. Recently, it was shown that oxo fatty acids can be coupled covalently to the receptor resulting in a more potent activation than noncovalently bound ligands (130).

PPARγ deficiency resulted in early embryonic lethality. Deficiency interferes with terminal differentiation of the trophoblasts and placental vascularization, leading to severe myocardial thinning and death by E10.0 (127). Tetraploid-rescued mutant exhibited another lethal combination of pathologies, including lipodystrophy, fatty liver and multiple hemorrhages (137) (138). Those any PPARγ null mice survived to term were deficient for all forms of fat providing evidence for the fundamental role of PPARγ in adipogenesis (127).

PPARγ was also shown to influence myeloid development (131, 132) (139). PPARγ has not appeared to regulate the formation of the monocytic lineage but modulates differentiation and metabolic functions of macrophages. Recent observations suggest that although PPARγ is not necessary for monocyte differentiation (140) (140) modulation of the level and activity of PPARγ has consequences in the fate and metabolism of a macrophage. Transplantation of PPARγ null bone marrow into LDLR-/- mice results in a significant increase in atherosclerosis (141). It was reported by the Glass laboratory that TZDs greatly inhibited the development of atherosclerosis in LDLR-deficient male mice (142). Similar results were shown by Chen and colleagues in apoE-/- mice, another murine atherosclerosis model (143). Targeted disruption of the PPARγ gene from macrophages resulted in reduced total plasma and HDL cholesterol levels and cholesterol efflux was significantly decreased from macrophages elicited by thioglycolate in mutant mice (144). Based on these observations PPARγ can be considered an anti-atherogenic molecule.

PPARγ is expressed in foam cells of atherosclerotic lesion and its expression could be further increased with oxLDL. PPARγ enhances uptake of oxidized but not native LDL (132) by inducing the transcription of scavenger receptor CD36. Two components from the lipids in oxLDL, 9-HODE and 13-HODE were identified as endogenous activators and bone fide ligands of PPARγ (131). These results suggested a novel model of

macrophage lipid metabolism. Macrophages internalize modified LDL via scavenger receptors (i.e., CD36), which unlike LDLR are not downregulated by high intracellular cholesterol levels. On the contrary, oxLDL increase the expression of a scavenger receptor CD36 by PPARγ and further induces its own uptake. Oxidative modification first allows LDL particle to bind to scavenger receptors and second it induces oxidation of its components, e.g., linoleic acid is converted into effective activators of PPARγ, 9-HODE and 13-HODE. Thus, the consequence of oxLDL internalization is the initiation of a positive feedback loop that enhances the expression of CD36.

There are other scavenger receptors, which can be also utilized for lipid uptake. OxLDL also induces SR-A expression via a PPARγ independent way. These findings provided an explanation for the formation of lipid-loaded macrophages (foam cells) and suggested the existence of a vicious cycle leading to atherosclerosis.

This model also implied that PPARγ might act as a proatherogenic factor. To further dissect the in vivo contribution of PPARγ to the process of atherosclerosis Chawla et al. generated mice lacking PPARγ in the macrophages. Since PPARγ total knockout animals are not viable chimeric mice were used for bone marrow transplantation into irradiated animals. Transplantation of PPARγ null bone marrow into LDLR knockout animals resulted in significant increase of the atherosclerotic lesion size. This contradiction was resolved by showing that activation of PPARγ not only results in cholesterol uptake but it can also increase cholesterol efflux from the macrophages. It was proven that PPARγ could directly induce transcription of the oxysterol receptor, LXR (15).

Seeking the molecular mechanism assigned a central role for PPARγ and LXR in regulating cholesterol uptake and efflux during foam cell formation.

Cholesterol influx and efflux in macrophages: role for PPARγ and LXR

PPARγ and LXR share some common features: both receptors form heterodimers with the retinoid X receptor (RXR), their endogenous activators reported so far are oxidized lipid molecules, oxidized fatty acids for the PPARγ and oxidized sterols for the LXR. Both ones have been involved in the regulation of lipid metabolism in adipocytes, macrophages or in the liver. 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 regulates macrophage response to oxLDL.

Remarkably, the result of LXR activation is cholesterol efflux from macrophages. Among LXR target genes there are transporter molecules, members of ABC transporter family, which are known to transport lipid molecules across the cell membrane and are involved in the reverse cholesterol transport. ABCA1 and ABCG1 are members of this family. They are highly expressed in lipid-loaded macrophages (104). Mutations in ABCA1 gene result in Tangier disease, a disease characterized by marked cholesterol accumulation in macrophages and other reticuloendothelial cells (145) (16, 146, 147). Cholesterol clearance is impaired in 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 ABCG4, ABCG5 and ABCG8 (30, 106) (104, 108, 148) (149) (109, 150) (110). 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 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 (15). There are several types of interactions by which one element regulates the other. These generally lead to further activation of subsequent reactions.

We distinguished four types of interactions: (i) protein–protein interaction, (ii) activation of a biological process, (iii) activation of transcription and (iv) endogenous ligand production that results in the activation of a nuclear receptor.

Based on these studies and results it seems that a coordinated lipid transport exists in macrophages orchestrated by basically two nuclear receptors, PPARγ and LXR. A series of molecules are involved in this cycle from the PPARγ side and the switch from this increased uptake towards the LXR-regulated lipid efflux. oxLDL induces PPARγ and lipid components activate it to increase scavenger receptor (e.g., CD36) levels. Increased CD36 causes further oxLDL uptake. PPARγ also induces the expression of LXRα, which once activated can induce transcription of cholesterol transporters e.g. ABCA1 and these lead to increased cholesterol efflux to ApoAI from macrophages. The existence of this transcriptional cascade 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. The model also suggests that during atherosclerosis when continuous supply of lipid molecules is maintained the decision maker molecule is the LXR. Its activity determines if lipids are eliminated through cholesterol efflux towards HDL or accumulate and form foam cells from macrophages to induce lesion formation.

It has been an appealing idea that lipid molecules from lipoproteins might act as activators or ligands for both PPARγ (131) and LXR (16). The fact that LXR signaling is activated in macrophages exposed to acetylated LDL (16), 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 sufficient for getting an activated LXR that induces cholesterol efflux. Most likely, it needs to be activated by its endogenous ligand. The search for real endogenous LXR activators placed oxysterol-producing enzymes in the focus of LXR research.

2.4. Lipid signaling and inflammation

Recent data suggest that both PPARγ and LXR participate in further regulatory processes in the macrophages and related cells. These include a complex cross-regulation between basic lipid metabolic pathways and inflammatory reactions. On one hand there are a few reports so far that raise the possibility that inflammatory conditions might be of major importance in the regulation of these receptors‘ activity and consequently lipid metabolism. On the other hand involvement of inflammatory reactions in basic metabolic disorders such as atherosclerosis and obesity attracts more and more attention.

LXR under inflammatory conditions

Macrophages play an essential role in innate and adaptive immunity. The discovery that activation of both LXR isotypes in cultured macrophages repressed the expression of

inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), IL-1b, monocyte chemoattractant protein-1 and -3 (MCP-1 and MCP-3) and the metalloprotease MMP-9 in response to bacterial infection or lipopolysaccharide (LPS) stimulation and at the same time kept its regulatory effect on the expression of genes involved in lipid homeostasis strongly indicated that LXRs play a pivotal role in translating signals of lipids to inflammatory responses (151) (124). By examining the phenomenon that mice lacking LXRα are highly susceptible to infection when exposed to intracellular bacteria Listeria monocytogenes, Joseph et al. identified AIM (also known as SPa, API6 and CD5L), a gene to promote macrophage survival, a target for LXRα but not for LXRβ, showing an important, but distinct roles of the two isoforms in macrophage biology.

Extending their studies they found that AIM protects macrophages from apoptosis too, when exposed to oxLDL during formation of atherosclerotic plaques through the LXR:RXR pathway (152). Valledor et al. reached a similar conclusion by showing the upregulation of other apoptotic regulators such as Bcl-XL and Birc1a and the inhibition of proapoptotic elements (caspases 1, 4/11, 7, and 12; Fas ligand; and DNase 1l3) by LXR:RXR in macrophages infected with Bacillus anthracis, Escherichia coli, or Salmonella typhimurium (153). As oxLDL induces not just the genes involved in cholesterol efflux from cells, but also the expression of AIM, promoting the expansion of the lesions leaves a question remained to be answer regarding the relative contribution of LXR in atherosclerosis formation. As a partial explanation given by Arai et al., the LXRα selective induction of AIM suggests that this isotype is predominantly atherogenic, while the b isotype is atheroprotective, noting however that LXRα is also involved in inducing the expression of genes that mediate cholesterol efflux (154).

Further work characterized an important crosstalk between LXR and TLR signaling in cultured macrophages as well as in aortic tissue in vivo. By showing that microbial ligands through the activation of TLR3 and TLR4 through a interferon regulatory factor 3-dependent (IRF3) pathway can block the induction of LXR target genes, such as ABCA1 and inhibit cholesterol efflux from macrophages proposed a mechanism via which pathogens may be involved in the formation of atherosclerosis through interacting with the LXR pathway (151). In vivo data further showed that activation of LXR reduced

inflammation in a mouse model of contact dermatitis and exerted inhibition of inflammatory gene expression in the aortas of atherosclerotic mice (112) (155).

As mentioned in the introduction LXRs and PPARγ can also exert their anti-inflammatory role in a ligand-dependent repression of overlapping but distinct set of inflammatory genes not as activators of transcription but as promoter specific repressors. The work primarily from the Glass laboratory demonstrated that the small ubiquitin-related modifier (SUMO)ylated forms of the ligand-activated both LXRs and PPARγ may be recruited to the promoters of inflammatory genes and inhibit the LPS induced corepressor (NCoR) clearance. Dissecting the role of LXRs and PPARγ in this novel type of transrepression a particular difference was found in different SUMO Acceptor Sites of the receptors. It was shown that while LXRs are SUMOylated by SUMO2 and SUMO3, PPARγ is by SUMO1 (6). The authors also demonstrated that activation and transrepression activities of LXRs can be chemically separated. While the naturally occurring LXR ligands such as 22(R)-HC, 24(S),25-EC, and 24-HC each repressed the activation of iNOS and induced the expression of ABCA1, 25-HC and 27-HC were able to activate ABCA1 but did not repress iNOS activation. The difference observed in the activating natural ligands could raise the possibility that by altering/regulating the expression of enzymes producing the ligands, different subsets of cells could be generated (6).

It is worth emphasizing that the role of LXRs is more complex in inflammation and it is a very simplified approach to assume that LXRs only have anti-inflammatory roles. Recently it has been shown that mice deficient in both LXR isoforms, (LXRα-/-LXRβ-/- mice), were more susceptible to M. tuberculosis infection, developing higher bacterial burdens and an increase in the size and number of granulomatous lesions showing that the role of LXR in inflammation is more complex and depends on the cellular and inflammatory context. Another observation, supporting a more pro- inflammatory role of LXR was published by Fontaine et al. showing that while in primary human macrophages short-term pretreatment with LXR agonists significantly reduced the inflammatory response induced by LPS as it is observed in mouse macrophages, but when pretreated with LXR agonists for 48 hours, macrophages exerted an enhanced LPS response leading to an increased MCP-1 and tumor necrosis factor (TNF) secretion and increased reactive

oxygen species generation (156). The observations that LXRs have distinct regulatory roles in human macrophages from those previously identified in mouse with a notable example of LXR activation leading to an increase in TLR4 expression and signaling in human but not in murine macrophages, calls for further studies to reconcile the results of mice models and in vitro/ex vivo human systems.

More recently, in work from the Tontonoz laboratory T cell activation was addressed from the T cell angle. In a mouse model ligand activation of LXR inhibited mitogen driven expansion, but not the activation of the T cells that could be achieved also by using 22(R)-HC in a dose dependent manner. The work showed that the loss of the b isoform – the form that is expressed in T cells – conferred a proliferative advantage of T cells that was suggested previously from the phenotype of the LXR null mice that exhibited age-dependent splenomegaly and lymphadenopathy. Furthermore the paper showed that the expression of LXR target genes (ABCA1, ABCG1 and SREBP-1c) was reciprocally regulated if compared to the synthesis of cholesterol, fatty acid and phospholipids, which processes are induced during activation of lymphocytes. This reciprocal regulation can be explained by the induction of sulfotransferase 2B (SULT) during activation of lymphocytes that catalyzes the transfer of sulfate groups to oxysterols thus inactivating them as LXR ligands. Among the LXR target genes ABCG1, but not ABCA1 was found to be the key player. As ABCG1 is a key component in cholesterol and oxysterol efflux the role of the SULT2B–LXR–ABCG1 axis is proposed to be responsible for coupling cholesterol metabolism and proliferation by maintaining the cholesterol within the cell (157).

PPARγ in inflammation

With the discovery that PPARγ controls lipid metabolism and inflammation at the same time more and more efforts were made to gain evidence on how PPARγ controled lipid metabolism and processing contributes to immunregulation. The role of PPARγ under inlammatory conditons has been widely studied in the cell types of the immune system where the receptor is expressed and can be avtivated, such as macrophages, DCs, T cells, B cells, NK cells, mast cells, eosionophils, neutrophils and basophils. Using knock out animal models its relation to diseases has also been characterized (158).

The macrophage is the most interesting cell type in understanding the cross-talk of lipid metabolism and inflammation. As discussed previously transformation of macrophages into foam cells is the initiating step in the development of atherosclerosis in which PPARγ plays a pivotal role. At the same time macrophages also provide the first line of defense against various microorganisms where the activity of PPARγ is affected not just by the lipid environment but also by inflammatory stimuli interlocking the two seemingly distant fields.

Studies on macrophages also identified novel natural activators of PPARγ and pathways that might be involved in the production of PPARγ ligands providing the complexity of the regulatory network where the receptor is not just present but could also be activated under (patho)physiological condtions. 9-HODE and 13-HODE that are present in both chemically oxidized LDL and LDL from atherosclerotic plaques, and their concentration correlate with the stage of lesion are great examples for such activators (131). The 15-lypoxygenase, an enzyme involved in the oxidation of LDL, and its orthologue in mouse, 12/15-lipoxygenase that generates 13-HODE and 15-HETE from linoleic and arachidonic acid, was found to be induced in cultured human monocytes upon IL-4 treatment in parallel with the receptor itself suggesting the potential of coordinated induction of both receptor and activating ligands (159) (160).

The first milestone in the anti-inflammatory effects of PPARγ ligands was the work of Jiang et al. demonstrating that both 15d-PGJ2 and TZDs could inhibit the elaboration of inflammatory cytokines (TNFα, IL-6 and IL-1β) from monocytes induced by phorbol myristyl acetate (PMA) (161). At the same time Ricote et al. showed that these compounds could also inhibit the expression of the inducible nitric oxide synthase (iNOS), gelatinase B (MMP-9) and scavenger receptor A (SR-A) mRNAs (162).

The debate on the exact role of PPARγ in inflammation is still open based on the fact, that 15d-PGJ2 can not be considered as a highly selective ligand for PPARγ, and that the concentration of TZDs required to exert anti-inflammatory effects were significantly higher than that for target gene activation (163) and that non-TZD PPARγ agonists failed to induce anti-inflammatory responses (164). These findings suggest the existence of at least partly, PPARγ-independent mechanisms that called for further studies on macrophages to distinguish PPARγ-dependent and -independent effects of 15d-PGJ2 and

TZDs. The studies of Chawla et al. (165) and Moore et al. (140) on PPARγ−/− murine monocytic lineage showed that PPARγ is not essential for myeloid development and both 15d-PGJ2 and TZDs have anti-inflammatory effects (inhibition of iNOS and COX2 expression, TNFα and IL-6 production) that are independent of PPARγ.

As a conclusion PPARγ still remains to be a key factor in regulating, at least in some aspects, macrophage lipid metabolism and plays a role as a repressor in inflammatory responses. The mechanisms how these two processes are connected and the contribution of macrophage specific PPARγ-induced gene expression to inflammatory responses in vivo remains to be explored.

Animal studies in models of colitis ulcerosa, rheumatoid arthritis (RA) and multiple sclerosis (MS) further supported the anti-inflammatory role of PPARγ carried out on various cell lines such as colon cancer cell lines Caco-2 and HT-29 (166) or in vitro cultures of synoviocytes from RA patients, where both 15d-PGJ2 and troglitazone was found to inhibit proliferation of synoviocytes and induced apoptosis at higher doses (167). In the experiments PPARγ +/- mice were more susceptible to induced colitis, as in these animals both the size of the histological lesions and the mRNA levels of inflammatory cytokines such as TNFα and IL-1β were greater. Moreover PPARγ agonists were effective not just in preventing development of inflammation but also in cases with already persisting inflammation (168). Setoguchi et al. also reported that PPARγ+/− mice showed an increased joint thickness shortly after antigen challenge in an antigen induced arthritis model in which B cells were found to have greater proliferative responses to stimulatory agents if compared to wild type and the hyperreactivity of B cells was suppressed when mice were treated with PPARγ agonists (169) suggesting that B cells might also be important in translating the effect of PPARγ in RA. Studies in a PPARγ+/− mouse model in which experimental allergic encephalitis (EAE) was induced with a protocol causing a phenotype that is very much like the human disease MS, PPARγ+/− mice developed an exacerbated and prolonged EAE that was associated with a more severe demyelinization and inflammation than in wild type. For the underlying mechanism the Th1 response is likely to be the candidate that was increased more in PPARγ+/− mice (170).

2.5. Dendritic cells

Dendritic cells are the most potent antigen presenting cells of the immune system playing important roles in the initiation and maintenance of the primary immune response, in mediating signals to activate adaptive immunity and are also involved in inducing tolerance (171). Several subtypes of DCs have been identified to date of both myeloid and lymphoid origin (the two main categories are conventional DCs such as interstitial DCs and Langerhans cells, and plasmacytoid DCs). Upon capturing antigens immature DCs (iDCs) transform into mature DCs (mDCs) and migrate to the lymphatic nodes from the periphery to present antigens and activate T cells. During this maturation process DCs almost completely loose their capacity to take up antigens and fulfill their ultimate functions: antigen presentation and activation of different subsets of lymphocytes. Their antigen-presentation function is not restricted to the presentation of peptides by MHC-II molecules, they also present glycolipids in complexes with CD1 molecules and endo- or exogenous antigens with MHC class I molecules (cross-presentation). Co-stimulatory molecules (CD80, CD86 and CD40) are also involved in activation coupled with cytokine production, resulting in the induction of different subsets of T lymphocytes regulating the inflammatory or anti-inflammatory cascade triggered by infectious agents and self antigens, respectively (172) (173) (Figure 4).

We and others have showed that lipid signaling has effects on the maturation and function of dendritic cells. Some of these effects are mediated by the nuclear hormone receptors.

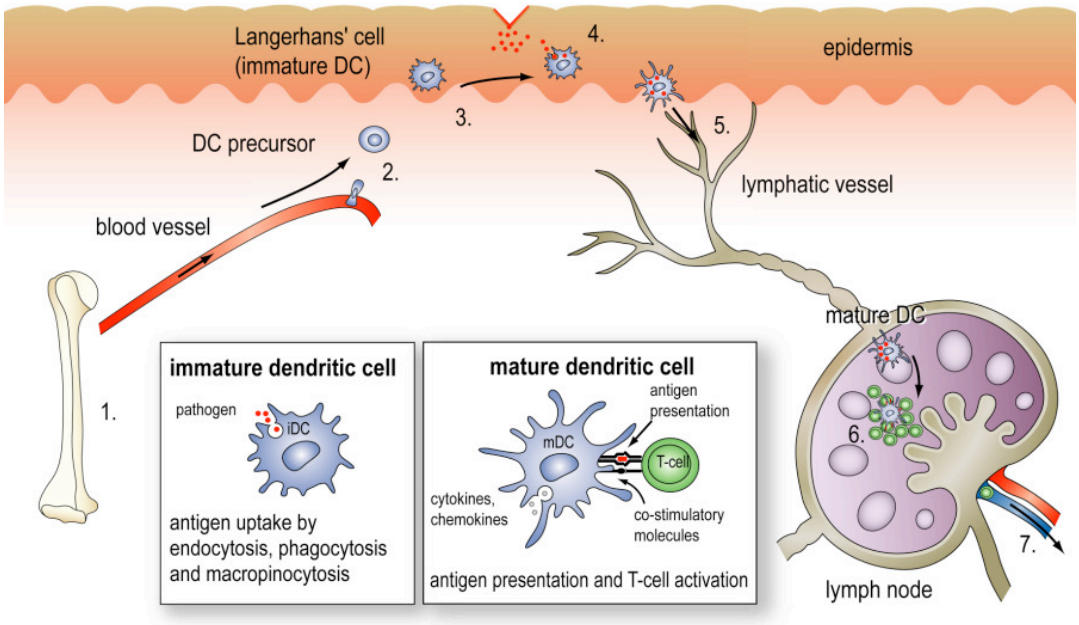

Figure 4. Stages of Langerhans' cells (LCs) differentiation. (1) LCs are bone-marrow-derived cells. (2) The precursors of LCs leave the blood vessel. (3, 4) LCs in the immature state are sentinels of the epidermal layer, well equipped for antigen uptake. (5) Antigen uptake associated with signals from surrounding tissues leads to maturation and migration to draining lymph nodes. (6) In the lymph node, mature LCs present antigens to naive T lymphocytes and activate them. (7) Activated T lymphocytes leave the lymph node to fulfill their function. (The figure is adopted from Széles L., Törőcsik D., Nagy L. At the crossroad of lipid metabolism and inflammation. The role of PPAR-g, a lipid activated transcription factor – review, Boehringer Ingelheim Fonds – FUTURA (2006) 21: 79-85.)

2.6. Role of nuclear receptors in dendritic cells

The role of nuclear receptors such as PPARs, RAR and VDR, that are found to be expressed at high levels in differentiating DCs and are activated upon exposure to various lipids such as fatty acids, retinoids or by active Vitamin D3, were extensively studied in DC differentiation and maturation by us and others. These studies showed that PPARs,

RAR and VDR, were found to support a tolerogenic DC phenotype, both through the regulation of surface molecules, interacting with stimulatory effects such as TLR ligands (2, 3, 4, and 7) and cytokine productions resulting in a reduced capacity to stimulate T-cell proliferation (174-180).

One of the most studied nuclear receptors in DC biology is the PPARγ, that just as in macrophages has an impact on both lipid homeostasis and immunoregulation. In our studies we found that during DC differentiation PPARγ primarily alters lipid metabolism and via this, indirectly modifies the immune phenotype. We found that only genes related to lipid metabolism are overrepresented among early induced genes leading to a diminished lipid accumulation, and genes related to immune response are regulated only after 24 hours. Similarly to glucocorticoids and 1α,25-dihydroxyvitamin D3, PPARγ also enhanced endocytosis in immature DCs (181) (182) (177). During maturation, DCs migrate to draining lymph nodes, principally due to changes in their chemokine receptor profile, including CCR7, that allows DCs to follow CCL19 and CCL21 chemokine signals released from the lymphatic vessels. CCR7 was found to be inhibited in DCs activated by PPARγ agonists (175). Our group and others also found that PPARγ activation of DCs changed the profile of CD1 molecules that bind and present glycolipids and are important in lipid–antigen presentation of DCs. Our group found that PPARγ ligand treatment not just down regulates the expression of CD1a, but also induced CD1d that resulted in an increased activation of iNKT cells (177).

It was also reported that PPARγ activators 15d-PGJ2 and/or rosiglitazone down-regulate the CD40-induced secretion of IL-12, a potent Th1-driving factor in murine and human DCs models suggesting that PPARγ activation favors a type 2 responses (183) (184). This is further supported by the papers on the decreased CD80 and induced CD86 levels, and the down-regulation of chemokines such as CXCL10 and RANTES upon PPARγ ligand treatment, which are involved in the recruitment of Th1 lymphocytes.

Despite the well-characterized role of PPARγ in dendritic cells and the importance of LXR in macrophage biology little is known about its effect on DC differentiation and function.

One report published on this issue so far reports that while LXRα is expressed in human myeloid DCs and is induced during differentiation LXRβ is expressed constitutively at a

very low level. The activation of LXR by synthetic activators at day 2 of differentiation resulted in the down regulation of the surface expression of CD86 on 5-day old iDCs. Mature DCs showed a suppressed IL-12 and an enhanced IL-10 secretion after LPS but not CD40 ligand-induced maturation. A retained adherence to the culture plate after LPS activation was also observed, explained by the regulation of actin-bundling protein fascin by LXR. This work also pointed out that LXR plays role in immunologic synapse formation and T cell activation that was found to be largely blocked after LXR activation in DCs although it should be noted that the work could not detect any change in the expression of various antigen-presenting and costimulatory molecules on DCs leaving many questions unanswered (185). Recent studies in mice also showed that tumor cells are capable of producing ligands to activate LXR, that control the migratory capacity of DCs to tumor tissues by down regulating CCR7 expression in maturing DCs. The finding strongly supports the role of LXR in favoring anti-tumor growth (186).

2.7. Aims of the studies

In our studies we aimed to find additional evidence supporting the cross-talk of inflammatory pathways and lipid activated nuclear receptors. For that we investigated the role of LXR in dendritic cell differentiation and functions and the interaction of PPARγ signaling with *M. bovis BCG* infection in macrophages.

These questions formed the basis of our works:

1. Is LXR present and could be activated in both monocyte derived and circulating DCs and does LXR signaling affect the phenotype and functions of DCs?
2. Is there an interaction between LXR signaling and inflammatory stimulus in DCs, what are the involved pathways in the interaction and what are the possible outcomes?
3. Can LXR be detected also under *in vivo* conditions?
4. Does lipid signaling and metabolism regulated by PPARγ interact with Mycobacterium infection in macrophages and what are the possible players in it?
5. What is the role of PPARγ in BCG infected macrophages?

3. MATERIALS AND METHODS

3.1. Ligands

Cells were treated with the following ligands: T090137 and BRL49653 (Alexis Biochemicals, San Diego, CA), GW3965, GW9662 (GlaxoSmithKline, Research Triangle Park, NC) and 22(R)-hydroxycholesterol (Sigma-Aldrich, St. Louis, MO).

3.2. Cell culturing and DC Generation

$CD14^+$ monocytes and blood myeloid DCs were obtained from platelet- free buffy coats from healthy donors by Ficoll gradient centrifugation followed by immunomagnetic cell separation with anti-CD14-conjugated or CD1c-conjugated microbeads, respectively (VarioMACS Separation System; Miltenyi Biotec, Auburn, CA). Blood myeloid DCs were cultured for 1 day at a density of 3.5×10^5cells/ml in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen, Karlsruhe, Germany) and 500 U/ml penicillin/streptomycin (Sigma-Aldrich). To obtain iDCs monocytes were cultured in 6-well culture dishes at a density of 1×10^6 cells/ml in RPMI 1640 supplemented with 10%FBS, 500 U/ml penicillin-streptomycin (Sigma-Aldrich), 2mM L-glutamine (Life Technologies, Paisley, UK), 500 U/ml IL-4 (PeproTech, Rocky Hill, NJ) and 800 U/ml GM-CSF (Leucomax; Gentaur Molecular Products, Brussels, Belgium). IL4 and GM-CSF were replenished on day 3. To obtain mDCs iDCs were challenged at day 5 of culturing for 48 hrs with various TLR ligands: LPS (E. coli and S. enterica serotype minnesota), LTA, Lipid A, CpG or Poly(I:C) (Sigma-Aldrich). To obtain macrophages monocytes were cultured in 6-well culture dishes at a density of 1×10^6 cells/ml in RPMI 1640 supplemented with 10%FBS (Invitrogen), 500 U/ml penicillin-streptomycin (Sigma-Aldrich) and 2mM L-glutamine (Life Technologies). Cells were maintained in a 37°C incubator venting 5% CO_2. Ligands or vehicle control (50% DMSO/ethanol) were added to the cell culture starting from the first day.

3.3. Microarray Experiment

Total RNA was isolated as described below. cRNA was generated from 5 μg of total RNA using the SuperScript Choice kit (Invitrogen , Paisley, UK) and the High Yield RNA transcription labeling kit (Enzo Diagnostics , Farmingdale, NY). Fragmented cRNA was hybridized to Affymetrix arrays (U133 Plus 2.0). Data analysis was performed using Gene Spring GX 7.3.1 software (Agilent, Santa Clara, CA). Signal for each transcript was normalized by comparing to the median signal (arbitrary value of 1.0). The microarray data presented in this article have been submitted to the Gene Expression Omnibus (GEO) under GEO accession number GSE8658.

3.4. Real-Time quantitative RT-PCR

To obtain total RNA cells were centrifuged and pelleted at the indicated times points. RNA was isolated with TRIZOL reagent (Invitrogen). The amount and quality of total RNA was determined by capillary electrophoresis analysis using an Agilent 2100 Bioanalyzer (Agilent). cDNA synthesises was performed at 42^{o}C for 120 min and 72^{o}C for 5 min (Invitrogen) using Superscript II reverse transcriptase and Random Primers (Invitrogen). Quantitative PCR was performed using real-time PCR (ABI PRISM 7900, Applied Biosystems, Darmstadt, Germany), 40 cycles of 95^{o}C for 10 sec and 60^{o}C for 1 min. The reactions were done in triplicate and comparative Ct method , which has been described by Applied Biosystems User Bulletin No.2, was used to quantify transcripts and to normalize for cyclophilin. Cylophilin expression levels did not vary between cell types or treatments. The sequences of the primers and probes are available upon request.

3.5. Immunodetection of LXRα on Cells and Tissue Sections

Human monocytes, and iDCs differentiated in the presence or absence of GW3962 were pelleted and fixed in 4% paraformaldehyde (pH 7.3) for 24 h at 4^{o}C. Each cell block was then embedded in paraffin followed by sectioning and mounting on the same glass slide. After deparaffinization, rehydration, and antigen (Ag) unmasking, immunohistochemical staining was performed by using a mAb to LXRα (PP-PPZ0412, R&D Systems).

Briefly, sections were dewaxed, rehydrated and treated with the antigen retrieval solution (pH 6.0), (Dako, Carpinteria, CA), according to the manufacturer's instructions.

Endogenous peroxidase activity was blocked by 1% H_2O_2 in absolute methanol for 30 min at room temperature. Non-specific IgG binding was prevented by preincubation of the sections in serum-free protein block solution (Dako). Mab to human LXRα was used as primary immunoreagent in 1:50 dilution (1 hour incubation at room temperature). Antigen-antibody reactions were detected with the use of the biotin-free Envision kit for mouse monoclonals containing HRP (horse raddish peroxidase) conjugated with the secondary antibodies on a polymer. The specific peroxidase activity was visualized with DAB (3,3'diaminobenzidine in 0.1 mol/l TRIS-HCl buffer, pH 7.2). Counterstaining was performed by methyl-green or Mayer's haematoxylin. Sections were dehydrated and mounted with Canada balsam then photographed with Leica DM2500 microscope equipped with a Leica DFC 500 12 Mpixel camera (Leica, Solms, Germany).

3.6. Immunodetection of PPARγ in human and murine macrophages

Human macrophages obtained from monocytes, noninfected or infected with GFP-BCG (6 X 10^6 cells/group), were pelleted and fixed in 4% paraformaldehyde (pH 7.3) for 24 h at 4^oC. Each cell block was then embedded in paraffin followed by sectioning and mounting on the same glass slide. After deparaffinization, rehydration, and Ag unmasking, immunofluorescent staining was performed by using a mAb to PPARγ (clone E8, 1/75 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, PPARγ was detected by incubating sections for 1 h at room temperature with the primary Ab followed by HRP- labeled anti-mouse secondary IgG-$F(ab)_2$ treatment. The visualization was made with a tyramide-conjugated red fluorescent amplification kit using tetramethylrhodamine (TSA-TMR System; PerkinElmer Life Science). The nuclear counterstain was made with DAPI (4,6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA). To ensure the staining specificities, negative controls were also included by using isotype-matched control IgG (DakoCytomation) in place of the primary Ab. Positive controls for PPARγ staining were made on normal human adult adipose tissue sections that exhibited nuclear staining in the majority of adipocytes. Fluorescence images were obtained using an Olympus BX51 microscope equipped with a narrowband tricolor excitation filter and DP71 digital camera. Fluorescent photomicrographs were captured with a single exposure, which simultaneously visualized both the green (the presence of GFP

Mycobacteria), the red (PPARγ protein), and the blue (DAPI) fluorescent lights. For transferring and editing images for documentation, Viewfinder and Studio Lite software version 1.0.136 of 2001 Pixera (Digital Imaging Systems) and Adobe Photoshop version 8.0 were used.

For the immunolocalization of PPARγ in murine macrophages, cells were stimulated with LAM (lipoarabinomannan; 300 ng/ml). PPARγ was detected by incubating formalin (3.7%)-fixed macrophage-containing coverslips for 1 h at room temperature with the primary pAb to PPARγ (clone H100; Santa Cruz Biotechnology). After a vigorous wash, cells were incubated with anti-rabbit Alexa Fluor 546-labeled secondary Ab (Molecular Probes). Nonimmune rabbit serum was used as negative control (The Jackson Laboratory). The slides were analyzed by confocal laser-scanning microscopy on a Zeiss LSM 510-META. The nuclear counterstain was made with DAPI (Sigma-Aldrich).

3.7. Quantification of Cytokine Production by ELISA

Supernatants of DC cultures were stored at −20°C until they were analyzed for the presence of TNFα, IL6, IL8, IL10, and IL12. Cytokine levels were measured by using BD OptEIA ELISA reagents (Beckton Dickinson), according to the manufacturer's instructions.

3.8. FACS Analysis

Cell staining was performed using FITC-, or PE-conjugated mAbs. Labeled antibodies for flow cytometry included anti-CD80-PE, CD83-PE, CD86-PE, CD206-PE, CD209-FITC, HLA-DR-PE, and isotype-matched controls (BD PharMingen, San Diego, CA). The cells were assessed for fluorescence intensity using FACS Calibur cytometer (BD Biosciences, San Diego, CA). Data analysis was performed using Cellquest software (BD Biosciences).

3.9. Endocytosis

FITC-dextran (Sigma-Aldrich) was used to measure mannose receptor-mediated endocytosis. Cells were incubated with 1 mg/ml FITC-dextran for 1 hr at 37°C and the uptake of FITC-dextran was determined by flow cytometry. Phagocytosis was measured

by the cellular uptake of Latex beads (Sigma-Aldrich) (carboxylate modified, mean diameter 1μm): cells were incubated with latex beads for 6 hr at 37°C and washed, and the uptake was quantified by FACS.

3.10. Western Blot Analysis

Cells were washed in phosphate-buffered saline, and then lysed in buffer A (150 mM NaCl, Tris-HCl, pH 7.5), 1mM EDTA, 15 mM b-mercaptoethanol, 0.1% Triton X-100, containing phosphatase inhibitor mix (Sigma-Aldrich) and protease inhibitors Aprotinin, Leupeptin, Pepstatin, Bestatin (20 ug/ml each) and 0.5mM PMSF (phenylmethylsulfonyl fluoride). 20 μg protein was separated by electrophoresis in 10% polyacrylamide gel and then transferred to PVDF membrane (Bio-Rad Laboratories, Hercules, CA). After blocking with 5% dry milk, membranes were probed with LXRα (PP-PPZ0412, R&D Systems), anti-Phospho-IκB alpha Ser32/36 (5A5; Cell Signaling Technology, Beverly, MA), anti-Phospho-NF-κB p65 Ser536 (3033; Cell Signaling), and anti-β-actin (Sigma-Aldrich) antibodies. For detecting ABCG1 western blot analysis was performed as previously described (187). PPARg was probed with polyclonal Ab anti-PPARγ (H100; Santa Cruz Biotechnology). The Ag-Ab complexes were labeled with appropriate HRP-conjugated secondary Abs (Sigma-Aldrich) and visualized by Immobilon Western HRP substrate kit (Millipore, Bedford, MA).

3.11. T-cell Proliferation Assay

The fluorescence dye CFSE (5-6-carboxyfluorescein diacetate succinimidyl ester), (Molecular Probes, Eugene, OR) was used for the analysis of T-cell proliferation. T-cells were isolated from buffy coats of healthy donors by Ficoll gradient centrifugation and immuno-magnetic cell separation (naive CD4+ T-cell isolation kit, Miltenyi Biotech). T-cells were washed once with sterile PBS, resuspended in diluted CFSE at the density of 5×106 cells/ml and labeled at 37°C for 15 minutes. CFSE-labeled cells were washed twice in RPMI-1640 culture medium (Sigma-Aldrich) supplemented with 10% FBS (Life Technologies) and seeded in a 96-well tissue culture plates. Immature DCs and LXR programmed iDCs were treated with 200ng/ml LPS for 10 hours, then washed three times in culture medium and co-cultured with CFSE-labeled allogeneic, naive CD4+ T-cells

cells in 96-well cell-culture plates for 5 days at a DC/T cell ratio of 1:15. As a control, untreated DCs were used. For the proliferation assays, anti-CD3 mAb was added to the culture media at day zero at 0.5 μg/ml final concentration. On day 5 fluorescence intensities were measured by flow cytometry and results were evaluated and proliferation index was determined using the Modifit LT software (Verity Software House, Topsham, ME)

3.12. Animals

C57BL/6 mice were obtained from the Fundacao Oswaldo Cruz breeding Unit, Rio de Janeiro, Brazil. TLR2 knockout (TLR2-/-) mice in a homogeneous C57BL/6 background were donated by Dr. S. Akira (Osaka University, Osaka, Japan). Animals were bred and maintained under standard conditions at the breeding unit of the Oswaldo Cruz Foundation. Animals were caged with free access to food and water in a room at 22–24°C and a 12-h light/dark cycle in the Department of Physiology and Pharmacodynamics animal facility until they were used. Animals weighing between 20 and 25 g from both sexes were used. All protocols were approved by the Fundac¸a˜o Oswaldo Cruz Animal Welfare Committee.

3.13. Bacterial strains

Mycobacterium bovis BCG (Moreau strain) vaccine was obtained from the Fundacao Athaulpho de Paiva, Brazil. The freeze-dried vaccine was stored at 4°C and resuspended in RPMI 1640 medium just before use. GFP-M. bovis BCG was provided by M. A. O'Donnell (Department of Urology, University of Iowa, Iowa City, IA).

3.14. Lipid body staining and enumeration

Macrophages were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+}-free HBSS (pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained in 1.5% osmium tetroxide (30 min), rinsed in water, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in water, rinsed in 0.1 M cacodylate buffer, reincubated in 1.5% osmium tetroxide (3 min), rinsed in distilled water, dried, and mounted for further analysis. The morphology of fixed cells

was observed and lipid bodies were enumerated by light microscopy with a X100 objective lens for 50 consecutive macrophages in each slide.

3.15. Mycobacterial viability determined by flow cytometry

A live/dead staining protocol based on the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) was applied to study the viable vs nonviable BCG obtained from GW9662-treated or vehicle-treated macrophages. In brief, peritoneal macrophages (1 X 10^6/well) in a 24-well plate were pretreated with either GW9662 (1 μM) or vehicle for 30 min at 37°C, then infected with BCG (MOI, 1:1) for 1 h, followed by three PBS washes to remove any noninternalized BCG. Macrophages were then incubated for 12 h after infection in RPMI 1640 cell culture medium containing 2% FCS and reconstituted with GW9662 (1 μM) or vehicle. Macrophages were lysed with 0.1% saponin and bacterial-containing suspensions were incubated with a LIVE/DEAD BacLight Bacterial Viability Kit according to the manufacturer's instructions. The percentages of live and dead bacteria were determined by flow cytometry as previously described. Flow cytometric measurements were performed on a FACSCalibur (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

3.16. Statistical Analysis

All data are presented as means ± standard deviations (SD). In real-time quantitative PCR experiments, the mean and SD 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 SD of the normalized values. For all experiments we made at least four biological replicates. In real-time quantitative PCR experiments we performed an unpaired (two-tailed) t test, in T-cell proliferation assays we performed a paired t test on normalized data. P value less than 0.03 was considered statistically significant.

4. RESULTS

4.1. LXRα is present and can be activated in monocyte derived DCs and also in CD1c+ circulating blood DCs

Our group and others have shown that LXRα was one of the nuclear receptors upregulated in human monocyte derived dendritic cells differentiated in the presence of GM-CSF and IL4 (19).

We have further validated this finding by carrying out a time course experiment in which the two LXR isoforms showed differential expression during DC differentiation. While α was one of the most strongly up-regulated amongst nuclear receptors, β was down-regulated and could be detected at a low level, suggesting that α is the dominant LXR isoform during DC differentiation (Figure 5).

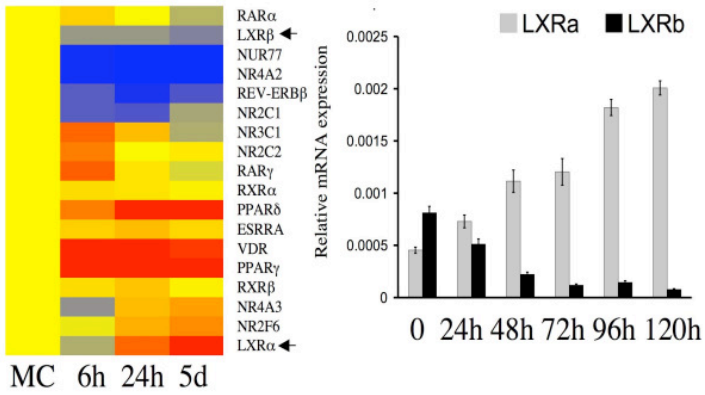

Figure 5. LXRα is Expressed at High Levels and is Inducible During DC Differentiation (A) Microarray transcript profiles for selected probe sets of nuclear receptors that were induced or repressed during differentiation of DCs. Heatmap shows expression levels. Raw data were normalized to the median expression. The mean of three determinations is shown. Arrows indicate the two isoforms of LXR. (B) Kinetics of LXRα and LXRβ mRNA expression was determined by RT-PCR as described in Experimental Procedures. Cells were harvested at the indicated points. All PCR data are expressed as a ratio of the

LXRα and LXRβ transcripts relative to cyclophilin expression. Error bars indicate the standard deviation of the relative expression.

Next we used immunohistochemistry to detect LXRα in monocytes and in monocyte derived iDCs. We found that while monocytes lack the expression of the LXRα protein, it is expressed in iDCs and markedly increased when DCs are differentiated in the presence of the synthetic agonist of LXR, GW3962 (GW) (Figure 6A-C). This is explained by the established autoregulatory activity of LXRα (17). Similarly, iDCs exposed to GW for various length of time show LXRα expression as detected by Western blotting (Figure 6D).

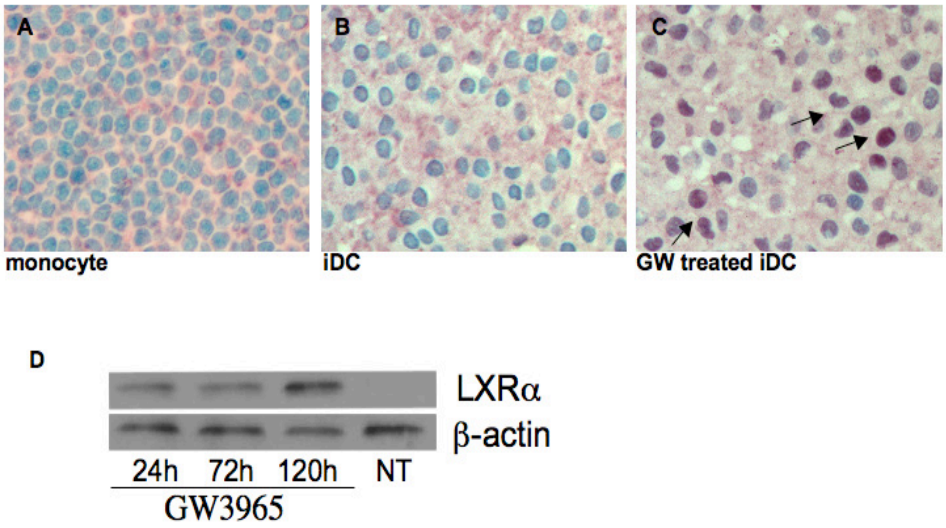

Figure 6. LXRα Can be Detected at ProteinLevels in DCs

(A-C) Immunohistochemical detection of LXRα in monocytes (A), and iDCs differentiated in the absence (B) or presence of GW3965 (C) for 5 days. Arrows on panel C indicate positive (brown) nuclear staining for LXRα. Note that no and remarkedly less staining intensities are found in panel A and B, respectively. Original magnifications: 20x.

(D) Western blot analysis of LXRα protein in iDCs that were differentiated in the presence or absence of 1uM GW3965 administrated for the indicated periods of time.

In order to further analyze whether LXR could become activated during in vitro DC differentiation another time course experiment was carried out in which ligand was added at various time points during the course of differentiation (Fig....) and expression levels of target genes including LXRα were measured 24 hours later. We found that synthetic ligands readily induced the expression of LXRα, ABCA1, ABCG1 and apoE and their inducibility was largely independent from the time point of treatment during DC differentiation (Figure 7A-E).

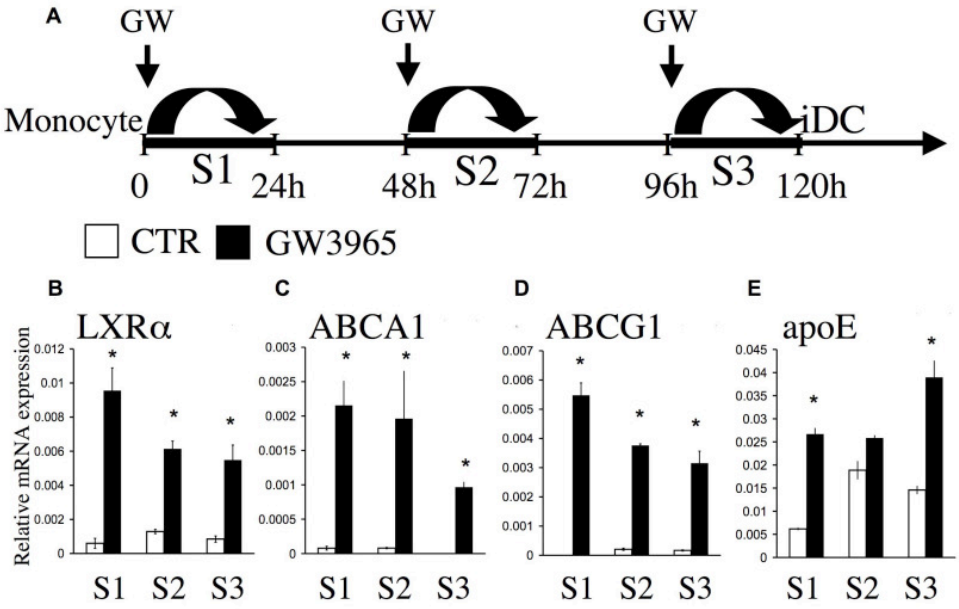

Figure 7. LXR can be Activated by LXR-specific Agonist During Differentiation of iDCs

(A) CD14^{+} monocytes were isolated from peripheral blood and cultured in the presence of IL-4 and GM-CSF. Effects of ligand treatment were tested by adding GW at the indicated timepoints and harvested 24 h later (S1, S2, S3). Samples for mRNA measurements were collected, processed and measured as described in Experimental Procedures.

*(B-E) Kinetics of transcript levels of LXRα (B), ABCA1 (C), ABCG1 (D) and apoE (E) were determined in monocyte-derived DCs during differentiation by RT-PCR as described. RNA was obtained from samples (S1, S2, S3) collected 24 hours after administration of 1uM GW3965 (filled bars) or vehicle (open bars) as controls at the indicated timepoints as described in Panel A. Data are expressed as a ratio of the transcripts relative to cyclophilin expression. Error bars indicate the standard deviation of the relative expression. *, P < 0.01; (compared to the respective control value).*

Similar results were obtained when using another synthetic LXR agonist, T090137 or the naturally occurring 22(R)-hydroxycholesterol (22ROH) (22) (23) (Figure 8).

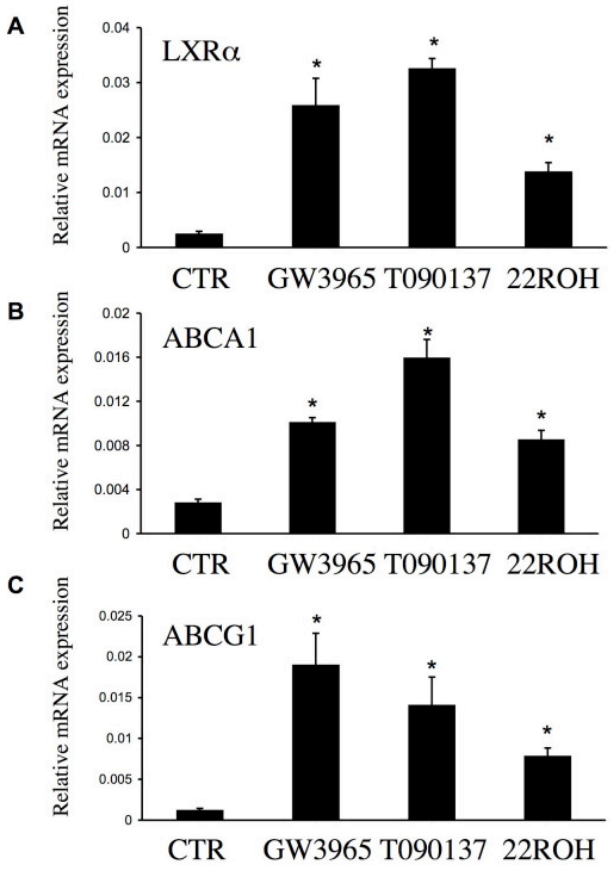

Figure 8. LXR Can be Activated with Various Ligands

*Transcript levels of LXRα (A), ABCA1 (B) and ABCG1 (C) respectively, were determined in differentiating monocyte-derived DCs treated with various ligands 1 μM T090137, 1 μM GW3965, and 4μM 22ROH after 24 hours. Data are expressed as a ratio of the transcripts relative to cyclophilin expression. Error bars indicate the standard deviation of the relative expression. *, P < 0.01; (compared to the respective control value).*

Next we tested whether LXR is present and can be activated in differentiated circulating DCs, therefore we separated CD1c+ DCs from peripherial blood and measured the expression levels of the two LXR isoforms and the levels of its target genes upon treatment with GW3965. We found that in CD1c+ blood DCs LXRα was also the dominant isoform (Figure 9A) and the expression levels of LXRα, ABCA1 and ABCG1 showed a marked increase when cells were treated with LXR ligand (Figure 9B-D).

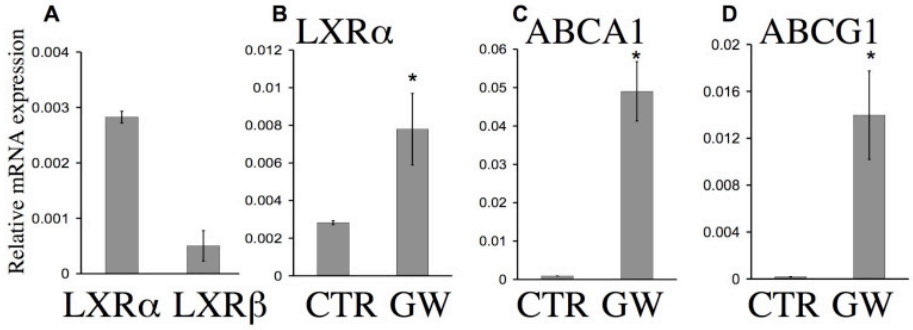

Figure 9. LXR is Present and can be Activated by LXR-specific Agonist in CD1c + blood DCs

*CD1c + blood DCs were separated as described in Experimental Procedures. The relative mRNA levels for LXRα and LXRβ (A) and kinetics of transcript levels of LXRα (B), ABCA1 (C) and ABCG1 (D) in the presence (GW) or absence (CTR) of 1uM GW3965 for 24 hours were determined by real-time quantitative RT-PCR as described. Data are expressed as a ratio of the transcripts relative to cyclophilin expression. Error bars indicate the standard deviation of the relative expression. *, P < 0.01; (compared to the respective control value).*

Taken together, these results suggest that both in blood DCs and in monocyte derived DCs α is the dominant LXR isoform present. Importantly, we could find no indication of LXR being transcriptionally active in the absence of added exogenous ligand in any of the differentiation states measured by target gene expression. However upon natural or synthetic ligand activation LXR target genes are readily inducible in both cell types. These findings were calling for further studies to address the role of LXR in both immature and mature DCs.

4.2. LXR agonists have minimal effects on the expression of iDC co-stimulatory and surface molecules

First we measured the expression of cell surface proteins on iDCs by flow cytometry upon administrating LXR activators at the beginning of differentiation. We found that surface expressions of the Mannose Receptor (MR) showed a slight decrease while CD80 was minimally increased. CD86 and MHCII were not affected on iDCs at day 5 (Fig. 2J). The measurement of CD14 levels, a marker of monocytes, also indicated that DC differentiation was complete and the entire cell population differentiated as indicated by the loss of CD14 positivity. By measuring the uptake of Latex bead, apoptotic bodies and FITC-dextran we couldn't find significant differences between the LXR programmed and the untreated DCs although in some cases the decreased levels of mannose receptor could be paired with a decreased uptake of FITC-dextran. These data suggested that activation of LXR has only slight effects on iDC maturation and function (Figure 10).

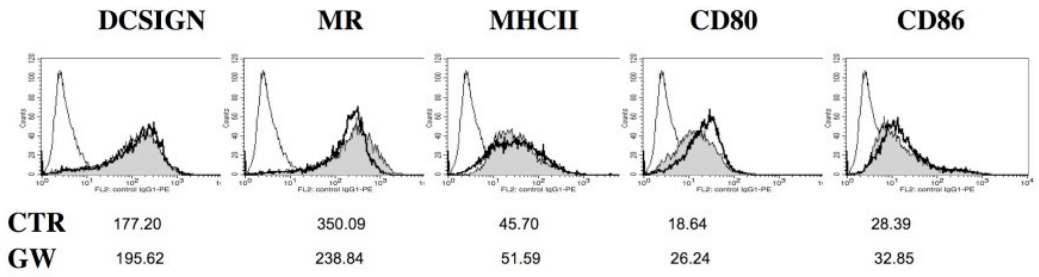

Figure 10. LXR Activation Affect the Expression of Co-stimulatory and Surface Molecules on DCs

Monocytes were cultured for 5 days as described in Experimental Procedures for the generation of iDC. DC-SIGN, Mannose Receptor (MR), MHC-II, CD80 and CD86 cell-surface expression was determined by flow cytometry on iDCs differentiated in the absence (CTR) (gray histograms) or presence of GW3965 1uM (GW) (solid line). Numbers are the mean flourescence intensity (MFI) values. Data obtained with specific monoclonal antibody (mAb) versus isotype-matched control. One representative experiment of eight performed is shown.

4.3. LXR signaling is enhanced during maturation of DCs and increases the expression of co-stimulatory molecules

Next we turned our attention to mature DCs. In response to stimulation iDCs transform into mDCs with characteristic changes in gene expression as well as in phenotype and function. We sought to identify how LXR signaling affects maturation and vica versa. Measuring the expression levels of mRNAs encoding ABCA1 and ABCG1 we found that LPS exposure by itself did not change target gene expression, however maturation resulted in an enhanced inducibility of the expression of these target genes upon exogenous ligand exposure (Figure 11A-B). The potentiating effect of LPS on LXR signaling was verified also at the protein level, where ABCG1 was detected in mDCs exposed to GW (Figure 11C).

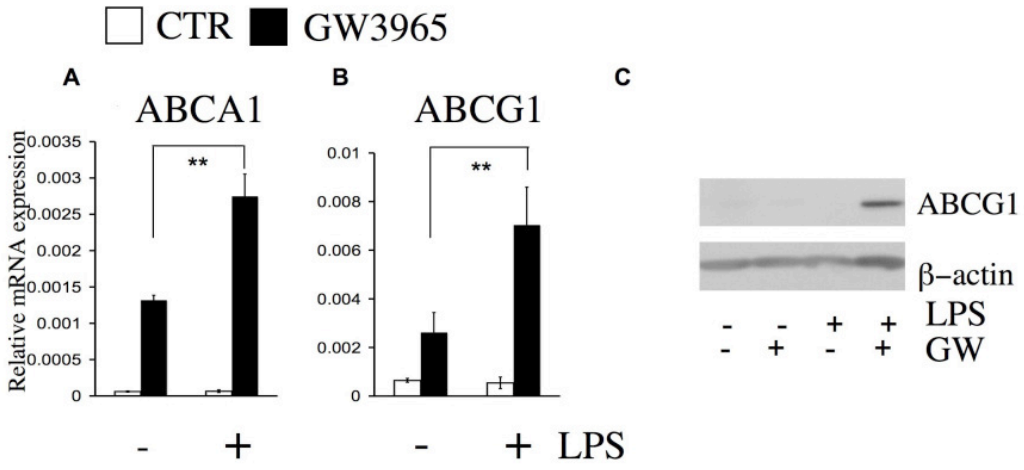

Figure 11. LXR Response is Enhanced During Maturation of DCs

*(A-B) Expression levels of ABCA1 (A) and ABCG1 (B) were determined by real-time quantitative RT-PCR from mDC samples cultured for 5 days in the presence or absence of 1uM GW3965 and stimulated with LPS for another 24 hours. Data are expressed as a ratio of the transcripts relative to cyclophilin expression. Error bars indicate the standard deviation of the relative expression. **, $P < 0.03$; (compared to the respective control value). (C) Western blot analysis of ABCG1 protein in LPS treated DCs that were differentiated in the presence or absence of 1uM GW3965 for 5 days and stimulated as described earlier.*

Expression levels of co-stimulatory molecules e.g. CD80, CD83, CD86 and MHCII that are required for activation and transmitting signals are increased during transformation of iDCs into mDCs. We next determined the effect of LXR activation on these markers. In the presence of LXR activators, the mRNA levels of both CD80 and CD86 showed a marked upregulation compared to untreated mDCs (Figure 12A-B). In parallel with the mRNA expression we detected the same effect when comparing the levels of surface expression of GW treated DCs, supporting that LXR programming affects the changes in the surface expression of CD80 and CD86 at the level of transcription and protein levels (Figure 11C).

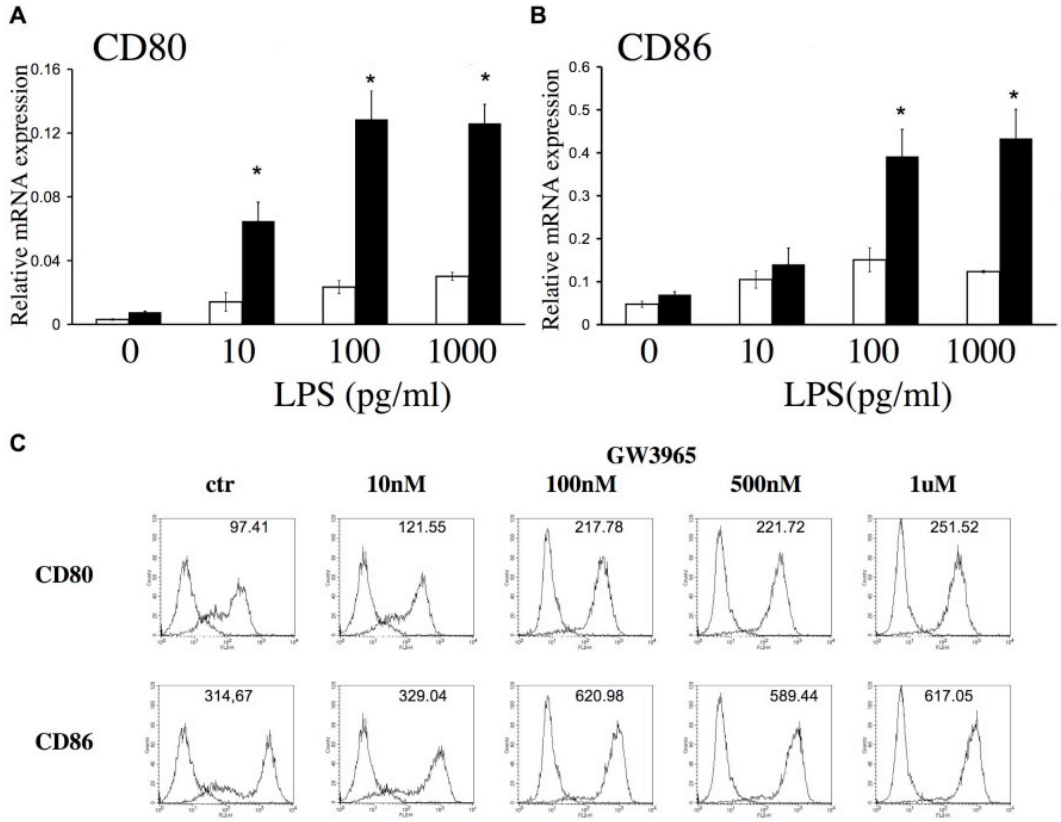

Figure 11. LXR Programming Increases CD80 and CD86 Expression in mDCs

*(A and B) The mRNA levels for CD80 (A) and CD86 (B) in mDCs differentiated in the presence or absence of 1uM GW3965 and stimulated with different doses of LPS for 24 hours. Expression levels were determined by real-time quantitative RT-PCR. Data are expressed as a ratio of the transcripts relative to cyclophilin expression. Error bars indicate the standard deviation of the relative expression. *, P < 0.01; (compared to the respective control value).*

(C) Characterization of CD80 and CD86 cell-surface expression by flow cytometry on mDCs that were differentiated in the presence of different doses of GW3965 and stimulated by LPS as described earlier. Numbers represent MFI values. Data obtained with specific monoclonal antibody (mAb) indicated (—) versus isotype-matched control (–). One representative experiment of five performed is shown.

Surface expression of CD83 showed no difference when compared to control mDCs (data not shown). There have been several endogenous oxysterols identified as ligands of LXR such as 24(S),25-epoxycholesterol, 27-hydroxycholesterol and 22(R)-hydroxycholesterol (22ROH). A common feature of all these compounds is that their affinity is much lower to the receptor than that of synthetic compounds like GW. We used 22ROH to activate the receptor. Similarly higher expression of CD86 was detected when mDCs were treated with a natural ligand, 22ROH, as compared to untreated cells (Figure 12).

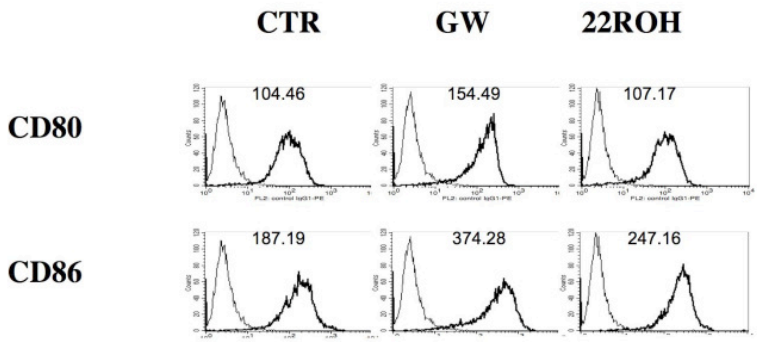

Figure 12. 22ROH Increases Surface Expression of CD86
DC differentiation in the presence of LXRα activator 22ROH results in increased cell-surface expression by flow cytometry (A) Cells were differentiated as described earlier and treated with LPS for 24 hours. Numbers on FACS represent MFI values. Data obtained with specific monoclonal antibody (mAb) indicated (–) versus isotype-matched control (—). One representative experiment of three performed is shown.

4.4.Increased expression of CD80 and CD86 on mDCs is not limited to LPS stimulus

To asses if LXR induced augmentation of mDCs' response is limited to LPS stimulus or the phenomenon is more general we tested other TLR activators such as LTA (TLR 2), lipidA (TLR 4), Poly(I:C) (TLR 3), and CpG (TLR 9). Measuring the expression of CD80 and CD86 revealed that the response of mDCs was also increased after lipidA and Poly(I:C) activators in the LXR programmed DCs (Figure 13).

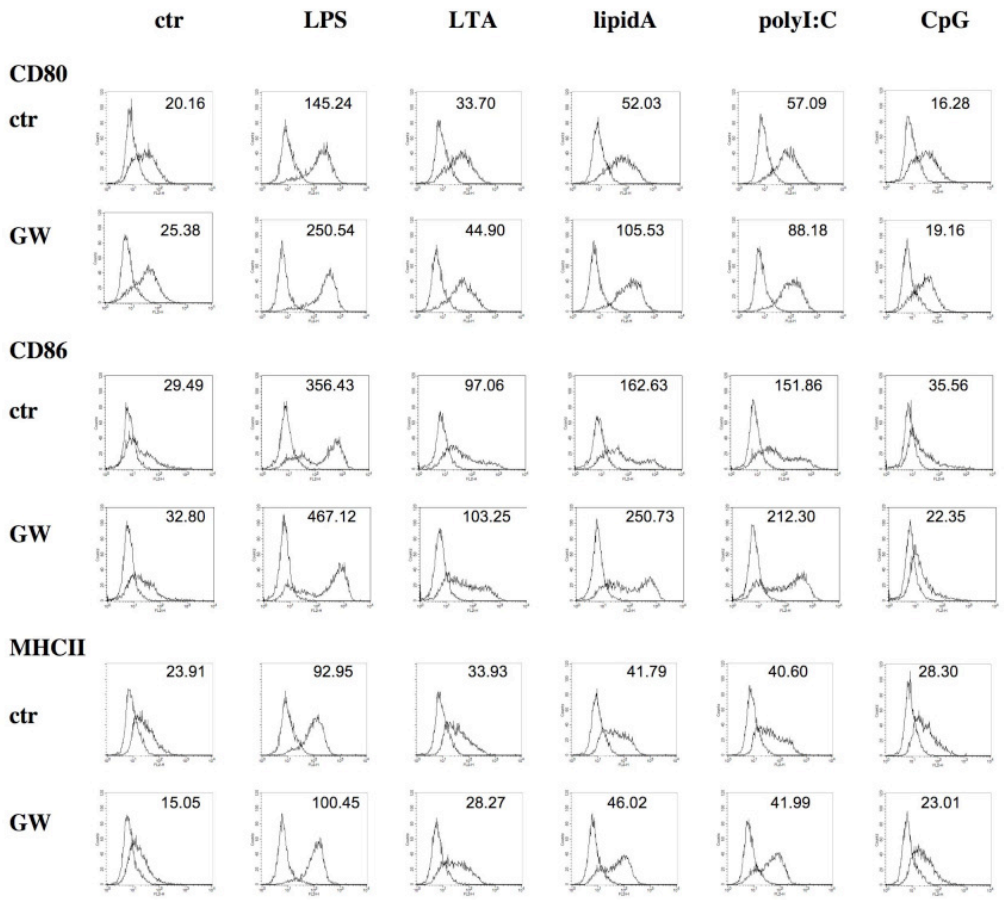

Figure 13. Increased Expression of CD80 and CD86 on mDCs Differentiated in the Presence of GW is not Limited to TLR4 activators

Cell-surface expression of CD80, CD86 and MHC-II were determined by flow cytometry on TLR specific activators (LPS and LipidA for TLR4, LTA for TLR2, poly:IC for TLR3 and CpG for TLR9) for 48 hours. Numbers are the mean flourescence intensity values. Data obtained with specific monoclonal antibody (mAb) indicated (–) versus isotype-matched control (—). One representative experiment of three performed is shown.

The increased induction of CD80 and CD86 by LPS and Poly(I:C) in LXR programmed DCs was also detectable at the mRNA levels. These data show that LXR programming is interacting with and enhancing not only TLR4 signaling, activated by LPS or lipidA, but also other signaling pathways such as TLR3. The expression levels of TLR3 and TLR4 in the GW and control iDCs showed no difference (Figure 14).

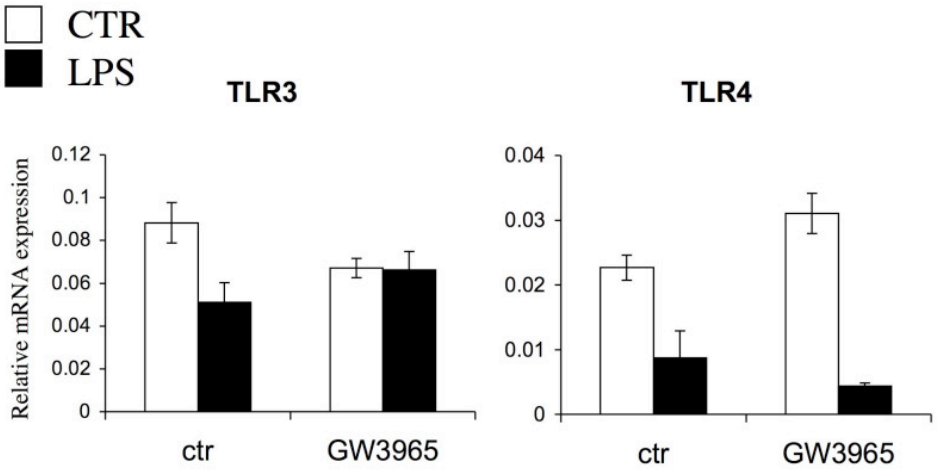

Figure 14. TLR3 and TLR4 are Expressed at Similar Levels in DCs Differentiated With or Without GW3965

Expression levels of TLR3 (A) and TLR4 (B) were determined by RT- PCR from RNA derived form mDCs differentiated in the absence (ctr) or presence of 1uM GW3965 (GW) and treated with or not LPS for 24 hours. Data are expressed as a ratio of the transcripts relative to that of cyclophilin. Error bars indicate the standard deviation of relative expression.

4.5. LXR activation results in increased cytokine production and prolonged NF-κB signaling in mDCs

One of the important functional consequence of increased TLR signaling is activation of NF-κB leading to increased cytokine production. Therefore we measured the levels of

secreted IL6, IL8, IL10, IL12, and TNFα, cytokines characterteristic of DC maturation.. We found that GW3965 alone has failed to induce the production of these cytokines in iDCs but following LPS stimulus the production of IL12, TNFα, IL6, and IL8 increased significantly in the LXR programmed DCs while the production of IL10 was unaffected (Figure 15).

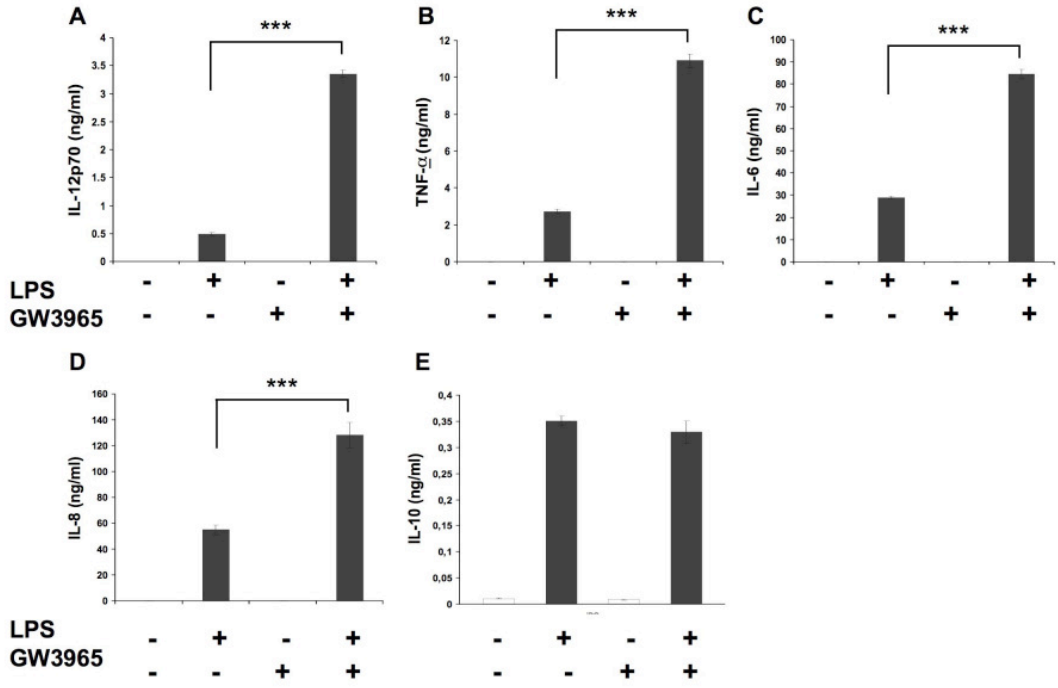

Figure 15. LXR Treatment Results in Increased Cytokine Production in mDCs and Prolonged NF-κB Signaling

*(A-E) Levels of cytokines IL-12 (A), TNFα (B), IL-6 (C), IL-8 (D) and IL-10 (E) from supernatants of mDCs differentiated in the presence or absence of GW3965 1uM and treated with LPS for 24 hours and measured by ELISA method as described in Experimental Procedures. Data show the means and SEM of four independent experiments. Significance versus untreated mDCs ***P < 0.03.*

22ROH differentiated and LPS activated DCs also produced more IL12, TNFα and IL8 (Figure 16).

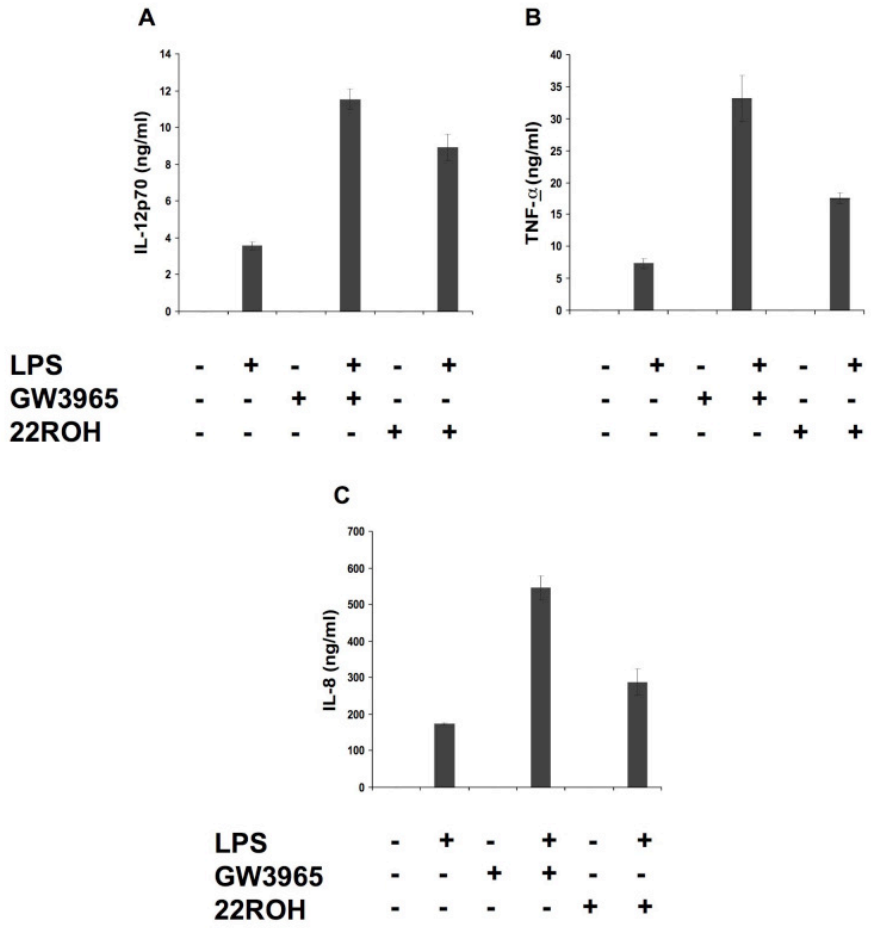

Figure 16. 22ROH Increases Citokine Producion of mDCs

DC differentiation in the presence of LXR activator 22ROH results in increased IL12, TNFα and IL8 production of mDCs measured by ELISA (A-C). Cells were differentiated as described earlier and treated with LPS for 24 hours. One representative experiment of three performed is shown.

To further explore the mechanism by which the LXR pathway modulates on DCs' response to LPS stimulus we examined its effect on NF-κB activation.

We measured and compared the intensity and length of the phosphorylation of IκBα and the phosphorylation of p65 in the control and LXR programmed DCs during a five-hour time course after LPS stimulus. In the control cells the low basal level of IκBα phosphorylation increased, but returned to basal levels by four hours. In contrast in the LXR ligand treated cells phosphorylation remained at high levels. The phosphorylation of p65 was also markedly higher in the GW treated cells (Figure 17). These data suggest that activation of LXR contributes to a prolonged NF-κB signaling that results in enhanced LPS signaling.

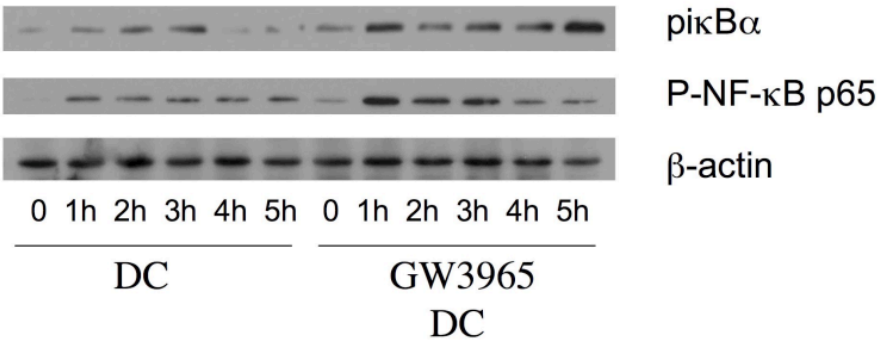

Figure 17. LXR Treatment Results in Prolonged NF-κB Signaling

Western blot analysis of IKB alpha Ser32/36, NF-KB p65 Ser536 phosphorylation and β-actin expression in LPS treated DCs that were cultured in the presence or absence of GW3965 as described above.

4.6. LXR programmed mDCs have an increased capacity to activate CD4+ T cell proliferation

The hallmark of mDCs is an increased capacity to activate T cells, in part by direct engagement of co-stimulatory molecules (e.g. CD80 and CD86) and through production of inflammatory cytokines (e.g. TNFα or IL12). To assess the effect of LXR signaling on

the capacity of DCs to promote T cell proliferation we used untreated or LXR-programmed DCs and cultured them with 5-6-carboxyfluorescein diacetate succinimidyl ester (CFSE) -labeled naïve allogeneic CD4+ T cells for 5 days. By using flow cytometry we found that the T-cell stimulatory capacity of LXR programmed iDCs was slightly, but significantly increased compared to that of non-treated iDCs. As detected by the more rapid dilution of the fluorescent signals in faster dividing T cells, LXR-programming of LPS-induced mDCs, had a clear stimulatory effect on T-cell proliferation. These results indicate that activation of the LXR pathway in DCs increases the expression of co-stimulatory molecules and inflammatory cytokines that results in improved costimulatory effect and thus, T cell activation (Figure 18).

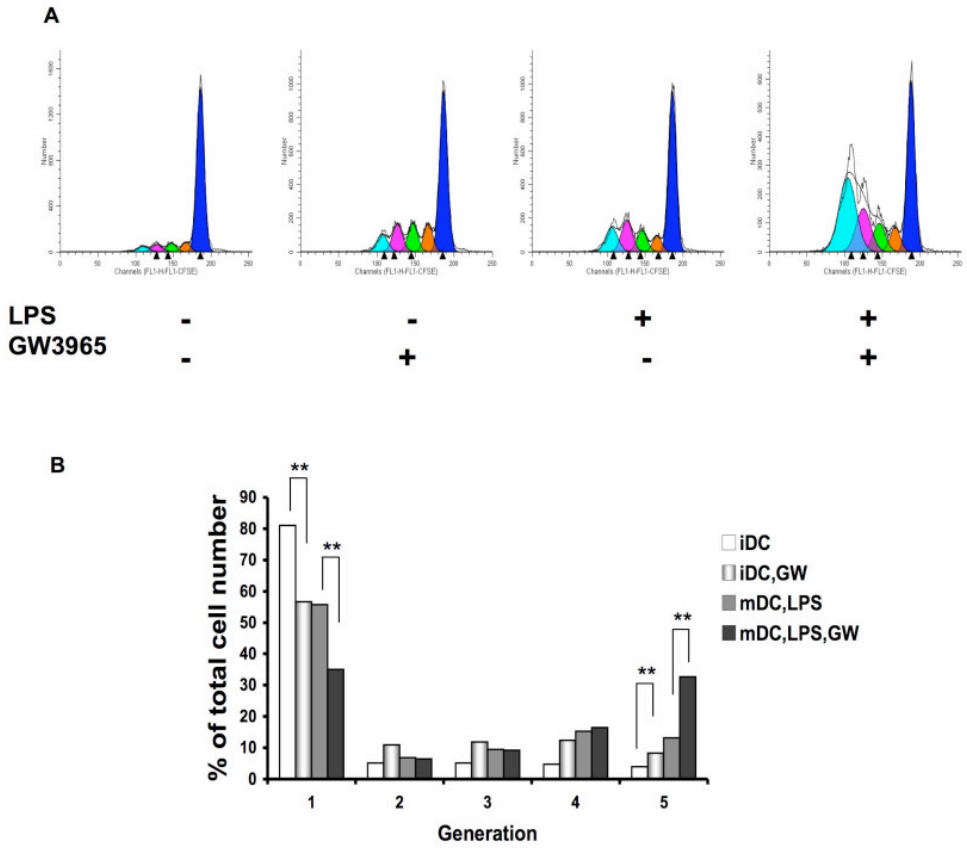

Figure 18. LXR Treatment Results in Increased T Cell Proliferation

*(A-B) (A) iDCs or LPS-activated mDCs preconditioned or not with GW (1 μM) were cocultured with CFSE-labeled allogeneic, naive CD4+ T-cells at 1:15 DC-T cell ratio. On day 5 fluorescence intensities of T cells were measured by flow cytometry. Representative histograms displaying the number of T cell divisions (arrowheads) are shown in Panel A. Panel B shows the percentages of cells in each generation. Results were calculated using the Modifit LT software. Identical results were obtained when LPS derived from E. coli and S. enterica serotype Minnesota were used (not shown). Results are represented as of seven independent experiments. **P < 0.02 for difference between GW-treated versus untreated iDCs or mDCs.*

4.7. LXRα is present in dendritic cells in reactive lymph nodes

The data presented so far revealed that oxysterol induced LXR signaling integrates into TLR-NFκB signaling by enhancing NFκB response (Figure 19A).

This suggests that LXRα is detectable in vivo in tissues such as dendritic cells of reactive lymph nodes. By carrying out immunohistochemistry analyses of such tissues we found that the receptor is expressed in dendritic cells of lymph nodes from patients with tuberculosis and sarcoidosis and was present also in dendritic cells of tumor associated lymph nodes. In contrast, non-reactive lymph nodes displayed very few LXR positive cells detected. These findings suggest that under inflammatory conditions LXR signaling is likely to be active in dendritic cells in vivo (Figure 19B-G).

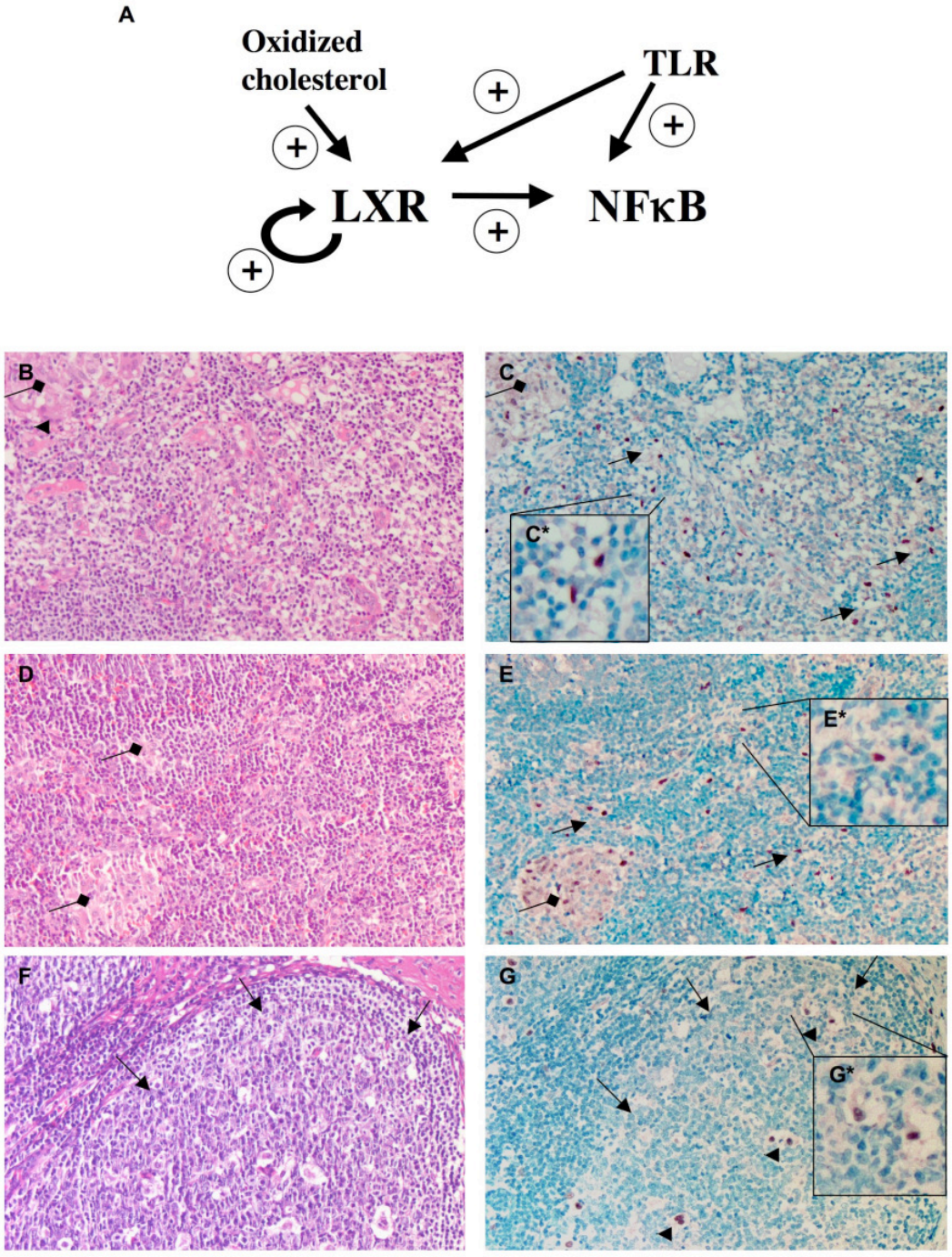

Figure 19. LXR Integrates Into the TLR-NFkB Signaling and is Present in Dendritic Cells

(A) A network where lipid activated LXR integrates into the TLR-NFkB signaling by enhancing NFkB response. TLR activators increase LXR response induced by activators such as oxidized cholesterols.

(B and C) Draining bronchopulmonary lymph node from a lung tissue of early active tuberculosis (with acid fast positivity for bacteria – not shown). (B) Haematoxylin & eosin (HE) stained section. Diamond arrow points to the edge of an early granuloma. Arrowhead indicates a Langhans' type of giant macrophage. (C) LXRα immunostained consecutive section from the identical microscopic field shown in B. Note the LXRα nuclear positivity of sinusoidal DCs/histiocytes (arrows). Few cells with same morphology are detectable in early granuloma epithelioid cells, also (diamond arrows). C insert demonstrates DCs with LXRα nuclear positivity (brown staining) that are digitally magnified.*

(D and E) Mediastinal lymph node harboring sarcoidosis showing non-necrotising granulomas (diamond arrows). (D) HE stained section; (E) the corresponding section from the same microscopic field that is immunostained with LXRα antibody. Note the LXRα nuclear positivities in DCs and histiocytes in association with the sinus system (arrows, brown staining) and the granuloma cells (diamond arrows). E insert shows DCs with LXRα nuclear positivity that are digtally magnified.*

(F and G) Tumor-associated reactive lymph node. (F) HE; (G) LXRα immunostained corresponding section of the lymph node from the vicinity of a seminoma tumor (the tumor is not shown). Note the positive nuclear LXRα staining of DCs (arrowheads, brown staining) located in the germinal center (arrows). G insert shows DCs with LXRα nuclear positivity that are digtally magnified.*

Note that cells in C, E* and G* are commonly detected with S100 protein also (not shown). Negative controls for C, E and G showed no tissue reactivities confirming the immunostaining specificities for LXRα (not shown, see material and methods).*

B-G, Original magnifications: 20x

4.8. Mycobacterium bovis BCG infection induces PPARγ expression in macrophages

Lipid-laden (foamy) macrophages are present in mycobacteria infection, but little is known about the molecular mechanisms in their formation. As PPARγ plays a central

role in lipid metabolism, macrophage differentiation, and inflammation control, our aim was to investigate the interaction of lipid body formation in the infected macrophages and PPARγ signaling.

First we investigated the PPARγ protein expression and nuclear localization upon BCG infection. Using human monocytes infected with fluorescent-labeled BCG (GFPBCG) we could confirm that although human peripherial blood derived monocytes lack the expression of the PPARγ protein, cells exposed to *M. bovis* BCG infection exert a marked upregulation in the protein levels of PPARγ. This observation was further confirmed by Western blot analysis in peritoneal macrophages from an experimental model of mouse infected by *M. bovis* BCG in which increased PPARγ protein content was observed within 2 h and was at its maximum within 24 h after BCG infection (Figure 20).

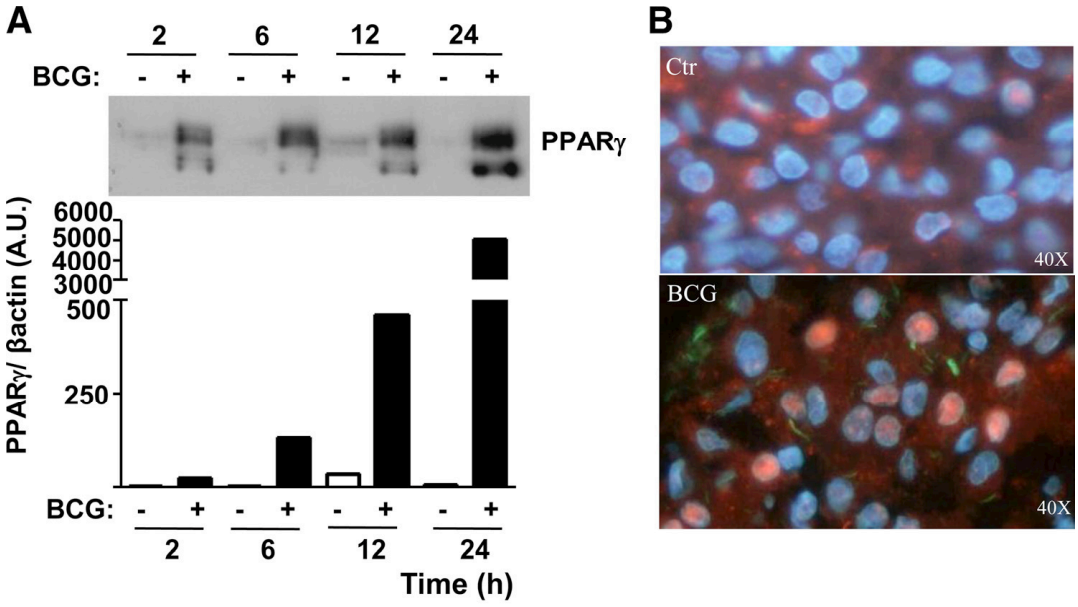

Figure 20. Kinetics of BCG-induced PPARγ expression

Peritoneal macrophages obtained from C57BL/6 mice (A) or monocytes obtained from human volunteers (B) were infected in vitro with BCG (MOI, 1:1). A, Total macrophage cell lysates (4X10^{6}cells/lane) were separated by SDS-PAGE (10%) and subjected to Western blotting for PPARγ or β-actin. The image is representative of at least two different blots. The graph represents the densitometric analysis (arbitrary units (A.U.)) of the Western blotting bands. B, PPARγ immunofluorescent staining of human macrophages that were noninfected (upper panel) or infected with GFP-mycobacteria.

As opposed to noninfected cells predominantly showing the blue nuclear counterstain, there is an increase in the amount of PPARγ-specific red nuclear fluorescence mainly associated with the presence of the engulfed mycobacteria (green fluorescence). Ctr, Control. Original magnification, X40.

4.9. Changes in Phenotype and Function Associated with Increased PPARγ Expression

Further exploring the changes in the phenotype and function of the infected macrophages we found that the increased expression of PPARγ paralleled the augmented lipid body formation and $PGE_{(2)}$ synthesis induced by BCG infection. The function of PPARγ in modulating BCG infection was demonstrated by the capacity of the PPARγ agonist BRL49653 to potentiate lipid body formation and $PGE_{(2)}$ production. Pretreatment with the PPARγ antagonist GW9662 inhibited BCG-induced lipid body formation and $PGE_{(2)}$ production (Figure 21).

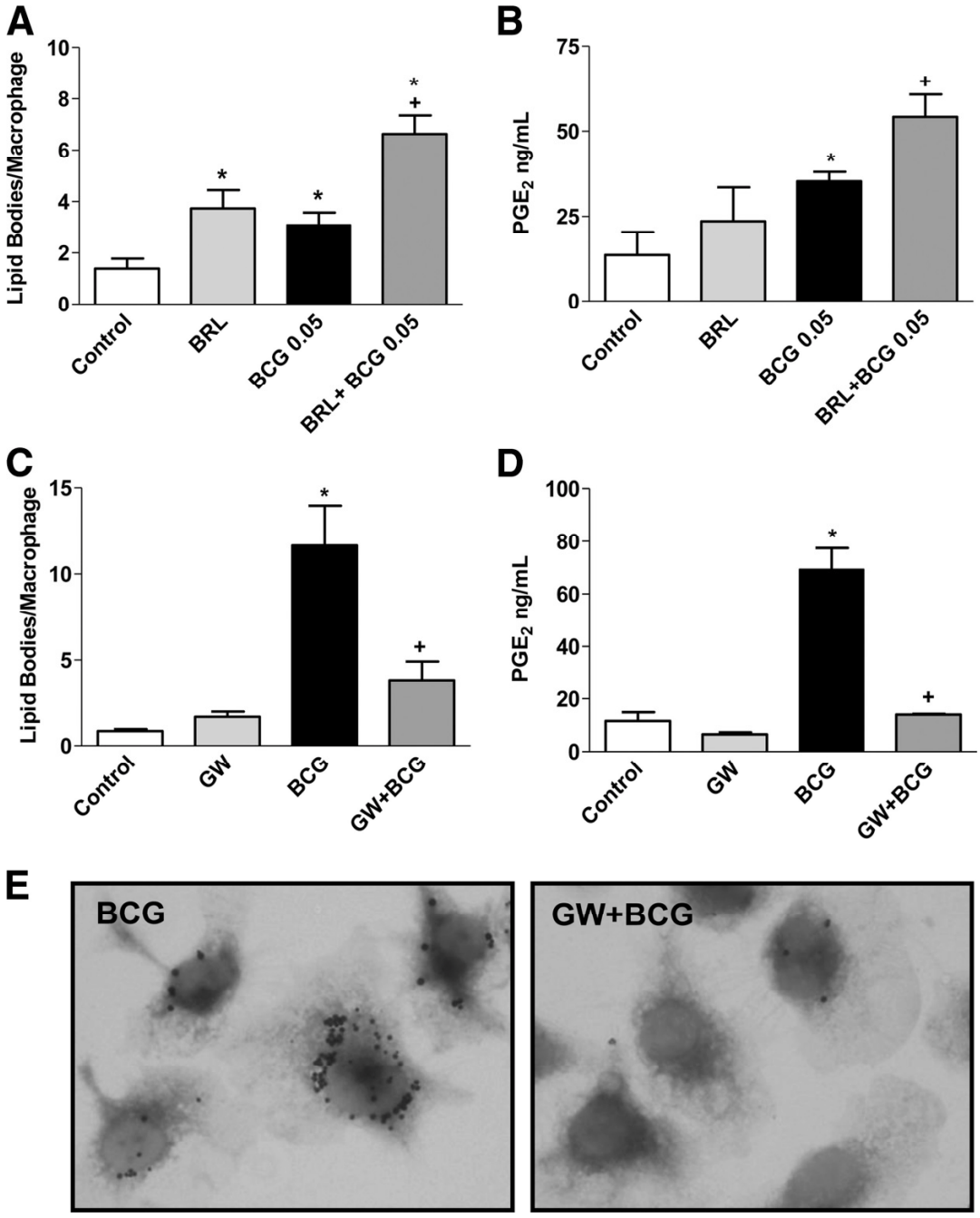

Figure 21. Effect of PPARγ agonist BRL49653 and PPARγ antagonist GW9662 on BCG-induced lipid body biogenesis and PGE2 production.

Peritoneal macrophage were treated with vehicle, BRL49653 (5 μM), or GW9662 (1 μM) for 30 min before infection with BCG. Lipid body counting (A) and PGE2 production (B)

in peritoneal macrophages infected with BCG (MOI, 0.05:1 bacterium:macrophage) treated with vehicle or BRL49653. Lipid body counting (C) and PGE2 production (D) in peritoneal macrophages infected with BCG (MOI, 1:1) treated with vehicle or GW9662. Each bar represents the mean SEM from three independent pools of 10 animals each. Differences between control and infected with treatment groups are indicated by asterisks ($p < 0.05$). _, Differences between BCG and BCG in the presence of BRL49653 or GW9662. E, Representative images of macrophages treated with vehicle or GW9662 followed by infection with BCG after osmium staining, as observed by light microscopy (original magnification, X100).

Using a TLR2-deficient mouse model we could show that these observations found in wild type were not present in TLR2-deficient mouse peritoneal macrophages (Figure 22).

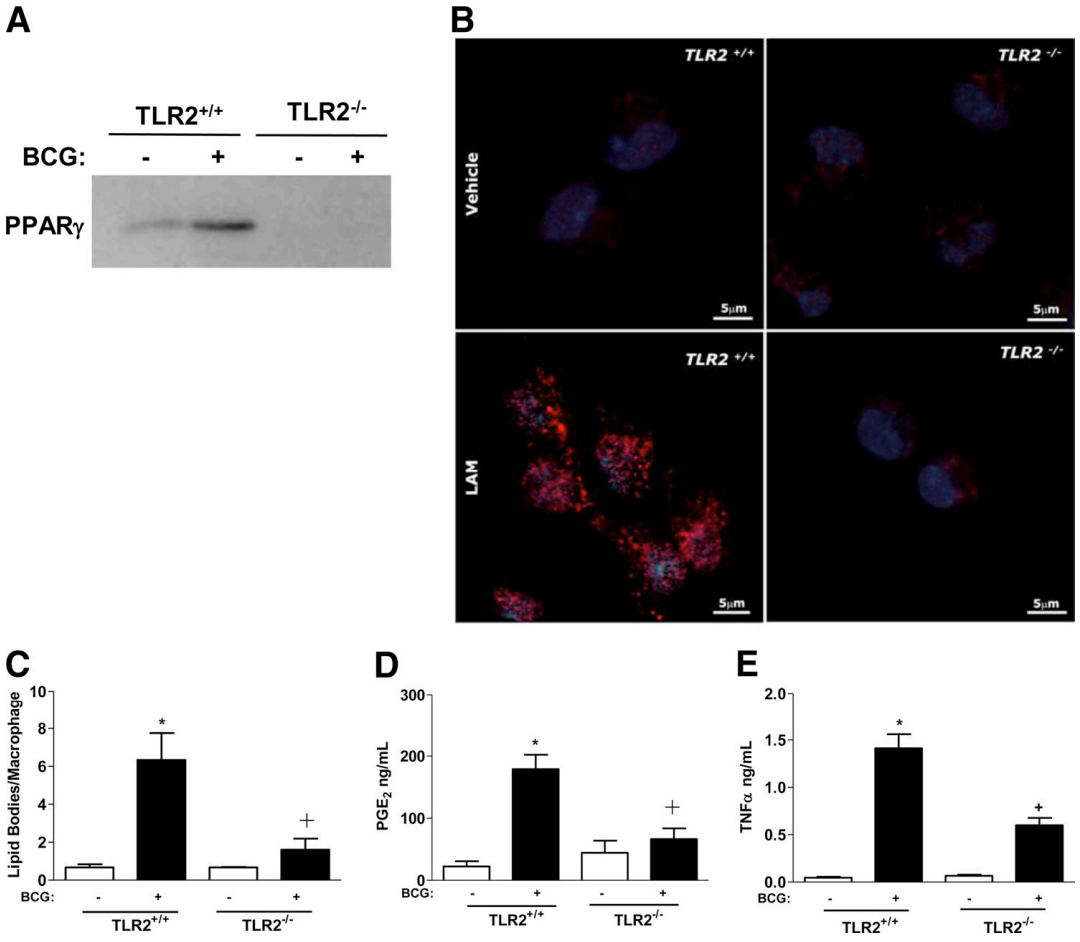

Figure 22. TLR2-dependent PPARγ expression in response to infection with BCG in peritoneal macrophages in vitro.

A, Analysis of PPARγ expression by Western blot in peritoneal macrophages obtained from TLR2+/+ and TLR2-/- mice 24 h after infection with BCG (MOI, 1:1). Total macrophage cell lysates (4 X 10^6cells/lane) were separated by SDS-PAGE (10%) and subjected to Western blotting for PPARγ. The image is representative of at least two different blots. B, TLR2-dependent PPARγ expression and nuclear localization 24 h after LAM (300 ng/ml) stimulation assessed by confocal laser microscopy analysis. As opposed to nonstimulated cells predominantly showing the blue nuclear counterstain, there is an increase in the amount of PPARγ-specific red nuclear fluorescence after LAM stimulation. PPARγ-specific red nuclear fluorescence after LAM stimulation was diminished in TLR2-/-. Lipid body formation (C), PGE2 synthesis (D), and TNF-_ production (E) were evaluated in macrophages from TLR2+/+ and TLR2-/- mice 24 h after infection in vitro with BCG (MOI, 1:1). Each bar represents the mean _ SEM from n = 3 pools of 10 animals in three independent experiments. Differences between control and infected groups are indicated by asterisks (p < 0.05). +, Differences between wild-type and deficient mice.

4.10. Inhibition of PPARγ leads to enhanced mycobacterial killing by macrophages

Evidence has suggested that lipid body formation may favor intracellular mycobacterial survival and/or replication (188) (189) (190) (188). According to the results that PPARγ activation was important in lipid body formation in BCG-infected macrophages, we asked whether PPARγ has a role in BCG pathogenesis. Inhibition of PPARγ with the pretreatment of its synthetic antagonist GW9662 we found that the capacity of macrophages to kill *M. bovis* BCG was significantly enhanced as assessed by live/dead bacterial staining by flow cytometry (Figure 23).

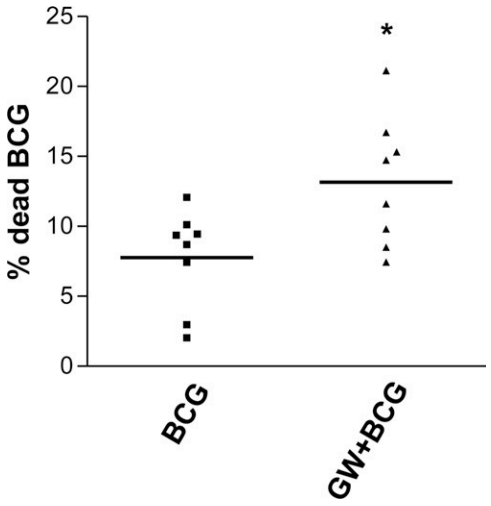

Figure 23. Pretreatment of macrophage with GW9662 enhances mycobacterial killing. Viable vs. nonviable BCG obtained from GW9662-treated or vehicle-treated macrophages were evaluated by a LIVE/DEAD BacLight Bacterial Viability Kit. The percentages of live and dead bacteria were determined by flow cytometry 12h after infection. Differences between treated and untreated groups are indicated by asterisks ($p < 0.05$), $n = 8$.

5. DISCUSSION

Immature dendritic cells are responsible for surveying their environment to detect and process antigens. Once activated by pathogen associated molecules and/or inflammatory cytokines they mature and migrate to the draining lymph nodes where they present the processed antigens and produce cytokines to activate T cells. This differentiation and maturation process provides a prime example how a cell type must change its behavior in response to its environment.

It has been established that tolerogenic DC subtypes are generated upon exposure to various lipids such as retinoids, fatty acids or by active Vitamin D3 resulting in changes of surface molecules, cytokine productions and a reduced capacity to stimulate T-cell proliferation. These effects have been tied to activation of some members of the nuclear hormone receptor superfamily. Retinoids activate the Retinoic Acid Receptor, modified fatty acids turn on PPARγ and Vitamin D3 activates the Vitamin D Receptor (174-180). In the past few years we and others have systematically mapped the mechanisms by which these receptors change the immunophenotype. It is intriguing to speculate that as a general principle, lipid signaling alone could polarize DCs into tolerogenic or immunogenic subtypes. We sought to determine how the LXR might play a role in DC differentiation and function within this context.

LXR is a receptor of oxysterols and as such regulates gene expression in response to changing lipid environment. While LXR has been extensively studied in macrophages, the role of this receptor in antigen presenting cells is not particularly well understood. In macrophages LXR appears to integrate lipid metabolism and inflammation. Its role in regulating cholesterol efflux from macrophages by the induction of the expression of cholesterol transporters (ABCA1, ABCG1, ABCG4, ABCG5 and ABCG8) has been established first (106) (30, 104, 108-110). Later studies primarily from the Tontonoz laboratory uncovered another important aspect of LXR in macrophage biology, and started a new chapter in LXR biology. In macrophages LXRs control both inflammation and immunity (112) (151) (121) (123) (21-24). It was shown that both LXR isoforms regulate the expression of their target genes involved in lipid homeostasis and at the same time repressed the expression of inflammatory mediators such as iNOS, COX-2, IL-6, IL-

1β, MCP-1, MCP-3 and MMP-9 in cultured macrophages in response to bacterial infection or LPS stimulation (151) (124) (22, 36). These results strongly suggest a role for LXR in downregulating inflammatory stimuli and make it a target in inflammatory conditions. However, recently it has been also shown that mice deficient in both LXR isoforms, LXRα and LXRβ (LXRα-/-LXRβ-/- mice), were more susceptible to M. tuberculosis infection, developing higher bacterial burdens and an increase in size and number of granulomatous lesions suggesting that the role for LXR in inflammation is more complex and depends on the cellular and inflammatory context (191) (37). Another observation, supporting a more pro- inflammatory role of LXR, was made in primary human macrophages. In these cells short-term pretreatment with LXR agonists significantly reduced the inflammatory response induced by LPS, but when pretreated with LXR agonists for 48 hours, macrophages exerted an enhanced LPS response leading to an increase in MCP-1 and TNF secretion and increased generation of ROS (156) (38).

The fact that lipid activated LXRs are also present in dendritic cells prompted us to explore the effect of ligand activation of this receptor.

A prior study published on the role of LXR in DCs already established that LXRα is the dominant isoform in differentiating DCs and that LPS induced maturation is impaired with a maintained adherence to the culture plate that was explained with the regulation of actin-bundling protein fascin by LXR (192) (19). However in that study no LXR-dependent gene expression analysis was carried out and therefore the correlation of receptor specific gene expression and changes in cellular phenotype upon ligand treatment cannot be easily made. In other words some of the effects might be receptor independent. Recent studies in mice also showed that endogenous ligands produced by the tumor activates LXR, which in turn controls the migratory capacity of DCs to tumor tissues by down regulating CCR7 expression. This finding strongly supports the role of LXR in favouring anti-tumor growth, moreover it further establishes the in vivo relevance of LXRs in dendritic cells (186) (39).

Our study presented here also provides support for an in vivo relevant function. We have carried out a comprehensive analysis of LXR expression and activation to provide further details on how the activation of LXR induces a program that affects the phenotype and response of DCs. This program includes induction of co-stimulatory molecules, increases

in pro-inflammatory cytokine production and enhanced T-cell activation. We also show that ligand treatment of circulating blood CD1c+ DCs separated from peripherial blood resulted in increased expression of LXR target genes showing that circulating DCs are also capable of LXR signaling. These experiments argue against a cell type restricted effect. DCs such as other professional APCs display the broadest repertoire and express the highest levels of TLRs ensuring the recognition of a wide array of pathogenic or damaged self structures resulting in the activation of NF-κB (193) (194) (40, 41). It is therefore likely that additional sensory mechanisms also participate in signal augmentation or attenuation. While in macrophages LPS interferes with the LXR signalling by inhibiting the expression of its target genes (123) (124) (24, 36) in our studes we found that LPS (and Poly(I:C)) in this human DC model increases the expression of the measured target genes. This suggests that LXR signaling is integrated in the TLR signaling pathway by a cell type or context dependent manner. One might speculate that DCs when exposed to specific TLR activators induce an increased LXR response, provided a ligand is available, indicating the existence of a potential mechanism for pathogen elimination by an enhanced inflammatory response (Fig. 6A). The source and origin of such ligand(s) are unknown. However the fact that a recent study documented the production of LXR activating lipids from tumors (186) (39) and our findings that DCs of reactive lymph nodes from patients with tuberculosis, sarcodiosis and of tumor associated lymph nodes also express LXRα suggest that the inflammatory environment has a role on the receptor‘s activity also in vivo.

For the mechanism of enhanced activation NF-κB signaling is a very likely candidate. The inhibitory IκB proteins are complexed with the NF-κB/Rel transcription factors in the cytosol. In its inactive state NF-κB molecules are associated with IκB proteins in the cytoplasm that prevents their nuclear localization and subsequent DNA binding. Its activation via phosphorylation at serine 32 and 36, is essential for release of active NF-κB resulting in the nuclear translocation of active NF-κB (195) (42). We measured the phosphorylation of the IκB and the phosphorylation of p65 after LPS stimulus in both the control and the LXR treated DCs and found evidence for a prolonged response in LXR activated cells. Concerning the mechanism of this prolongation it is conceivable that activation of yet unidentified kinases might be involved (Figure 24).

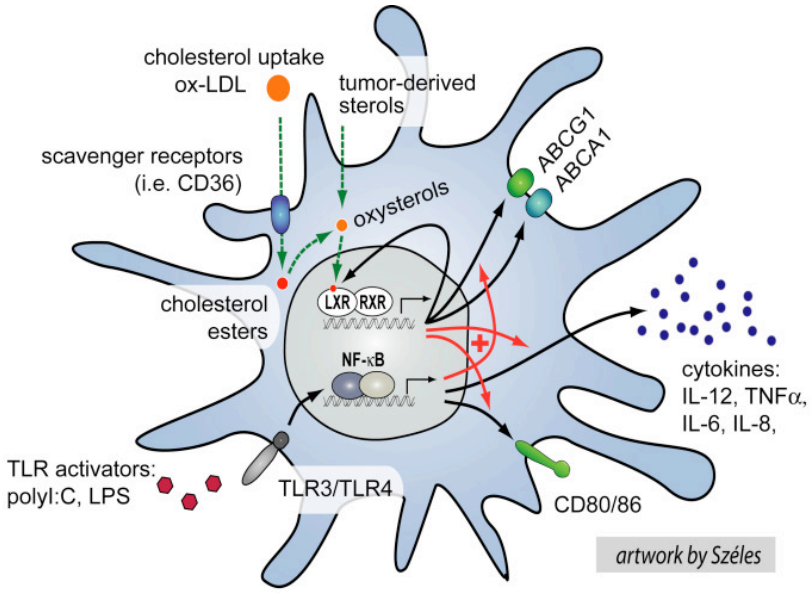

Figure 24. LXR pathway and TLR signaling cross reacts in dendritic cells
LXRs are present and can be activated by natural or synthetic activators in dendritic cells. Upon its activation the induced target gene expression (ABCA1, ABCG1) is accompanied by increased expression of DC maturation markers such as CD80 and CD86. In mature DCs LXR activation augmented the production of inflammatory cytokines (IL-12, TNFa, IL-6 and IL-8) underpinned by prolonged NFkB signaling that resulted in an increased capacity to activate CD4+ T cell proliferation upon ligation with TLR4 or TLR3 ligands. Activation of the NFkB pathway resulted in augmented LXR signaling.

In our work we provide data and support to suggest that the role of LXR in inflammation is more complex than previously anticipated and further work is needed to sort out cell, context and species specific differences (196) (43). Nonetheless the concept that lipids can both positively and negatively modulate the immunophenotype of antigen presenting cells provides an intriguing model worth further exploring.
In our extended studies on the cross-talk of inflammatory pathways and lipid activated nuclear receptors we detected that although human peripherial blood derived monocytes

lack the expression of the PPARγ protein, cells exposed to BCG infection exert a marked upregulation in the protein levels of PPARγ. This observation provided a starting point for the characterization of the possible signaling mechanisms for the induction of the receptor and the related biological functions through examining lipid accumulation and bacteria survival. We found that BCG infection induced increased expression of PPARγ paralleled the augmented lipid body formation and $PGE_{(2)}$ synthesis in wild type but not in TLR2-deficient mouse peritoneal macrophages. The function of PPARγ in modulating BCG infection was demonstrated by the capacity of the PPARγ agonist BRL49653 to potentiate lipid body formation and $PGE_{(2)}$ production. Pretreatment with the PPARγ antagonist GW9662 inhibited BCG-induced lipid body formation and $PGE_{(2)}$ production. Moreover, inhibition of PPARγ by specific synthetic inhibitor (GW9662) enhanced the mycobacterial killing capacity of macrophages.

As PPARγ coordinates lipid metabolism and inflammation in BCG-infected macrophages, potentially affecting mycobacterial pathogenesis, the role for LXR in tuberculosis associated dendritic cells is also of definite interest calling for further studies.

5. SUMMARY

DCs respond to changes in their lipid environment by altering gene expression and immunophenotype. Some of these alterations are mediated via the nuclear receptor superfamily. In previous works nuclear receptors such as PPARs, RAR and VDR, that are found to be expressed at high levels in differentiating DCs and are activated upon exposure to various lipids such as fatty acids, retinoids or by active Vitamin D3, were found to support a tolerogenic DC phenotype. Their effect on DC functions involved interaction with stimulatory effects such as TLR ligands (2, 3, 4, and 7) and cytokine productions resulting in a reduced capacity to stimulate T-cell proliferation. Despite the well-characterized role of LXR in macrophage biology little is known about its the contribution to DC biology. In our work we investiagted the role of LXR in dendritic cell differentiation and functions. We carried out a systematic analysis of LXR, activated by synthetic ligands or naturally occurring oxysterols in developing human monocyte derived dendritic cells. We found that LXRs are present and can be activated throughout dendritic cell differentiation in monocyte as well as blood derived DCs. Administration of LXR specific natural or synthetic activators induced target gene expression accompanied by increased expression of DC maturation markers such as CD80 and CD86. In mature DCs LXR activation augmented the production of inflammatory cytokines IL-12, TNFα, IL-6 and IL-8 and resulted in an increased capacity to activate CD4+ T cell proliferation upon ligation with TLR4 or TLR3 ligands. These effects appear to be underpinned by prolonged NFκB signaling. Supporting such an inflammatory role we found that LXR positive DCs are present in reactive lymph nodes from patients with tuberculosis and sarcoidosis and was present also in DCs of tumor associated lymph nodes. In our work based on similar histological findings where PPARγ was induced in monoctyes upon BCG infection we found that PPARγ coordinates lipid metabolism and inflammation in BCG-infected macrophages, potentially affecting mycobacterial pathogenesis. We propose that activation of LXR represents a novel lipid-signaling paradigm that alters the inflammatory response of human DCs, therefore the role for LXR activation of dendritic cells in vivo calls for further studies.

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9. PUBLICATIONS

Publications included in the PhD thesis:

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