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Lipid Signaling and Inflammation in Macrophages and Dendritic Cells

Role of Liver X Receptor in Dendritic Cell Biology

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INTRODUCTION

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). 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, LXRs and also

PPARs have large hydrophobic cavities that enable receptors to bind several different kinds of ligands but with a considerably lower affinity.

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.

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.

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, RARs are associated with certain types of leukaemia, ER with breast cancer and glucocorticoid receptors with inflammation control.

2. Liver X Receptor

Liver X receptors α and β (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. 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.

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 where PPARγ can induce its expression. In human but not in mouse cells another autoregulatory loop exists: LXR can induce its own transcription.

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).

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. There are also two synthetic LXR agonists existing and both of them have been widely used during the last several years GW3965 and T0901317.

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.

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 synthetic activators 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. LXR agonists also 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. In LDLR as well as apoE knockout mice LXR activators decreased atherosclerotic lesion development. On the other side 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. These mice develop severe atherosclerosis.

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.

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. In mice it is essential for placental development and vascularization. PPARγ can be activated by natural ligands such as oxidized fatty acids, oxLDL components, 11,13-hydroxyoctadecadienoic acid, 15D-PGJ2 and the thiazolidinedione (TZD) class of insulin-sensitizing drugs that have been developed and used in the treatment of type II diabetes mellitus.

PPARγ was also shown to influence myeloid development by modulating differentiation and metabolic functions of macrophages. In foam cells of atherosclerotic lesions PPARγ is expressed and its expression could be further increased with oxLDL. PPARγ enhances uptake of oxidized but not native LDL 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. 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.

Transplantation of PPARγ null bone marrow into LDLR-/- mice results in a significant increase in atherosclerosis. It was reported by the Glass laboratory that TZDs greatly inhibited the development of atherosclerosis in LDLR-deficient male mice. Similar results were shown by Chen and colleagues in apoE-/- mice, another murine atherosclerosis model. 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. Furthermore it was proven that PPARγ could directly induce transcription of the oxysterol receptor, LXR.

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

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. With the discovery that PPARγ and LXR controls lipid metabolism and inflammation at the same

time more and more efforts were made to gain evidence on how lipid metabolism and processing controled by these receptors contributes to immunregulation.

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. 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. 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. 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 β isotype is atheroprotective, noting however that LXRα is also involved in inducing the expression of genes that mediate cholesterol efflux.

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. 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.

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.

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.

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.

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). 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.

Studies on macrophages also identified novel natural activators of PPARγ (e.g. 9-HODE and 13-HODE) and pathways (e.g. 15-lypoxygenase) 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.

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 and that non-TZD PPARγ agonists failed to induce anti-inflammatory responses.

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.

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. 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.

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.

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.

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. 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. 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 and showed that ligand treatment not just down regulates the expression of CD1a, but also induced CD1d that resulted in an increased activation of iNKT cells (177).

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. 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.

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?

MATERIALS AND METHODS

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).

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. 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.

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.

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).

6. Immunodetection of PPARγ in Human and Murine Macrophages

Human macrophages obtained from monocytes, noninfected or infected with GFP-BCG (6×10^6 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).

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.

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).

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.

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).

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)

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.

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).

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.

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).

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.

RESULTS

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. We have further validated this finding by carrying out a time course experiment in which the two LXR isoforms showed differential expression during the 5 day long 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. Using 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). This is explained by the established autoregulatory activity of LXRα.

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

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 and the expression levels of LXRα, ABCA1 and ABCG1 showed a marked increase when cells were treated with LXR ligand.

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.

2. LXR Agonists Have Minimal Effects on the Expression of iDC Co-stimulatory and Surface Molecules

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 showed a slight decrease while CD80 was minimally increased. CD86 and MHCII were not affected on iDCs at day 5. 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.

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. The potentiating effect of LPS on LXR signaling was verified also at the protein level, where ABCG1 was detected in mDCs exposed to GW.

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. 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. Surface expression of CD83 showed no difference when compared to control mDCs. Similarly higher expression of CD86 was detected when mDCs were treated with a natural ligand, 22ROH, as compared to untreated cells.

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.

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.

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. 22ROH differentiated and LPS activated DCs also produced more IL12, TNFα and IL8.

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. These data suggest that activation of LXR contributes to a prolonged NF-κB signaling that results in enhanced LPS signaling.

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.

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.

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.

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.

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.

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.

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. 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.

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. 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 has been established first. 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. 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. 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. 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..

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. 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.

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. 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 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. 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 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. 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.

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 difference. 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.

SUMMARY

Dndritic cells (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 extended studies on the cross-talk of inflammatory pathways and lipid activated nuclear receptors we found that PPARγ was induced in monoctyes upon *Mycobacterium bovis BCG* infection and showed that PPARγ coordinates lipid metabolism and inflammation in BCG-infected macrophages, potentially affecting mycobacterial pathogenesis. We propose that activation of LXR and PPARγ represents a novel lipid-signaling paradigm that alters the inflammatory response of human DCs and plays a pivotal role in the host vs. pathogen interactions.

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