The crosstalk between LXR and JNK pathways:

Mechanisms and mediators

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Benim guzel aileme,

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Abstract

This project was carried out in the Cell Signaling Research Group headed by Dr. Carme Caelles at IRB Barcelona. As a part of the research-line that deals with physiological and pharmacological (anti-inflammatory and/or anti-diabetic) actions conducted by some nuclear receptor (NR) ligands through negative interference with the c-Jun N-terminal kinase (JNK) signaling pathway, this project was focused on studying the mechanism of cross-talk between those pathways.

The results of the study show the ligand-dependent LXR inhibition of the LPS-activated SAPK (JNK and $p38^{MAPK}$) pathways. Moreover, PP5, a serine/threonine phosphatase previously shown to regulate MAPK pathways, is suggested as a novel target of LXR that negatively regulates LPS-induced activation of SAPK pathways. Furthermore, it is proposed that through the inhibition of SAPK activity, and thereby cJun/AP-1 activity, PP5 is mediating negative regulation of LPS-induced *Mmp13* gene expression by LXR in murine primary macrophages.

Resumen

Este proyecto se llevó a cabo en el Grupo de Investigación en Señalización Celular del IRB Barcelona y fue dirigido por la Dra. Carme Caelles. El trabajo se centra en el estudio del mecanismo de interferencia entre las vías de los receptores nucleares (NR) y la señalización de la quinasa c-Jun N-terminal Kinase (JNK). Esta inhibición forma parte de la línea investigación sobre las acciones fisiológicas y farmacológicas (anti-inflamatorias y / o anti-diabéticas) realizadas por los ligandos de algunos NR.

El estudio demuestra la inhibición de las vías SAPK (JNK y $p38^{MAPK}$) en respuesta a LPS a través de la activación dependiente de ligando de LXR. Además, PP5, una fosfatasa serina/treonina que previamente se demostró que regula las vías de las MAPKs, se sugiere como el mediador de esta inhibición. Esta interacción estaría inhibiendo la expresión en respuesta a LPS del gen Mmp13 en macrófagos de ratón.

Preface

Increasing amount of evidence states a close link between metabolism and immunity. Certain nuclear receptor pathways (particularly PPARs and LXRs) and stress activated protein kinase (SAPK) pathways are both shown to be important regulators of glucose and lipid metabolism as well as immune response. Since they have opposite effects on these processes, the existence of a biologically relevant negative crosstalk between these signaling pathways is very likely. As an example of this interaction, in our laboratory, it has been previously shown that thiazolidinediones, which are synthetic PPARγ ligands used as insulin-sensitizing agents in medicine for treatment of type 2 diabetes, mediate their hypoglycemic action by inhibition of the JNK cascade. This work extends the research to LXRs, analyzing their ability to inhibit the LPS-induced activation of the JNK and $p38^{MAPK}$ pathway. LXR ligand inhibits LPS-induced activation of the JNK and $p38^{MAPK}$ pathway in an LXR dependent manner in murine primary macrophages. PP5, another phosphatase that has been previously shown to inhibit JNK activity through direct interaction and dephosphorylation of ASK1 (MAP3K that activates SAPK activity upon stimuli such as LPS), mediates this negative crosstalk in murine primary macrophages. Moreover, the results suggest PP5 as a novel transcriptional target of LXR that is required for the transrepression of LPS-induced gene expression of *Mmp13* but not *Il-1β*, *Tnfα* or *Il-6*. Physiological relevance of this interference, in particular, in the context of inflammation should be further studied since foam cell formation assays failed to demonstrate involvement of PP5 in LXR ligand protection against AcLDL-induced foam cell formation.

INTRODUCTION

Inflammation

Inflammation is the multicomponent, protective tissue response to harmful stimuli such as tissue stress, injury or infection which serves to destroy, dilute or wall off both the injurious agent and the injured tissues. A complex network of coordinated cellular responses underlies inflammation that acts as part of the innate immune system (Medzhitov, 2008).

A controlled inflammatory response is clearly beneficial for the organism such as protection from exogenous pathogens and the repair of tissue damage that results from infection or trauma, but there are settings in which inflammatory response fails to achieve the physiological purpose and results in different pathological conditions depending on the inflammatory trigger (Figure 1).

Inflammation can be classified as acute or chronic according to the duration and kinetics of the response reaction. Acute inflammation is a short term process, usually appearing within a few minutes or hours and ceasing upon removal of the injurious stimuli. Triggered by infection or tissue injury, it involves the coordinated delivery of plasma and leukocytes, especially granulocytes, from the blood to the site of infection or injury. In case of chronic inflammation, the response is prolonged and this leads to a progressive shift in type of cells present at the site of inflammation. Chronic inflammation is characterised by simultaneous destruction and healing of the tissue as a result of the inflammatory process.

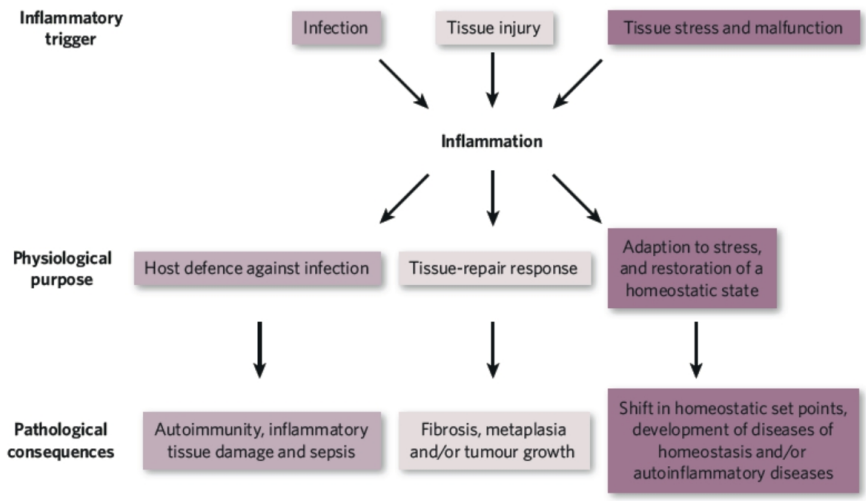

Figure 1. Causes, and physiological and pathological outcomes, of inflammation. Depending on the trigger, the inflammatory response has a different physiological purpose, however, when it is not properly regulated it has different pathological consequences. Adapted from (Medzhitov, 2008).

A number of widespread and devastating chronic diseases, including atherosclerosis, type 2 diabetes, and Alzheimer's disease, has an important inflammatory component at the origin and/or during the progression of the disease. In these diseases the precise identity of the stimulus is often either unknown or, if known, is very difficult to remove. Thus, there is strong interest in therapeutically targeting the inflammatory response (Tabas & Glass, 2013).

Inflammatory pathway

At the signaling level, the pro-inflammatory pathways consist of inducers, receptors, transducers and effectors and, therefore, can be targeted at different levels (Figure 2). Recognition of the inflammatory trigger by the specific receptor leads to the activation of protein kinases such as inhibitor of kappa B kinase (IKK) and c-Jun N-terminal

kinase (JNK) that in turn activate the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the activator protein 1 (AP-1), respectively. These regulators act in a combinatorial and cell-specific manner to induce the expression of genes that initiate the inflammatory response (e.g., tumor necrosis factor α (*TNFA*), interleukin 1 β (*IL1B*)), exert antimicrobial functions (e.g., inducible nitric oxide synthase), and recruit additional immune cells (e.g., chemokines), thereby setting into motion both innate and adaptive immune responses. Moreover, there are cytokine-mediated feed-forward loops that amplify the initial inflammatory response. Many receptors, transducers and effectors are existing or candidate targets for therapeutic intervention (Figure 2).

A reasonable alternative to inhibiting the inflammatory response by therapeutic intervention can be utilizing and activating the naturally existing anti-inflammatory pathways of the cell. Examples to such pathways are cyclic adenosine monophosphate (cAMP) pathway, cytokines such as interleukin 10 (IL-10) and transforming growth factor-β (TGF-β), certain nuclear receptors (NRs) such as glucocorticoid receptor (GR), liver X receptors (LXRs), peroxisome proliferator-activated receptors (PPARs) and vitamin D receptor (VDR) (Medzhitov & Horng, 2009). Among those negative regulators of inflammation, LXRs have the ability to suppress inflammatory signaling in macrophages and have shown benefit in mouse models of atherosclerosis, Alzheimer's disease and stroke (Morales *et al*, 2008; Calkin & Tontonoz, 2011; Cui *et al*., 2012), therefore, they constitute a favoured candidate for the enhancement of inflammation resolution.

As stated above JNK and LXR pathways both have important roles on the progress of inflammation with opposite outcomes. In the present work, the crosstalk between these two main regulatory pathways is studied; thereby a closer look into those pathways, in particular, as regulators of the inflammatory response is essential.

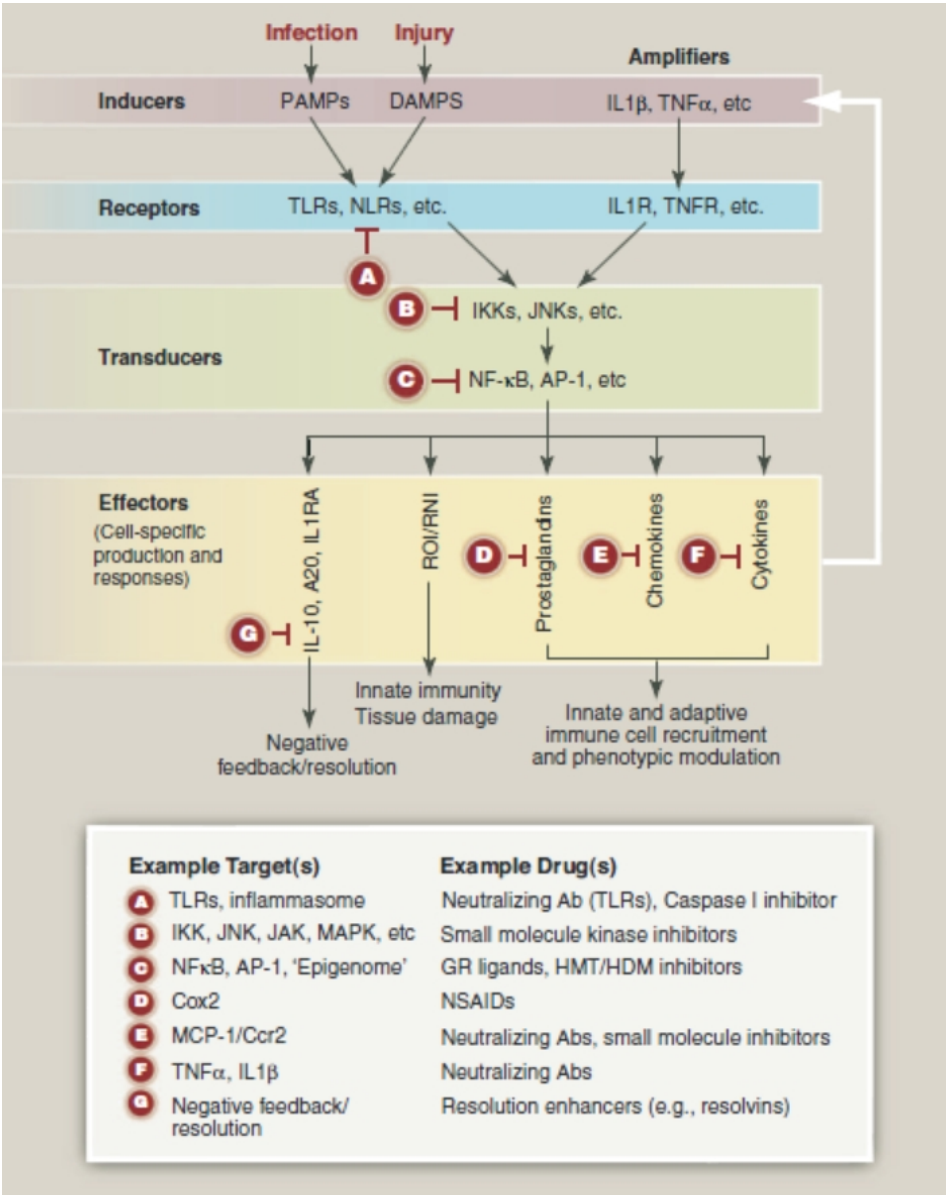

Figure 2. Inflammation at a signaling level and candidate therapeutic targets. Inflammation is typically initiated by pattern recognition receptors, such as TLRs and NLRs, that recognize PAMPs and/or DAMPs. These receptors typically couple to signal transduction pathways that activate latent transcription factors that include members of the NF-κB and AP-1 families. These factors in turn act in a combinatorial and cell-specific manner to induce the expression of a large number of genes that exert antimicrobial activities (e.g., generate ROI and RNI). Chemokines regulate the recruitment of additional immune cells. Production of bioactive lipids, such as prostaglandins (PG), also regulates pro- and anti-inflammatory cell functions. Expression of inflammatory cytokines provides a feed-forward loop for amplification of the initial response. The production of anti-inflammatory/resolution mediators, such as IL-10 and PGE2, in response to proinflammatory signals suggests that resolution programs are an inherent aspect of inflammation. Letters in red "stop signs" represent examples of points in proinflammatory signaling pathways that are currently existing or potential targets for therapeutic intervention. Ab, antibody; GR, glucocorticoid receptor; HDM, histone demethylase; HMT, histone methyltransferase; IKK, IkB kinase; MAPK, mitogen-activated protein kinase; MCP1, monocyte chemotactic protein–1; TNFR, tumor necrosis factor receptor; Cox2, cyclooxygenase 2; NSAIDs, nonsteroidal anti-inflammatory drugs. Adapted from (Tabas & Glass, 2013).

Introduction to MAPK family

JNK belongs to the family of mitogen-activated protein kinase (MAPK) family, which are proline-directed, serine/threonine protein kinases involved in signal transduction. MAPKs are ubiquitously expressed and highly conserved throughout evolution in eukaryotes. A very wide range of stimuli from hormones, growth factors, cytokines, agents acting through G protein-coupled receptors to environmental stress, activates MAPK pathways; which in turn regulate the coordinated and integrated cellular responses to such stimuli. MAPK pathways, once

activated, exert major effects on cell physiology by regulating the gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation (Kyriakis & Avruch, 2012).

The members of the MAPK family that are best described are the conventional MAPKs; namely extracellular signal-regulated kinases 1 and 2 (ERK1/2), JNK 1, 2 and 3, p38 mitogen activated protein kinase α, β, γ, and δ (p38^{MAPK} α, β, γ, and δ), and extracellular signal-regulated kinase 5 (ERK5). In addition to the conventional MAPKs, there are atypical MAPKs; ERK3/4, ERK7/8, and Nemo-like kinase (NLK) with distinct regulation and functions (Cargnello & Roux, 2011). Among those kinases, JNK and p38^{MAPK} subfamilies are activated by cellular stress and thereby, collectively known as stress-activated protein kinases (SAPKs).

Activation of MAPK depends on dual phosphorylation of threonine (T) and tyrosine (Y) residues on the signature sequence –TXY– where X is glutamate, proline or glycine in ERK, JNK and p38^{MAPK}, respectively (Kyriakis & Avruch, 2012). This dual phosphorylation is achieved by sequential phosphorylation events in a signaling cascade involving At least two other protein kinases, MAPK kinase (MAPKK or MAP2K) that is responsible for the dual phosphorylation of the appropriate MAPK, and a MAP2K kinase (MAPKKK or MAP3K) that phosphorylates and activates the corresponding MAP2K (Figure 3) (Huang *et al*., 2009).

Once activated, MAPKs regulate key cellular events in the cytoplasm by phosphorylation of membrane-associated and cytoplasmic proteins including other kinases and cytoskeletal elements. Activated MAPKs

also translocate to the nucleus to phosphorylate transcription factors such as c-Jun, c-Fos, ETS domain containing protein 1 (Elk-1) and c-Myc as well as other components of the transcriptional machinery to coordinate the expression of downstream target genes (Kyriakis & Avruch, 2012).

MAPKs are shown to have high a degree of substrate selectivity whereas MAP2K and MAP3K seem to function promiscuously in several pathways and are often subject to regulation by multiple stimuli (Kallunki *et al*., 1994; Biondi & Nebreda, 2003; Tanoue & Nishida, 2003). Therefore, scaffold proteins that select the specific MAPK pathway elements and bring them together are essential for the preservation of the selectivity and the efficiency of the MAPK signaling pathways. Scaffold proteins can be distinct polypeptides that bind specific MAPK pathway components or alternatively, core MAPK signaling components themselves can also possess intrinsic scaffolding properties (Morrison & Davis, 2003).

MAPK activity is also subjected to negative regulation by dephosphorylation by MAPK phosphatases (MKPs) or dual specificity phosphatases (DUSPs). These phosphatases can act on both the phosphothreonine and phosphotyrosine residues on activated MAPKs. The dual dephosphorylation renders MAPKs inactive, effectively ensuring a fast and efficient control of the signaling pathway (Barr & Bogoyevitch, 2001; Kyriakis & Avruch, 2012). MKPs may have one or several target MAPKs, such as the ERK specific inactivation by the cytoplasmic MKP-3/DUSP6 or MKP-1/DUSP1, which is able to dephosphorylate ERK, JNK and $p38^{MAPK}$ (Owens & Keyse, 2007).

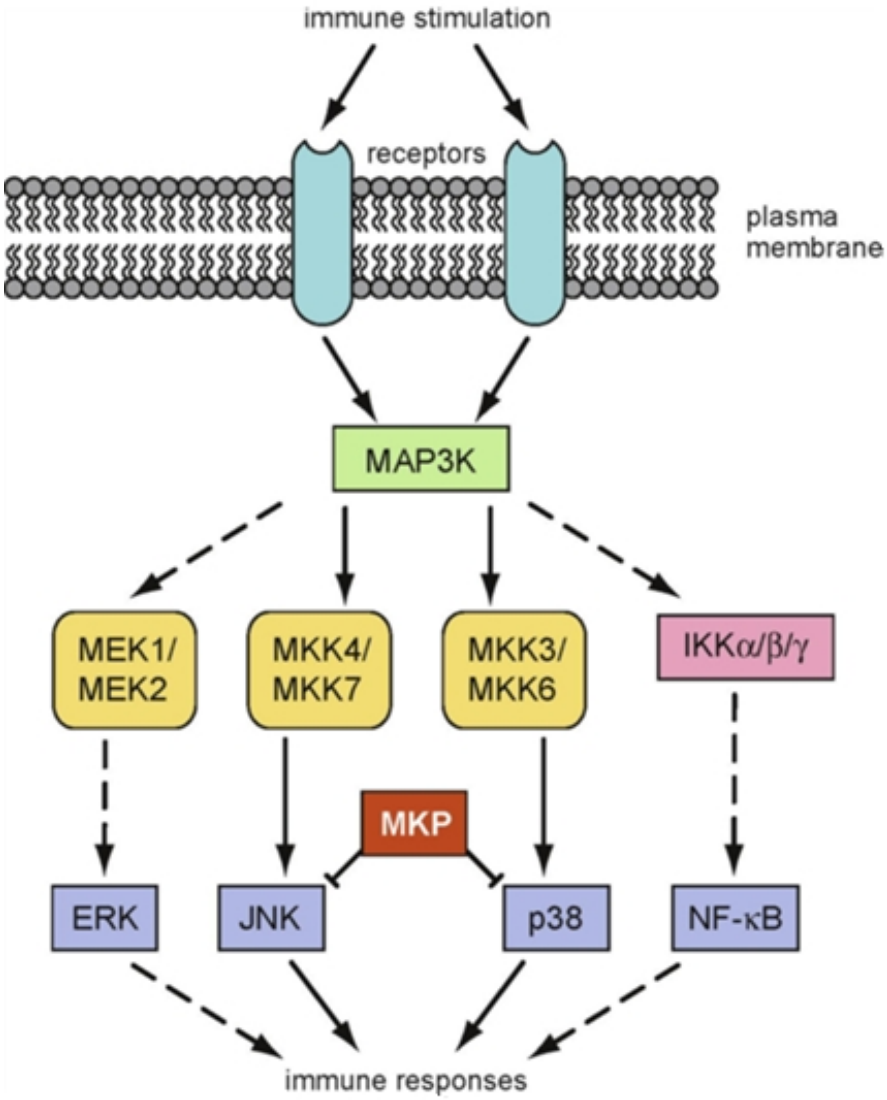

Figure 3. Organization of the MAPK cascade in the immune system. Stimulation of receptors in the immune system results in the activation of the three-tiered kinase module comprised of MAP3K, MAP2K and MAPK through sequential protein phosphorylation. Activated MAPKs are dephosphorylated by MKPs. Certain MAP3Ks such as TAK1 and MEKK3 also contribute to the activation of the IKK/NF-κB pathway. Although not depicted here, $p38^{MAPK}$ can be also activated by MAP2K-independent mechanisms. Adapted from (Huang *et al*., 2009).

Stress activated protein kinases (SAPKs)

As stated earlier, JNK and p38^{MAPK} together are called SAPKs because of their ability to respond to a wide range of cellular stresses. JNK was initially identified and purified as a protein kinase that was activated in the liver of rodents exposed to cycloheximide and therefore named Stress Activated Protein Kinase (SAPK) (Kyriakis & Avruch, 1990). Later studies identified JNK as a ultraviolet (UV) activated kinase that phosphorylated the c-Jun transcription factor in two residues (Ser63 and Ser73) of the transactivation domain (Pulverer *et al*., 1991; Adler *et al*., 1992; Hibi *et al.*, 1993). JNK can be vigorously activated by a variety of environmental stresses (heat shock, ionizing radiation, oxidants), genotoxins (topoisomerase inhibitors and alkylating agents), mechanical shear stress, vasoactive peptides, proinflammatory cytokines, ER stress, PAMPs/DAMPs and translational inhibitors such as cyclohexamide and anisomycin in addition to mitogens (Kallunki *et al.*, 1994; Weston & Davis, 2007).

The first p38^{MAPK} defined was identified as a stress and IL-1 activated kinase that could phosphorylate and activate MAPK-activated protein kinase 2 (MK2) that is a member of a Ser/Thr kinase family that targets heat shock protein 27 (Hsp27) (Kyriakis & Avruch, 2012). Similar to JNK, p38^{MAPK} is also activated by environmental stress such as osmotic and oxidative stress, inflammatory cytokines such as IL-1β and TNFα, PAMPs and DAMPs (Kyriakis & Avruch, 2012).

The JNKs are encoded by three different genes, *JNK1* to *3*, which are also named *MAPK8* to *10* respectively (Kyriakis *et al.*, 1994). On the

other hand, there are four genes encoding for p38^{MAPK} α, β, γ and δ; *MAPK14*, *MAPK11*, *MAPK12* and *MAPK13*, respectively (Han *et al*., 1996; Lechner *et al*., 1996; Li *et al*., 1996; Kumar *et al.*, 1997). As stated earlier, both JNKs and p38^{MAPK} contain the characteristic Threonine-X-Tyrosine MAPK dual phosphorylation motive, X being Proline and Glycine respectively (Kyriakis & Avruch, 2012).

JNK1 and *JNK2* are expressed ubiquitously, whereas *JNK3* is expressed specifically in heart brain and testis (Gupta *et al.*, 1996). Among p38^{MAPK} kinases, p38α and β are ubiquitously expressed while p38γ and δ display a more restricted pattern of expression. P38γ is expressed in skeletal muscle, heart, lung, thymus and testis, whereas p38δ is expressed in lung, pancreas, small intestine, kidney, testis and epidermis (Han *et al*., 1996; Lechner *et al*., 1996; Li *et al*., 1996; Kumar *et al.*, 1997; Dashti *et al.*, 2001; Court *et al.*, 2002).

Similar to other members of the MAPK family, SAPKs are subject to activation by several MAP2Ks (Figure 4). JNKs are activated through dual phosphorylation by two MAP2Ks, MKK4 and MKK7 (Rana *et al*., 1996; Yao *et al*., 1997). MKK7 selectively activates only JNK (Moriguchi *et al*., 1997) whereas MKK4 can also activate p38^{MAPK} (Dérijard *et al*., 1995; Lin *et al*., 1995). In addition to MKK4, p38^{MAPK} is also activated through phosphorylation by MKK3 and MKK6 which are both selective activators of p38^{MAPK} and do not activate JNK (Dérijard *et al*., 1995; Cuenda *et al*., 1996; Raingeaud *et al*, 1996; Cuenda *et al*., 1997).

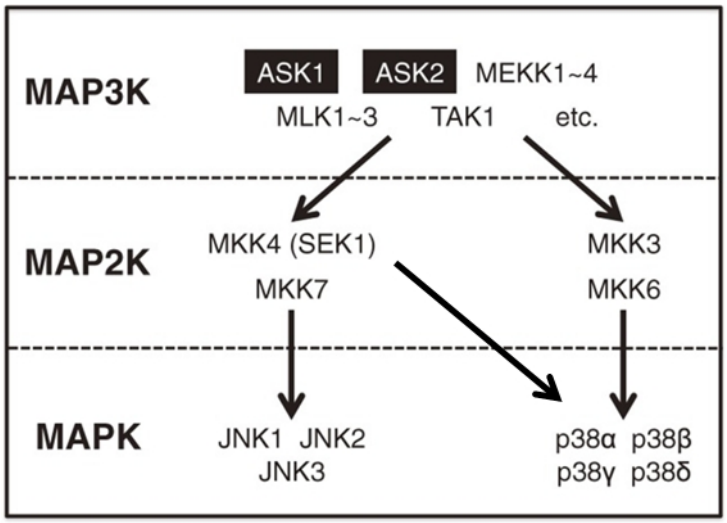

Figure 4. SAPK cascades. Apoptosis signal-regulating kinase 1 and 2 (ASK1 and ASK2) are among a number of MAP3Ks that activate three isoforms of JNK and four isoforms of $p38^{MAPK}$ through activation of MKK4/MKK7 and MKK3/MKK6, respectively. Adapted from (Hayakawa *et al*., 2012).

ASK1 as a regulator of SAPKs

The MAP2Ks that regulate the activation of SAPKs are also subject to regulation by serine/threonine phosphorylation by MAP3Ks (Figure 4). Among those kinases, ASK1 is able to activate both SAPKs through activation of MKK4,3 and 6 (Hayakawa *et al*., 2012).

ASK1 is activated in response to various stresses, such as reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, lipopolysaccharide (LPS), and calcium overload and is responsible for the activation of both SAPKs under these circumstances (Figure 5). The activation mechanism of ASK1 involves homo-oligomerization and depends on autophosphorylation of a threonine residue (Thr838 or Thr845 in human and mouse ASK1, respectively) within the activation

loop of the kinase domain (Matsukawa *et al*., 2004). The activating auto-phosphorylation event, thereby ASK1 activity, is highly regulated by ASK1 interacting proteins among which thioredoxin (Trx) plays an important role (Figure 5). Trx is a redox protein that changes structure depending on the cellular redox state. The reduced form of Trx binds to ASK1 oligomer inhibiting its autophosphorylation. ROS stimulation results in Trx oxidation and concomitant dissociation of Trx from ASK1 resulting in tight oligomerization and autophosphorylation of ASK1. ASK1 activation in response to TNFα and LPS signaling was reported to depend on ROS generation, suggesting a key role of ROS in the regulation of ASK1 activity (Hayakawa *et al*., 2012). In addition, other post-translational modifications play important roles in regulation of ASK1 activity. For instance, Akt negatively regulates ASK1 activity by direct phosphorylation of ASK1 on serine 83. A recent study suggests that ubiquitination of ASK1 is important for its stability and, therefore, has a role in the net activation of ASK1 and ROS-induced cell death. Moreover, ASK1 activity is also negatively regulated by serine/threonine protein phosphatase 5 (PP5) through direct binding and dephosphorylation of Thr845 (Morita *et al*, 2001; Zhou *et al*., 2004). This mechanism will be discussed in further detail in following sections. Regulation through MAP3Ks and MAP2Ks together with regulation by MKPs as stated earlier ensures a defined, specific and fast SAPK activation in response to specific stimuli.

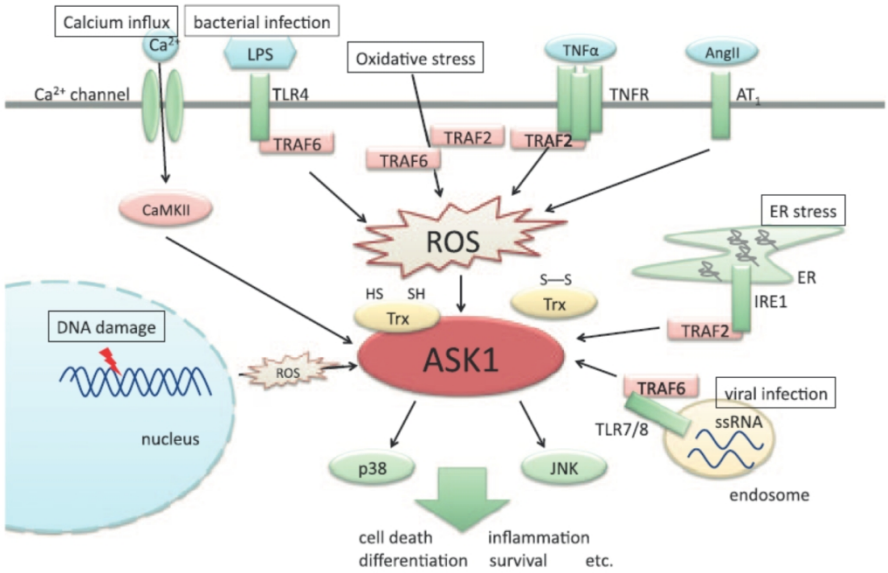

Figure 5. Overview of the functions of ASK1. ASK1 is activated by various stimuli, such as oxidative stress, ER stress, calcium influx, DNA damage-inducing agents, and receptor-mediated signaling through TNF receptor (TNFR), AngII receptor type 1 (AT1), and Toll-like receptors TLR4, 7, and 8. Intracellular signaling molecules, such as TNFR-associated factor (TRAF) family proteins, TRAF2 and TRAF6, and CaMKII, act as activators of ASK1. In addition to such ASK1-activating molecules, many stimuli that activate ASK1 use reactive oxygen species (ROS) as signaling intermediates. Thioredoxin (Trx) is a redox protein that changes its structure depending on the cellular redox state. Only the reduced form of Trx binds to the N-terminus of ASK1 and inhibits ASK1 activity. Upon ROS stimulation, Trx is converted to the oxidized form and dissociates from ASK1, leading to ASK1 activation. Activated ASK1 in turn activates the downstream $p38^{MAPK}$ and JNK pathways and, thereby induces various cellular responses, including cell death, inflammation, differentiation, and survival. Adapted from (Hayakawa *et al*., 2012).

AP-1, a major transducer of inflammatory response and a target of SAPKs

Once activated, SAPKs exert part of their effects through activation of transcriptional regulators (Figure 6). Among those, AP-1 transcriptional complex stands out as an important transducer of inflammatory response (Figure 2). AP-1 is a dimeric complex that is formed by members of the families JUN, FOS, activating transcription factor (ATF) and musculoaponeurotic fibrosarcoma oncogene homolog (MAF). These proteins interact to form heterodimers and/or homodimers with distinct affinities to bind different response elements in the DNA such as the heptameric TPA response elements (TREs) and/or the octomeric cAMP response Elements (CREs) (Eferl & Wagner, 2003). Target genes of AP-1 mainly encode proteins recruited by stress and inflammation such as IL-2, CD40, CD30, TNFα and c-Jun itself. In addition, AP-1 also participates in the transcriptional induction of extracellular proteases and cell adhesion proteins, late response genes that are important to inflammation (Shaulian & Karin, 2002).

The $p38^{MAPK}$ and JNK are the major kinases responsible for the recruitment of AP-1 (Kyriakis & Avruch, 2001; Kawai & Akira, 2010). Regulation of AP-1 activity by these kinases is quite complex and occurs at different levels including gene transcription, mRNA and protein stability and post-translational modifications of the components, thereby regulating dimer composition and activity (Figure 6).

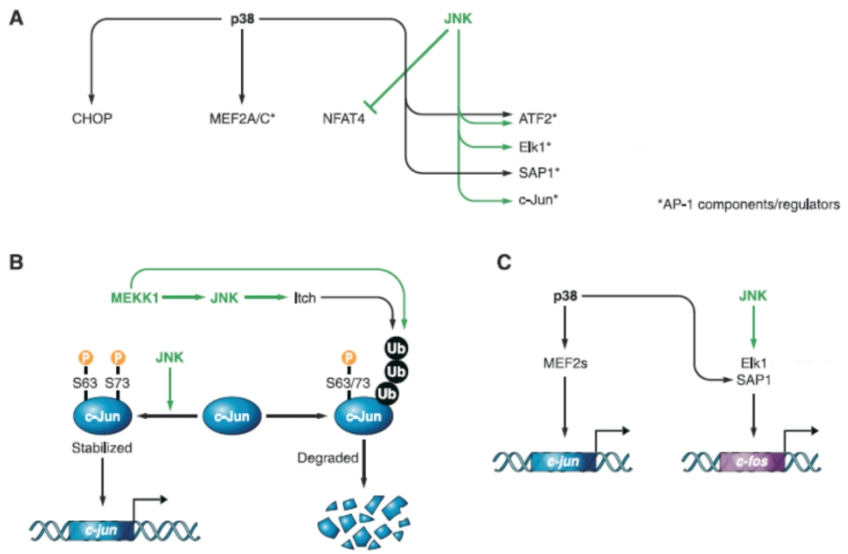

Figure 6. Regulation of transcriptional factors by SAPKs. **A.** Representative SAPK transcription factor substrates. Note that the complex regulation of AP-1 involves direct phosphorylation (c-Jun) as well as phosphorylation of elements that induce components (detailed in C). **B.** c-Jun phosphorylation by JNK can modulate c-Jun stability. JNK and MEKK1 (independently or through JNK) can recruit the Itch E3 ligase to foster c-Jun ubiquitination that leads to c-Jun degradation. **C.** MAPKs phosphorylate and activate transcription factors that upregulate AP-1 component genes. Note also that *c-jun* has an AP-1 element in its promoter and can be induced by activated c-Jun/AP-1. Adapted from (Kyriakis & Avruch, 2012).

Biological roles of SAPK

Since SAPKs control a great variety of cellular responses such as cell proliferation, differentiation, survival and migration of specific cell types, they are implicated in a wide range of biological progresses such as development, metabolism and immune response.

Studies of single and combinatorial *Jnk* isoform knockout mice have revealed specific stress responses and developmental roles for different JNK isoforms expressed in the brain. *Jnk3* deficient mice are resistant to hippocampal neuronal apoptosis which is an important clinical indicator in Alzheimer's disease, thereby, suggesting a role for JNK3 in the disease (Yang *et al*., 1997). Double *Jnk1* and *Jnk2* knockout mice die early in development due to massive neuronal apoptosis (Kuan *et al*., 1999).

JNKs were shown to exert pro- and anti-oncogenic functions depending on the cell type and stage of cancer development. For instance, *Jnk1* deficiency, but not *Jnk2* deficiency was shown to decrease susceptibility to hepatocellular carcinoma. In contrast to this, *Jnk1* deficiency results in more susceptibility to skin tumors (Kyriakis & Avruch, 2012). $P38^{MAPK}$ is also implicated in several types of cancer. Mice lacking p38α are more sensitive to K-ras-induced lung tumorigenesis. Moreover, hepatocyte specific deletion of p38α promotes chemically induced liver cancer. In contrast, mice lacking p38δ shows reduced susceptibility to development of skin carcinoma and Kras-induced lung tumors (Wagner & Nebreda, 2009).

JNK is also implicated in obesity and insulin resistance. In fact, obesity is associated with a low grade chronic inflammation that is a result of infiltration of adipose tissue by macrophages that become activated and, in turn, produce inflammatory cytokines such as TNFα which interfere with insulin signaling, thereby inducing insulin resistance (Gregor & Hotamisligil, 2011). The primary evidence came when it was shown that obese mice, either genetically or induced by diet, showed

increased JNK activity in skeletal muscle, adipose tissue and liver. Moreover, mice lacking JNK1 were protected from high fat diet (HFD)-induced obesity and had improved insulin signaling (Hirosumi *et al.*, 2002). Afterwards, several conditional *Jnk1* knockouts were used to better understand the role of JNK as a metabolic regulator. Adipocyte specific deletion of *Jnk1* was enough for protection against obesity and the development of systemic HFD-induced insulin resistance (Sabio *et al*., 2008). The role of macrophage JNK1 in obesity and insulin resistance was initially studied by bone marrow transplantation experiments. These studies showed that JNK1 deficiency in bone marrow was enough to protect mice from HFD-induced insulin resistance, but not from obesity, underlining the inflammatory component of the insulin resistance (Solinas *et al*., 2007). Moreover, a recent study of macrophage specific deletion of both *Jnk1* and *Jnk2* corroborates that JNK expression in macrophages is required for the establishment of obesity-induced insulin resistance and inflammation (Han *et al*., 2013).

The involvement of JNK in atherosclerosis, yet another pathological condition with an important inflammatory component, has also been reported. Atherosclerosis prone *ApoE* knockout mice simultaneously lacking JNK2, but not JNK1 developed less atherosclerosis than mice lacking only ApoE. Moreover, same study showed that macrophages lacking JNK2 displayed suppressed oxLDL-induced foam cell formation *in vitro* (Ricci *et al*., 2004).

Anti-Inflammatory Pathways: Nuclear receptors

Inflammatory gene induction is subject to negative regulation by many different pathways and mechanisms that are essential in protecting the organism against detrimental consequences of excessive inflammation. As mentioned briefly earlier, an alternative way of targeting inflammation in pathological conditions is to activate the naturally occurring anti-inflammatory pathways instead of using inhibitors targeting components of the inflammatory pathways. NRs seem to be convenient targets for such attempts since members of this superfamily such as PPARs, LXRs and GR show outstanding anti-inflammatory effects (Medzhitov & Horng, 2009). After a general introduction of the NR superfamily of transcription factors, the LXR pathway and its anti-inflammatory role will be discussed in more detail.

Introduction to Nuclear Receptors

The LXRs belong to the NR superfamily of transcription factors that control the expression of genes involved in diverse processes such as reproduction, development and metabolism in a ligand-dependent manner. The NR superfamily includes receptors for steroid hormones, such as the estrogen (ER) and GR receptors, receptors for nonsteroidal ligands, such as the thyroid hormone (TR) and retinoic acid (RAR) receptors, as well as receptors that bind diverse products of lipid metabolism, such as PPAR and LXR receptors. The NR superfamily includes several orphan receptors for which ligands have not been identified so far (Glass & Saijo, 2010).

Members of the NR superfamily have a common modular architecture organised into functional domains. The structure consists of a variable N-terminal activation domain containing the activation function 1 (AF1), a highly conserved DNA-binding domain (DBD) containing two zinc fingers and a conserved carboxy (C)-terminal ligand binding domain (LBD) (Figure 7) (Valledor & Ricote, 2004).

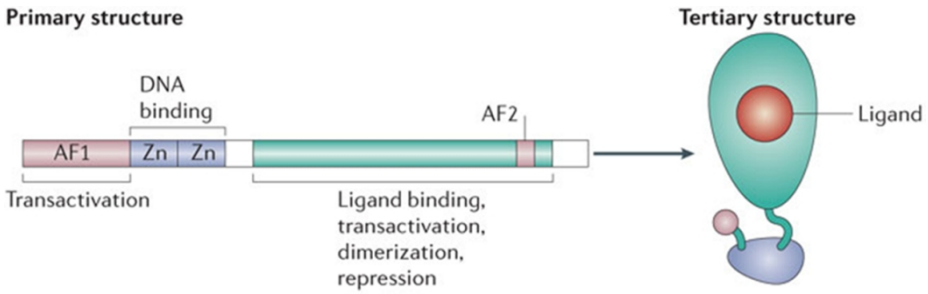

Figure 7. Domain Structure of NRs. Most members of the NR superfamily have a common domain structure consisting of an amino-terminal activation domain (often containing the activation function 1 (AF1)), a central DNA-binding domain (DBD), and a carboxy-terminal ligand-binding domain (LBD). The LBD specifies the ligand-binding properties of each receptor and determines ligand-regulated interactions with co-activators and co-repressors through allosteric changes mainly but not exclusively in a short helical region known as AF2. Together with the DBD, the LBD also contributes to homodimerization and heterodimerization of many NRs. Zn, zinc-finger domain. Adapted from (Glass & Ogawa, 2006).

NRs can be classified into three groups according to their modes of action. Some NRs act as homodimers, some as obligatory heterodimers with the retinoid X receptor (RXR) and some as monomers (Figure 8). The DBD mediates specific binding of these monomers, homodimers and heterodimers to specific DNA response elements located in enhancer or promoter regions of target genes.

These response elements mostly contain two loosely located core recognition motifs and each of these motifs contact a single DBD (Glass & Ogawa, 2006).

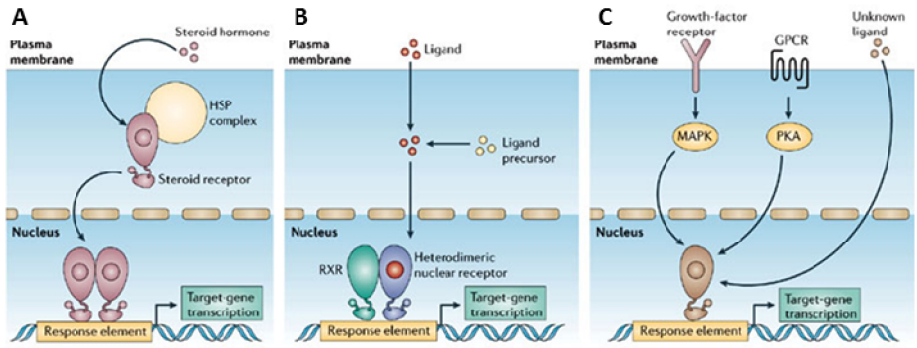

Figure 8. Mechanisms of DNA binding and transcriptional activation. The NR superfamily can be subdivided into three classes: the classical steroid-hormone receptors, the so-called 'adopted' orphan receptors that bind DNA as heterodimers with retinoid X receptors (RXRs), and the orphan receptors that have not been linked to naturally occurring ligands. **A.** Steroid receptors are synthesized in inactive forms that are associated with heat-shock protein (HSP) complexes in the cytoplasm and/or nucleus. The primary sources of ligands for this class of receptors are steroid hormones that are produced in steroidogenic tissues under the control of feedback control systems such as the hypothalamic–pituitary–adrenal axis. Hormone binding causes dissociation of steroid receptors from HSP complexes and allows binding to specific response elements in target genes. **B.** Heterodimeric nuclear receptors, including peroxisome-proliferator-activated receptors (PPARs) and liver X receptors (LXRs), bind constitutively to DNA with RXRs as obligate partners. Although some heterodimeric receptors, for example the thyroid-hormone receptors, are regulated by endocrine systems, most ligand-activated members of this group are regulated by molecules that are produced in an autocrine or paracrine manner. **C.** A considerable subset of the NR superfamily seems to primarily bind to DNA as monomers. In most cases, these receptors are designated as 'orphans'. Orphan receptors might mediate regulated transcription through changes in their expression or post-translation

modification (for example phosphorylation or sumoylation). GPCR, G-protein-coupled receptor; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase. Adapted from (Glass & Ogawa, 2006).

LXRs fall into the group of heterodimeric NRs together with the PPAR, farnesoid X receptor (FXR) and retinoic acid receptor (RAR). Unlike some other members of the family (such as steroid receptors) that shuttle between the cytoplasm and nucleus, most RXR heterodimers including those with LXRs are constitutively nuclear (Figure 8). In the absence of the ligand, these NRs are bound to the target DNA sequence in a complex with corepressor proteins so that the target gene transcription is repressed. Binding of the ligand induces a conformational change in the receptor that leads to the release of the corepressors and the recruitment of coactivators resulting in the transcriptional activation of the target gene (Figure 8).

Liver X Receptors

The LXR subfamily of NRs includes two members, LXRα and LXRβ with the NR nomenclature symbols NR1H3 and NR1H2, respectively. LXRα is highly expressed in liver and is also found in adipose tissue, intestine, kidney, spleen and macrophages, whereas LXRβ is expressed ubiquitously. Both members of the subfamily have the typical functional domains of the NRs that were mentioned above (Zelcer & Tontonoz, 2006). LXR/RXR heterodimers bind to LXR response elements (LXREs) that contain the hexamer core AGGTCA separated by four nucleotides. LXREs can be located in enhancer or promoter regions of the target genes (Figure 9). However, genome wide

mapping studies showed that LXRs, as well as other NRs, can bind to regions of DNA distant from transcriptional start sites, the functionality of some of these sites have been established and some remain to be assigned (Carroll *et al*., 2005; Lefterova *et al*., 2008; Lupien *et al*., 2008; Nielsen *et al*., 2008, Heinz *et al*., 2010).

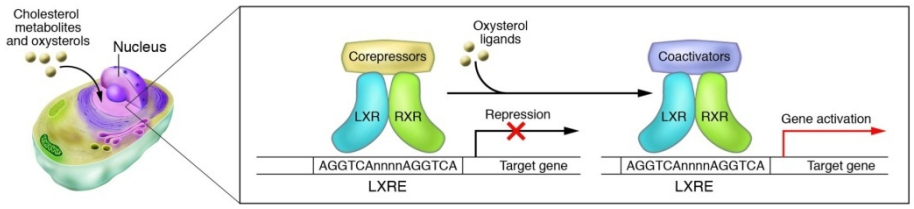

Figure 9. LXRs are cholesterol sensing transcription-factors. Within the nucleus, LXR/RXR heterodimers are bound to LXREs in complex with corepressors (e.g., SMRT, N-CoR). In response to the binding of oxysterol ligands, the corepressor complexes are exchanged for activator complexes, and target gene expression is induced. Adapted from (Zelcer & Tontonoz, 2006).

Endogenous ligands of LXRs are oxysterols such as 24(S),25-epoxycholesterol, 22(S)-hydroxycholesterol, and 24(S)-hydroxycholesterol that reflect the cellular cholesterol content. Consistent with this, LXRs have been shown to be essential in the maintenance of cholesterol homeostasis. Important evidence came from the study where mice lacking LXRα fed with high-cholesterol diet developed massive hepatic accumulation of cholesterol, whereas wild-type mice were highly resistant to the effect of this particular diet (Peet *et al*., 1998). The study demonstrated the role of LXRα as an effector of feed-forward mechanism that protects cells from increased cholesterol levels. In addition LXR is able to induce several hepatic and intestinal genes that are essential for cholesterol excretion such as the

ATP binding cassette (ABC) genes that are involved in cholesterol transport in liver and intestine (*ABCG5, ABCG8*) (Repa *et al*., 2002) as well as in macrophages (*ABCA1, ABCG1*) (Tangirala *et al*., 2002). Moreover, LXR was also shown to directly bind to the promoter and activate the expression of genes encoding sterol regulatory element binding protein 1c (SREBP-1c) (Repa, 2000; Schultz *et al*., 2000) and carbohydrate response-element binding protein (ChREBP) (Cha & Repa, 2007). Together these proteins activate most of the genes required for hepatic lipogenesis and triglyceride secretion. Nonetheless, biological roles of LXRs are not restricted to maintenance of cholesterol metabolism but also of immune system homeostasis. LXR has been shown to play an important role in apoptotic cell clearance that is essential for the maintenance of immune homeostasis (Gonzalez *et al.*, 2009) as well as inhibiting inflammatory signaling (Castrillo *et al.*, 2003; Joseph *et al.*, 2003) as will be discussed in detail in the following section.

LXRs as anti-inflammatory agents

LXRs are defined as the cholesterol-sensing NRs of the cell as discussed above. However, it is starting to be clear that they are not only key regulators of lipid metabolism and transport but they also play a very important role in inflammation by suppressing the inflammatory signaling in macrophages (Figure 10). It was shown that activation of LXR antagonises expression of a set of inflammatory genes after bacterial, LPS, TNFα or IL-1β stimulation in macrophages (Joseph *et al.*, 2003). Examples to such inflammatory genes are those involved in

generation of bioactive molecules such as iNOS and COX2, IL-6 and IL-1β, the chemokines monocyte chemoattractant protein-1 (MCP-1) and MCP-3 and MMP9 (Castrillo *et al.*, 2003; Joseph *et al.*, 2003). Moreover, other inflammatory genes such as tissue factor and osteopontin were also shown to be subject to similar repression by LXR agonists in macrophages (Ogawa *et al.*, 2005; Terasaka *et al.*, 2005).

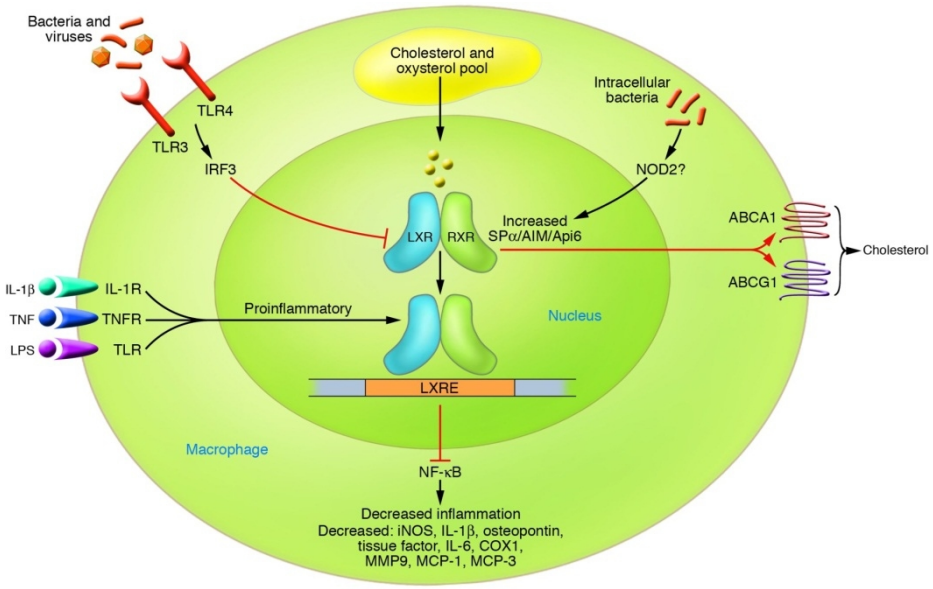

Figure 10. Integration of lipid metabolic and inflammatory signaling in macrophages by LXRs. Recognition of cytokines, bacterial components, or intact pathogens by their corresponding receptors initiates expression of proinflammatory genes. Activation of the TLR3/4 receptors by these signals blocks LXR-dependent gene transcription and cholesterol efflux from macrophages via an interferon (IFN) regulatory factor 3 (IRF3) dependent pathway. On the other hand ligand activation of LXRs inhibits inflammatory gene expression. Also intracellular bacteria induce LXR expression, possibly through a NOD2-dependent pathway, and promote macrophage survival through targets such as Api6. Adapted from (Zelcer & Tontonoz, 2006).

The anti-inflammatory effect of LXRs is not limited to isolated macrophages as demonstrated by several *in vivo* studies. In an example study, it was shown that LPS challenge led to an exacerbated systemic inflammatory response in LXR double knockout (LXR DKO) mice with increased hepatic expression of inflammatory genes encoding iNOS, TNFα and IL-1β (Joseph *et al.*, 2003). In addition, LXRs exhibited anti-inflammatory effects in two different models of dermatitis in mice; irritant contact dermatitis and oxazolone-induced allergic dermatitis (Fowler *et al*., 2003).

Studies have also demonstrated that chronic administration of LXR agonists dramatically decrease lesion formation in two different atherosclerosis mouse models; the low-density lipoprotein receptor (LDLR) and the ApoE knockout mice (Joseph *et al*., 2002). Moreover, it is strongly suggested that the anti-atherogenic properties depend on LXR expressed in macrophages. Combinatorial transplantation studies where bone marrow from wild type and LXR DKO mice was used to repopulate bone marrow from irradiated LDLR and ApoE knockout mice showed that the disease protection was mainly provided by LXR activity in bone-marrow derived cell lineages (Levin *et al*., 2005; Tangirala *et al*., 2002). Macrophages are derived from the bone marrow and are known to have key roles in lipid metabolism and progression of atherosclerosis as well as the inflammatory response. Excessive accumulation of cholesterol within macrophages at sites of atherosclerotic lesions converts macrophages into foam cells and accounts for the major fraction of lesion-deposited cholesterol (Im & Osborne, 2011). LXR agonists increase reverse cholesterol transport

from macrophages by increasing expression of macrophage ApoE and cholesterol efflux transporters; ABCA1 and ABCG1, thereby, reducing foam cell formation and cholesterol content directly (Tangirala *et al*., 2002).

LXR mediated transrepression and mechanisms

As discussed earlier, LXRs, that bind to DNA as heterodimers with RXRs act as transcriptional repressors in the absence of the ligand by interacting with co-repressor complexes that contain the NR co-repressor (NCoR) or the silencing mediator of retinoic acid and thyroid receptors (SMRT) (Figure 9). In addition to this repressive mechanism, LXRs are able to inhibit transcription of inflammatory genes that are targets of other transcription factors such as AP-1 and NF-κB in an LXRE-independent manner. In fact, a transcriptome profiling study showed that LXR ligands suppressed expression of a subset of important TLR4 target genes that contained AP-1 and NF-κB binding sequences but did not contain LXR binding sequences (Ogawa *et al*., 2005). Consistently, the repression activity does not require direct binding of LXR/RXR heterodimer to specific DNA sequences and the term 'transrepression' is used to define this activity (Glass & Saijo, 2010).

A considerable amount of data now is available that suggests a mechanism for transrepression of inflammatory genes by LXR and is mainly focused on the involvement of co-repressor NCoR turnover. The first and unexpected insight to the involvement of NCoR came from a study showing that NCoR deficient macrophages exhibit

deprepression of a subset of genes that are normally activated by AP-1 and NF-κB in response to inflammatory stimuli such as LPS and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Ogawa *et al.*, 2004). It was also shown that PPARγ ligands inhibit TLR4 mediated activation of the gene encoding iNOS by preventing the turnover of NCoR complex (Pascual *et al.*, 2005). This and further studies provided evidence for a model for transrepression by PPARγ in which ligand binding leads to an allosteric change that enables sumoylation of PPARγ. Sumoylated PPARγ is targeted to the NCoR complex and prevents the recruitment of the ubiquitination machinery necessary for NCoR clearance, so the NCoR complex remains bound to the promoter region repressing transcription (Figure 11). These findings suggested the possibility of LXR utilizing a similar mechanism to exert inhibitory effects on inflammatory gene expression. NCoR turnover was also shown to be important in transrepression by LXRs as well as the sumoylation of the ligand binding domain of LXRs through a slightly different mechanism (Ghisletti *et al*., 2007) (Figure 11).

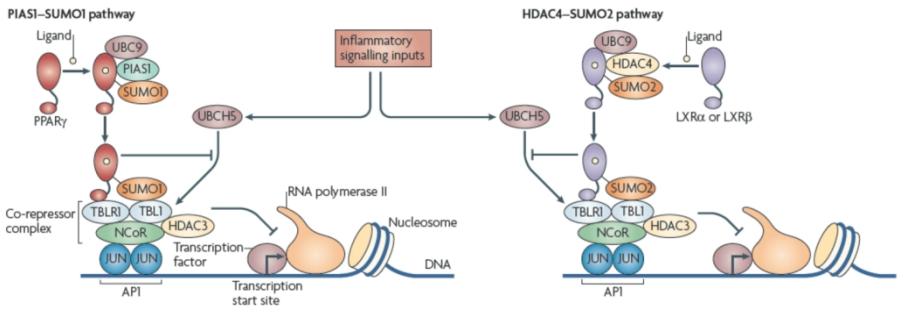

Figure 11. Sumoylation-dependent transrepression model for PPARγ and LXRs. PPARγ ligands induce sumoylation of a fraction of cellular PPARγ by the small ubiquitin-like modifier 1 (SUMO1). This is dependent on ubiquitin-conjugating enzyme 9 (UBC9) and protein inhibitor of activated STAT 1 (PIAS1) as the SUMO E2 and E3 ligases, respectively. Sumoylated PPARγ interacts with the NCoR complex to prevent signal-dependent recruitment of ubiquitin-conjugating enzymes (such as UBCH5) and the 19S proteasome components required for NCoR clearance. LXR ligands induce sumoylation of a fraction of LXRs with SUMO2 or SUMO3 (not shown), dependent on UBC9 and histone deacetylase 4 (HDAC4) as the SUMO E2 and E3 ligases, respectively. Sumoylated LXRs also interact with the NCoR complex to prevent signal-dependent recruitment of UBCH5 and the 19S proteasome components required for NCoR clearance. Adapted from (Glass & Saijo, 2010).

Nuclear receptors as regulators of SAPK activity

In addition to the suggested mechanisms to explain anti-inflammatory actions of NRs, some NRs were shown to act on the level of SAPK activity. For instance, hormone-bound GR can inhibit JNK activation, and therefore AP-1 activation, by a direct interaction with JNK (Caelles *et al.*,1997; Bruna, *et al.*, 2003). JNK inhibition by GR can also be transcriptionally mediated, as GR can induce *MKP1* gene transcription (Kassel *et al*., 2001). Regulation of JNK activity through *MKP1* gene transcription is not restricted to GR since RAR has also been shown to

stimulate *MKP1* gene expression (Lu *et al*., 2008). GR can also regulate SAPK activity through activating serum and glucocorticoid induced kinase (SGK) that phosphorylates, and concomitantly inhibits MKK4 (Kim *et al.*, 2007). Moreover, GR ligand has been shown to destabilize COX-2 mRNA through inhibiting $p38^{MAPK}$ activity (Lasa *et al*., 2001).

Another NR whose activation leads to decreased JNK activity is PPARγ. Thiazolidinediones (TZDs), a group of synthetic PPARγ ligands with insulin sensitizing activity were shown to inhibit JNK activity in adipocytes and pancreatic β cells, respectively (Díaz-Delfín *et al.*, 2007; Lanuza-Masdeu *et al*., 2013). Moreoevr, it was also shown that the inhibition of JNK activity was essential for the hypoglycemic action of TZDs *in vivo* (Díaz-Delfín *et al.*, 2007).

A member of the protein serine/threonine phosphatase (PSP) superfamily: PP5

As stated earlier, the reversible phosphorylation of proteins plays a crucial role in regulating cell signaling such as inflammatory responses or in particular, the activity of MAPK pathways. Therefore, protein phosphatases have an important role on regulating the activity of these kinases. For example, it has been shown that MKP1 has a prominent role in the negative regulation of JNK and $p38^{MAPK}$ activities and MKP1 knockout mice show exacerbated inflammatory response and increased susceptibility to endotoxic shock (Hammer *et al*., 2006; Zhao *et al*., 2006). In this context, a relatively recently identified serine/threonine phosphatase, PP5, is emerging as a regulator of MAPK signaling.

PP5 belongs to the superfamily of protein serine/threonine phosphatases (PSPs). PSPs comprise three major families; phosphoprotein phosphatases (PPPs), metal dependent protein phosphatases (PPMs) and the aspartate based phosphatases (Figure 12). The catalytic subunit of the most PPP family members associates with a wide variety of regulatory subunits. Representative members of the PPP family are protein phosphatase 1 (PP1), PP2A, PP2B (also known as calcineurin), PP4, PP6 and PP7. The PPM family members such as PP2C and pyruvate dehydrogenase phosphatase are dependent on manganese/magnesium ions (Mn^{2+}/Mg^{2+}). Unlike PPP, PPMs do not have regulatory subunits but they contain additional domains and conserved sequence motifs that contribute to substrate specificity. Metal ions play a crucial role in the catalysis of the dephosphorylating reaction for both PPP and PPM family members. The third family, the aspartate based phosphatases, uses an aspartate-based catalysis, as the name implies (Shi, 2009).

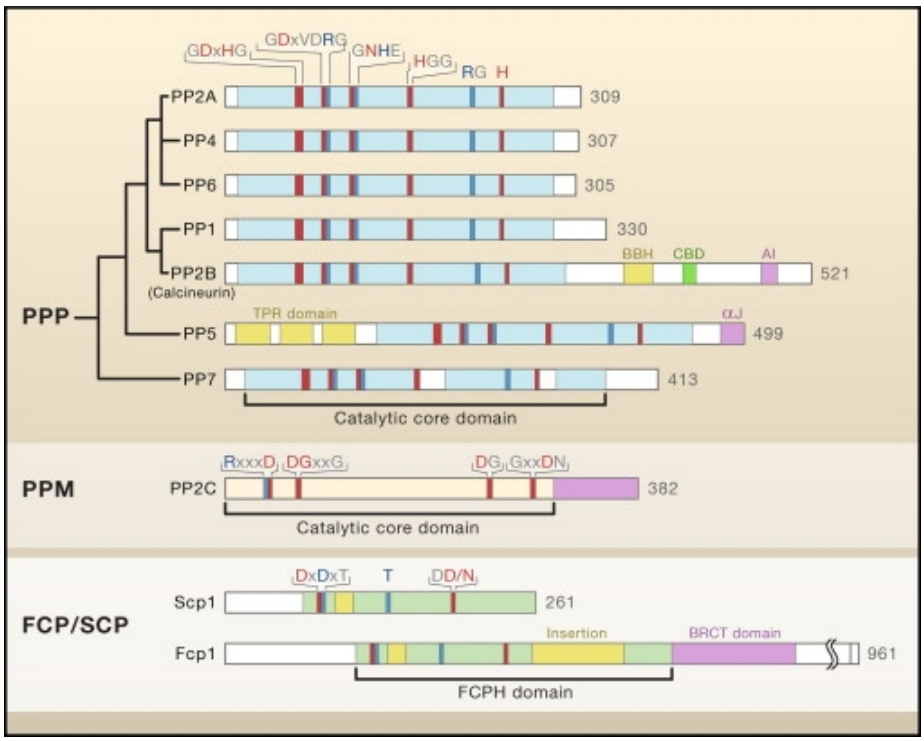

Figure 12. Protein serine/threonine phosphatases (PSPs). The three families of PSPs can be categorized into three families—phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases such as FCP (TFIIF-associating component of RNA polymerase II CTD phosphatase) and SCP (small CTD phosphatase). Representative members of each family are presented here. The catalytic core domains of each protein are indicated below the diagram. Signature sequence motifs are labeled above the diagram. Residues that contribute to metal coordination and phosphate binding are coloured in red and blue, respectively. The PPP family contains three characteristic sequence motifs within the conserved 30 kD catalytic domain: GDxHG, GDxVDRG, and GNHE (G, glycine; D, aspartic acid; x, any amino acid; H, histidine; V, valine; R, arginine; N, asparagine; E, glutamic acid). BBH, CNB-binding helix; CTD, carboxy-terminal domain; CBD, Ca2+-calmodulin-binding motif; AI, autoinhibitory sequence; TPR, tetratricopeptide repeat; FCPH, FCP-homology domain. All proteins here are from *Homo sapiens* except for PP7 (from the model plant *Arabidopsis thaliana*). Adapted from (Shi, 2009).

Protein Phosphatase 5

As stated above members of PPP family generally consist of separate catalytic and regulatory subunits whereas PP5 contains catalytic, regulatory and subcellular targeting functions within a single polypeptide chain. This and other structural properties of PP5 such as the regulatory tetratricopeptide repeat (TPR) domain makes it a unique serine/threonine phosphatase (Golden *et al*., 2008).

PP5 was discovered much later than the related enzymes; PP1, PP2A and PP2B not only because it is less abundant but also its basal activity is unusually low under typical protein phosphatase assay conditions. The catalytic domain of PP5 was first purified from a bovine brain extract and originally designated as PP3. Shortly after, the cDNA encoding PP5 was identified in cDNA libraries derived from rat adipocytes, yeast, a human teratocarcinoma and mouse embryos. The human gene encoding PP5 (*PPP5c*) was then identified on chromosome 19. PP5 differs from the other PPP members in this sense since it is encoded by a single gene throughout *Eukaryota* while most PPP members have isoforms encoded by different genes. PP5 is expressed in all tissues tested up to now with particularly higher expression in brain and neurons (Hinds & Sanchez, 2008).

Structure of PP5

PP5, as stated earlier, is structurally unique among the protein phosphatases. Although, the phosphatase domain resides in the C-terminal region and contains all the relevant motifs of the PPP family

of phosphatases, PP5 contains another domain not found in any other member of the PPP family, three consecutive TPR domains located in the N-terminal region (Figure 12). TPR domains are imperfect 34 amino acid sequences that mediate protein-protein interactions. The crystal structure of the PP5 shows strong similarity to TPR regions of other proteins. Each TPR motif consists of a pair of antiparallel α-helices, with adjacent TPRs packing together to form a series of paired-helices, generating an amphipathic groove that serves as the binding surface. HSP90; a molecular chaperone known to bind other TPR containing proteins, is up to now, the best known binding partner of PP5. PP5 binds the C-terminal region of HSP90 and four key residues (lysine 32 (Lys32), arginine 74 (Arg74), Lys97, and Arg101) in the TPR domain of PP5 are essential for this interaction (Russell *et al*., 1999). The TPR domain is also responsible for the low basal phosphatase activity and it is shown that long fatty acids such as arachidonic acid and HSP90 directly bind the TPR domain and activate phosphatase activity. The TPR domain engages with the catalytic channel of the phosphatase domain, restricting access to the catalytic site. This autoinhibited conformation of Ppp5 is stabilised by the C-terminal αJ helix that contacts a region of the HSP90-binding groove on the TPR domain. HSP90 activates Ppp5 by disrupting TPR-phosphatase domain interactions, permitting substrate access to the constitutively active phosphatase domain, whereas arachidonic acid prompts an alternate conformation of the TPR domain, destabilising the TPR-phosphatase domain interface (Yang *et al*., 2005).

Biological roles of PP5

PP5 is ubiquitously expressed in all mammalian tissues examined with higher levels in brain and neurons. Inside the cell, PP5 is broadly distributed defined as both nuclear and cytoplasmic. It has been implicated in the regulation of several cellular processes such as proliferation, migration, differentiation, electrolyte balance, apoptosis, survival, and DNA damage repair (Hinds & Sanchez, 2008).

PP5 appears to play a role in cell cycle progression in several ways. Treatment of cells with PP5 antisense RNA leads to hyperphosphorylation of p53 and subsequent G1 growth arrest (Chinkers, 2001). PP5 also binds to two proteins, CDC16 and CDC27, which are members of the anaphase-promoting complex (APC); a complex required for anaphase initiation and the exit from mitosis (Chinkers, 2001). Lastly, it is now known that PP5 plays an important role in DNA-damage repair and cell cycle arrest by attenuating the activities of two closely-related checkpoint kinases, ataxia telangiectasia mutated kinase (ATM) and ATM and Rad3 related kinase (ATR). These early studies relied on PP5 knock-down by RNAi. More recent work utilizing cells from PP5 deficient mice have confirmed the role of PP5 in ATM signaling (Yong *et al*., 2007).

PP5 also controls the activity of steroid receptors such as ER and GR through interaction with by HSP90. PP5 was identified as a binding partner and a negative regulator of ER (Ikeda *et al*., 2004). Interaction of PP5 with GR is highly studied and there is controversial data in the literature stating PP5 as a positive or negative regulator of GR (Somers

& DeFranco, 1992; Chen *et al.*, 1996). A recent study suggests a role for PP5 as a mediator of lipid metabolism through reciprocal regulation of GR and PPARγ activity (Hinds *et al.*, 2011).

PP5 was identified as a key effector for inactivation of MAPK signaling. Three major MAPK signal components affected by PP5 are Rac GTPase, Raf and ASK1 (Hinds & Sanchez, 2008). Prolonged hypoxia and treatment with reagents that induce oxidative stress result in higher expression of PP5 (Morita *et al.*, 2001; Zhou *et al.*, 2004). This increase in expression under low oxygen conditions is mediated by the activation and stabilization of a transcription factor, hypoxia inducible factor-1 (HIF-1), which binds to a HIF-1 response element in the PP5 promoter (Zhou *et al.*, 2004). Both, hypoxia and acute oxidative stress also induce the association of PP5 with ASK1 (Morita *et al*., 2001; Zhou *et al.*, 2004). After exposure to oxidative stress (e.g. treatment with hydrogen peroxide (H_20_2)) ASK1 is transiently activated by autophosphorylation at Thr845. *In vitro*, PP5 can dephosphorylate ASK1 at Thr845, suggesting that PP5 can inactivate ASK1 (Morita *et al.*, 2001). However, the ablation of PP5 expression using siRNA or antisense oligonucleotides results only in the prolonged activation of the ASK1/MKK4/JNK arm of ASK-signaling, without affecting the phosphorylation of $p38^{MAPK}$ (Zhou *et al*, 2004). Related to these findings, PP5 was shown to help suppress apoptosis on pancreatic β-cells by a mechanism involving regulation of JNK activity. Moreover, same study defines a novel phenotype for the PP5 KO mice, with reduced weight gain, lower fasting glycemia and improved glucose tolerance (Grankvist *et al.*, 2012).

OBJECTIVES

The general objective of this thesis project is to study the negative crosstalk between SAPK and LXR pathways.

Detailed objectives are:

1- To demonstrate the negative crosstalk between LXR and SAPK pathways.
2- To explore the mechanism underlying this crosstalk by identifying and validating the mediators.
3- To study the physiological relevance of this crosstalk.

MATERIALS&METHODS

Cell culture

HEK293T and HeLa cells were cultured in a 5% CO_2 atmosphere and 37^{o}C in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum), L-glutamine (2 mM), penicillin and streptomycin (100 I.U./mL and 100 µg/mL, respectively). Thioglycollate-elicited peritoneal and bone marrow derived macrophages were also cultured in a 5% CO_2 atmosphere and 37^{o}C in their corresponding growth mediums. See 'Obtaining primary macrophages' for the corresponding growth media.

Animals and obtaining primary macrophages

In this study, primary macrophages were obtained from animals between 8-12 weeks old; wild-type and knockouts of MKP1, JNK2 or PP5 as well as double knockout of LXRα/β. All animal procedures were approved by the Animal Care Research Committee of the University of Barcelona.

Thioglycollate-elicited peritoneal macrophages

1ml of 10% Brewer Thioglycolate preparation was injected into the peritoneal cavity of 8 to 12 weeks old mice 3 days before cell harvest with a 25-G needle. Third day, animals were sacrificed and the abdomen was sprayed with 70% ethanol (EtOH). The outer skin of the peritoneum was cut carefully and pulled back gently to expose the inner skin lining the peritoneal cavity. Using a 20-G needle, 10ml of growth medium (Roswell Park Memorial Institute (RMPI) 1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin

and streptomycin (100 I.U./mL and 100 µg/mL, respectively)) was injected gently into the cavity in order not to puncture any organ. At this step the needle should be facing up. After this, the needle was turned around to face down and gently as much liquid as possible was collected. The samples contaminated with visible blood were not used. The liquid was placed in pre-cooled falcons and kept on ice. After centrifugation 10' at 400 RCF and 4^{o}C, the supernatant was discarded and the pellet was gently resuspended in the growth medium, cells were counted and plated in the desired density for further use. After 2-3 hours, medium was changed to fresh medium. The typical yield is 7-10x10^{6} cells per animal.

Bone Marrow Macrophages

Mice between 8 to 12 weeks were used to obtain BMM. Animals were sacrificed and sprayed with 70% EtOH. The lower skin of the animal was removed and the exposed muscle and fat tissue of the legs was dissected away from the body. Femurs of the animals were isolated and put on a separate plate with pre-warmed DMEM medium. The remaining tissue on the femoral bones was carefully removed and the bones were cut near the extremes. The bone marrow was separated from the bone by passing approximately 2ml of DMEM medium through the medullar hole with a 25-G needle onto a fresh plate. The exposed bone marrow was disaggregated by passing twice through the needle and plated on non-treated culture plates with the growth medium for 7 days for the maturation to macrophages. At the end of the maturation process, cells firmly attached to the culture plate were

counted and seeded in the density required for the upcoming experiment. Growth medium of BMM is DMEM supplemented with 20% FBS, L-glutamine (2 mM), penicillin and streptomycin (100 I.U./mL and 100 μg/mL, respectively) and 30% L929 cell conditioned medium. L929 cells secrete the M-CSF growth factor that is necessary for the proliferation and maturation of the progenitor cells to macrophages. The L929 conditioned medium was prepared by seeding $5x10^5$ cells in a p150 with 40ml of DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin and streptomycin (100 I.U./mL and 100 μg/mL, respectively) and culturing them for 7 days. On the 7^{th} day, the medium was recovered and after centrifugation for 5' at 1000rpm, 4^0C, supernatant was stored in -20^0C for further use.

Foam cell formation assay

Thioglycollate-elicited peritoneal macrophages were seeded 200.000 cells per well on a 24 multi well plate. The following day, a pre-induction with 5μM TO901317 (Sigma) was done. 4 hours after, acetylated low density lipoprotein (AcLDL) (Kalen Biomedical) was added to get a concentration of 20μg/ml. 12 hours after the addition of AcLDL, another addition of 5μM TO901317 was done. 24hours after the addition of AcLDL, cells were fixed with 4% Paraformaldehyde and stained with Oil Red O to detect the accumulation of lipid inside the cells. Cells that were stained red were counted as positive. The experiment was carried out in triplicate and approximately 300 cells were counted and labeled positive or negative for each condition.

Transfection

HEK293T and HeLa cells were transfected using different expression plasmids (Table 1) with Polyethyleneimine (PEI) (Polyscience, Inc) (Boussif et al, 1995). Briefly, the plasmid DNA and the PEI were mixed in the right proportion in 150 mM NaCl and after 30' of incubation, the transfection mixture was added onto the cells. After at least 5 hours of transfection and up to 24 hours, the medium was changed and cells were kept at least 24 hours up to 72 hours to enable gene expression.

Insert	Tag	Species	Backbone
ASK1	Ha	mouse	pcDNA3.1
MKK7D	-	mouse	pCAGGS
PP5	Flag	mouse	pCMV5
$MKK6^{EE}$	-	mouse	pCAGGS

Table 1. Expression plasmids used in overexpression studies.

Transient depletion of PP5 by RNAi technique

BMMs were transfected with PP5 specific (Thermo Scientific, ON-TARGETplus SMART pool siRNA, PPP5C) and non-targeting (Thermo Scientific, ON-TARGETplus Non-targeting pool) siRNA by electroporation. Cells were scraped, washed with growth medium and counted and diluted to $10x10^6$/ml. 1,5µM siRNA was added per condition and $4x10^6$ cells were electroporated. The Neon Transfection

System (Invitrogen, Life technologies) was used for transfection following the manufacturer's instructions.

JNK immunocomplex assay

JNK was immunoprecipitated with the specific antibody (sc-474, Santa Cruz Biotechnology) and the JNK activity associated with the immunoprecipitates was determined by its capacity to phosphorylate in vitro the substrate GST-cJun.

The cells were lysed with a buffer that contained 20 mM HEPES-Na (pH 7.5), 10 mM EGTA, 40 mM β-glycerolphosphate, 2.5 mM $MgCl_2$, 1% NP-40, 2 mM Na_3VO_4, 1 mM DTT, 0.5 mM PMSF and 1 µg/L aprotinin. 1 mg of cell extract was immunoprecipitated with the JNK antibody and after three washes with the lysis buffer, the immunoprecipitate was incubated with the substrate (GST-cJun) for 30 minutes at 30ºC in presence of 20µM of ATP and 0.5µCi of [γ -^{32}P] ATP in a buffer containing 20 mM HEPES-Na (pH 7.5), 20 mM β-glycerolphosphate, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4 and 2 mM DTT.

Reactions were terminated by addition of 5X Laemli buffer and boiling, and proteins were resolved by SDS-PAGE. Coomassie staining was used to visualize proteins and kinase activity was measured with a Phospho Imager system.

Cellular, nuclear extracts and immunoblotting

Cells were lysed with lysis buffer that contained 50 mM of Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerolphosphate, 2 mM Na3VO4, 25 nM calyculin A, 1% TX100, 0.5 mM PMSF, 1 μg/mL leupeptin and 1 μg/mL aprotinin. Whole cell extracts were obtained by centrifugation at 13200 rpm for 10 minutes.

In order to obtain nuclear extract, cells were partially lysed in NPBT buffer (10mM Tris-HCl pH 7.4, 140nM NaCl, 2mM $MgCl_2$, 0.1% Triton X-100, 1mM DTT, 20mM β-glycerophosphate, 100μM Na_3VO_4, 0.5mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin). They were kept 10min. on ice and then centrifuged through a 50% sucrose cushion prepared in NBP (NPBT minus Triton) at 4^oC, 12000 rpm for 10min. Supernatant was discarded and nuclear pellets were suspended in DC buffer (20mM HEPES-NaOH, pH 7.9, 25% glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT, 20mM β-glycerophosphate , 100μM Na_3VO_4, 0.5mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin). Nuclear extracts were obtained by centrifugation at 13200 rpm for 10 minutes.

Protein concentrations were determined using the Bradford reagent (BioRad). Immunoblotting was carried out after separation of proteins by SDS-PAGE and transfer to PVDF membranes (Immobilon-P Transfer Membrane, Millipore).

Antigen	Company / Reference
PP5	BD Biosciences/611020
JNK	Santa Cruz / sc-474
Phospho-JNK	Invitrogen/44682
cJun	Santa Cruz/sc-45
Phospho-cJun	Santa Cruz/ sc-822
ASK1	Cell Signaling/ 3762&7931
Phospho-ASK1	Cell Signaling/ 3765
Flag	Sigma / F3165
Nek-9	(Roig *et al*, 2002)
myc	9e10 hybridoma
HA	9e10 hybridoma
$P38^{MAPK}$	Cell Signaling/ 9212
Phospho-$p38^{MAPK}$	Cell Signaling/ 9211

Table 2. Primary antibodies used.

Protein expression in bacteria

GST fusion protein, GST c-Jun (chicken) was expressed using pGEX-4T1 in E. coli RossetaTM 2 (DE3) or E. coli BL21 (DE3) induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 hours at 18^{o}C or 3 hours at 30^{o}C. It was purified with glutathione-sepharose (GE Healthcare) following standard protocols, and eluted with 25 mM reduced glutathione. Purified proteins were resolved in SDS-PAGE gels and stained with Coomassie blue (Sigma) to check protein size and purity.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted with TRIzol reagent (Invitrogen) following Manufacturer's Instructions. RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen) and quantified by qRT-PCR using SYBR Green (Applied Biosystems). The pairs of primers that were used are shown in Table 3.

Statistical analysis

Data were analyzed with a two-tailed unpaired Student's test. Values are presented as means + SEM.

Cloning

Ppp5c coding sequence was cloned into pCMV5-flag expression vector. The sequence was obtained and amplified by PCR from mouse cDNA library and was cloned into pCR2.1-TOPO vector and then subcloned into pCMV5-flag by using with TOPO TA Cloning (Invitrogen) and the restriction enzymes EcoRI and XhoI. All constructs were sequenced after generation with the commercial kit ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequences of primers used can be found in Table 3 (Ppp5c_cloning).

Ppp5c	forward	cacagacgctctgtcgtggactctc
	reverse	gcacttccggtgcagtttcttctg
Srebp-1	forward	aggccatcgactacatccg
	reverse	atccatagacacatctgtgcctc
Abca1	forward	gcggacctcctgggtgtt
	reverse	caagaatctccgggctttagg
Abcg1	forward	aaggcctactacctggcaaaga
	reverse	gcagtaggccacagggaaca
Cd36	forward	tccagccaatgcctttgc
	reverse	tggagattacttttcagtgcagaa
Sra	forward	catgaacgagaggatgctgact
	reverse	ggaagggatgctgtcattgaa
Tnfa	forward	caaagggatgagaagttccc
	reverse	tggtggtttgctacgacgt
Il-1b	forward	tgggcctcaaaggaaagaat
	reverse	caggcttgtgctctgcttgt
Il-6	forward	ccagagatacaaagaaatgatgg
	reverse	actccagaagaccagaggaaat
Mmp13	forward	agagggagaaaattctgggc
	reverse	ctcagagaagaagagggtct
L14	forward	gcgctggctgaatgctctg
	reverse	gcgctggctgaatgctctg
Ppp5c_cloning	forward	gaattccatggcgatggcggaggg
	reverse	gtcgacggctcactgctgctgacagc
Ppp5c_ChIP	forward	gtctcacgtcactggttgga
	reverse	aagactagcaggcagcgaag
Ppp5c_EMSA	forward	ataatgttcagaaatgggcaatat
	reverse	atattgcccatttctgaacattat
Abcg1_EMSA	forward	gctttggtcactcaagttcaagtt
	reverse	aacttgaactcgagtgaccaaagc

Table 3. Sequences of oligos used.

Gene reporter assay

Alterations in transcriptional activity of AP-1 complex were analysed by a gene reporter assay using Dual-Luciferase Reporter Assay System (Promega). The construct used for assessing AP-1 activity was 73-col-luc that includes an AP-1 binding site of *Mmp13* gene. Renilla expression vector served as the transfection control. The transfections and the assays were carried out according to manufacturer's instructions and the luciferase activity was monitored using a Berthold Technologies, Lumat LB 9507 Single Tube Luminometer.

Chromatin immunoprecipitation (ChIP)

ChIP assay was used to assess binding of LXR and RXR receptor to the intragenic region (+3439 - +4186) of *Ppp5c.* Binding of LXR and RXR to these genomic region has been also shown by ChIP-sequencing data deposited at (http://genome.ucsc.edu) (Heinz *et al*., 2010). The chromatin immunoprecipitation (ChIP) assay was conducted as previously described (Wagner *et al*., 2003). Briefly, 20 x 10^6 macrophages were fixed with 1% formaldehyde. Cross-linked adducts were resuspended and sonicated. Immunoprecipitation was performed by using 1.5µg rabbit anti-LXR antibody (generated as described in (Jakobsson *et al*., 2009)) or 1.5 µg rabbit anti-RXRα antibody (Santa Cruz Biotechnology). Rabbit IgG (Sigma-Aldrich) was used as a control for nonspecific binding. Protein-bound, immunoprecipitated DNA was reverse cross-linked at 65°C overnight and then purified by using a PCR purification kit (Qiagen). 1µl of a 30µl DNA extraction volume was used for either quantitative real time PCR

analysis as decribed earlier (primers can be found in Table 3). Input samples were diluted 1:64 before PCR analysis.

Electrophoretic mobility shift assay

Proteins LXRα, LXRβ and RXRα were transcribed and translated in vitro by TNT T7 Quick Coupled Transcription/Translation System (Promega) following the manufacturers' instructions. The double stranded DNA probes were labeled by terminal phosphorylation with [γ-^{32}P] ATP with the enzyme T4 PNK. The proteins were pre-incubated 10min. on ice in binding buffer (10mM Tris-HCl pH 8.0, 40mM KCl, 0.05% (v/v) Nonidet P-40, 6% glycerol and 1mM DTT), then 4ng of probe was added and 15min. of incubation was done at room temperature. Samples were resolved by gel electrophoresis, the gel was dried and analysed in a PhosphoImager. Sequences of probes used can be found in Table 3.

RESULTS

LXR ligand TO901317 inhibits LPS-induced JNK activation in primary macrophages.

Several NRs whose ligands have anti-inflammatory activity such as GR and PPARγ have the ability to inhibit SAPKs (Caelles *et al*., 1997; Lasa *et al*., 2001; Bruna *et al*., 2003; Díaz-Delfín *et al*., 2007; Lanuza-Masdeu *et al*., 2013). Since LXR ligands also have anti-inflammatory activity (Joseph *et al*., 2003), the ability of the synthetic LXR ligand TO901317 to inhibit JNK activity was tested. TO901317 is a potent, high affinity agonist of LXR (Repa *et al*., 2000). Bone marrow macrophages (BMMs) derived from wild-type mice were treated with different concentrations of the ligand TO901317 O.N. and the following day LPS induction was carried out. Activity of JNK was assessed by JNK immunocomplex assay using GST cJun as the substrate.

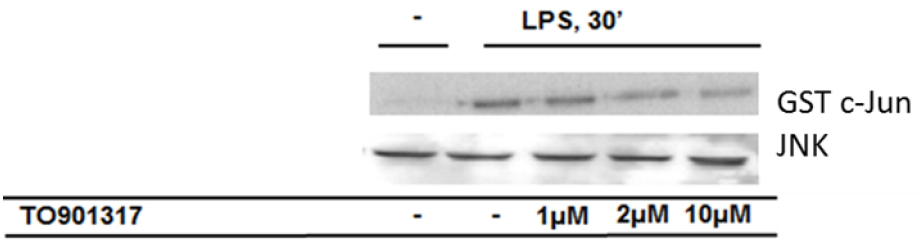

Figure 13. Effect of increasing concentration of LXR ligand on LPS-induced JNK activation. Upper panel shows JNK immunocomplex assay from extracts of BMMs treated O.N. with DMSO (-), 1, 2 or 10μM TO901317 and with 100ng/ml LPS for the last 30 min. as indicated. Lower panel shows the immunoblot analysis of total JNK protein in those extracts. This figure is representative of at least 3 independent experiments.

The results showed that LPS treatment effectively increased JNK activity (Figure 13). LXR ligand TO901317 at concentrations 2 and 10μM was able to inhibit JNK activity whereas at 1μM TO901317 did not lead to an efficient inhibition (Figure 13). The lowest effective concentration, 2 μM was selected for use in subsequent experiments.

JNK inhibition by LXR ligand is dependent on LXR.

In order to be sure that JNK inhibition by the ligand is through the activation of LXR itself and not an out of target action of the ligand, the ability of the LXR ligand TO901317 to inhibit JNK was analysed in macrophages derived from bone marrow of LXR double knockout animals (LXR DKO) lacking both LXRα and LXRβ. BMMs from both wild-type and LXR DKO animals were treated with the ligand TO901317 O.N. and activated by LPS during the last 30 minutes. The activity of JNK was assessed by western blotting with the specific (Thr183/Tyr185) phospho-JNK antibody as shown in Figure 14A.

An increase in the phosphorylation levels of JNK was observed similarly in wild-type and LXR DKO cells due to LPS treatment (Figure 14A). In wild-type cells, the ligand treatment led to a decrease in the phosphorylation levels indicating decrease in JNK activity whereas in the LXR DKO this effect was almost abrogated. It should also be pointed out that basal levels of JNK phosphorylation were noticed to be consistently higher in LXR DKO animals suggesting that LXRs might be negative modulators of JNK activity in basal conditions. The quantification of these experiments showed that JNK activity was highly inhibited by TO901317 treatment in wild-type cells (48%)

whereas this inhibition was significantly impaired in LXR DKO cells (11.5%), (Figure 14B).

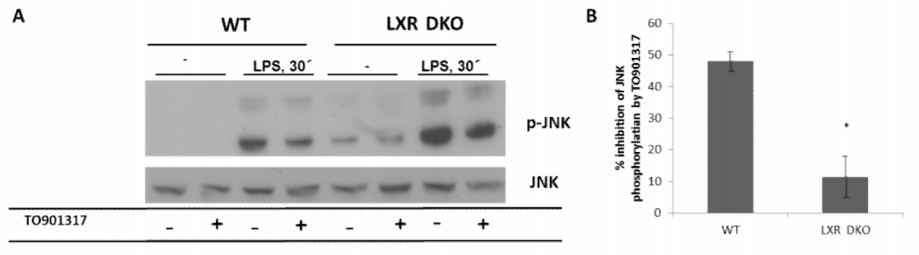

Figure 14. LXR ligand TO901317 inhibits JNK activity in an LXR dependent manner. **A:** The immunoblot analysis of phospho JNK and total JNK from extracts of wild type (WT) and LXR double knockout (LXR DKO) BMMs treated O.N. with DMSO (-) or 2μM TO901317 and with 100ng/ml LPS for the last 30 min. are shown in upper and lower panel, respectively. This figure is representative of three independent experiments. **B:** Percentage inhibition of JNK activity by TO901317 in wild-type and LXR DKO cells. The graph shows the quantification of immunblots represented in A. Phosphorylation levels were calculated as the sum of p46 and p54 isoforms and the phosphorylation levels were normalized according to total JNK protein levels. LPS-induced JNK phosphorylation level was assigned as 100% in each experiment and percentage of JNK inhibition was calculated. Data are expressed as the mean ± SEM and $^{*}P \leq 0.05$ compared to wild-type.

The ability of LXR ligand to inhibit another member of the SAPK family; $p38^{MAPK}$ was also analysed in a similar manner. Specific phosphorylation level of $p38^{MAPK}$ (Thr180/Tyr182) was used as an indicator of its activity.

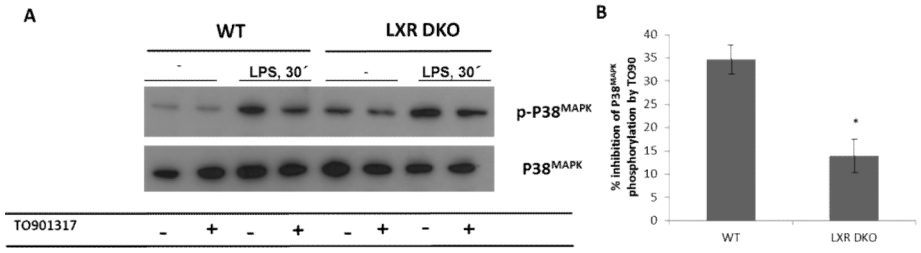

Figure 15. LXR ligand TO901317 inhibits p38^{MAPK} activity in an LXR dependent manner. A: The immunoblot analysis of phospho p38^{MAPK} and total p38^{MAPK} from extracts of wild type (WT) and LXR double knockout (LXR DKO) BMMs treated O.N. with DMSO (-) or 2μM TO901317 and with 100ng/ml LPS for the last 30 min. are shown in upper and lower panel, respectively. This figure is representative of two independent experiments. **B:** Percentage inhibition of p38^{MAPK} activity by TO901317 in wild-type and LXR DKO cells. The graph shows the quantification of immunblots mentioned in A. Phosphorylation levels of p38^{MAPK} were normalised according to total p38^{MAPK} protein levels. LPS-induced p38^{MAPK} phosphorylation level was assigned as 100% in each experiment and percentage of p38^{MAPK} inhibition was calculated. Data are expressed as the mean ± SEM and $^{*}P \leq 0.05$ compared to wild-type.

Similar to the results obtained with JNK, an increase in the phosphorylation levels of p38^{MAPK} was observed in both wild-type and LXR DKO cells due to LPS treatment (Figure 14A). Again in the wild-type condition, the ligand treatment led to an efficient decrease in the phosphorylation levels indicating decrease in p38^{MAPK} activity whereas in the LXR DKO this effect was highly impaired. It was also observed that basal levels of p38^{MAPK} phosphorylation were higher in LXR DKO animals. The quantification of these experiments showed that inhibition of p38^{MAPK} activity by TO901317 treatment was significantly impaired in LXR DKO cells (14%) compared to wild-type cells (35%) (Figure 14B).

Based on these results, it can be concluded that the LXR ligand negatively regulates pro-inflammatory SAPK activity; meaning the activity of both JNK and $p38^{MAPK}$ when induced with LPS. Moreover, this inhibitory action is mediated by LXR.

MKP-1 is not involved in the negative crosstalk between JNK and LXR pathways.

MKP-1 is a MAPK phosphatase that is known to mediate the negative regulation of JNK activation by GR. MKP-1 was shown to be a GR target gene required for JNK inhibition by glucocorticoids in macrophages and some other cell types (Kassel *et al*., 2001). After establishing the negative crosstalk between JNK and LXR pathways, the possible involvement of MKP-1 in this crosstalk was tested.

In order to test this hypothesis, BMMs from wild-type and MKP-1 knockout animals were treated with TO901317 O.N. and LPS for the last 30min. as indicated in Figure 15. Activity of JNK was assessed by immunocomplex JNK assay using GST cJun as the substrate.

Figure 15 shows that JNK pathway was activated efficiently by LPS similarly in wild-type and MKP-1 KO cells. Remarkably, inhibition of JNK activity by the ligand is not impaired in the MKP-1 KO cells. Since the effect of the ligand was not diminished in the absence of MKP-1 protein, it can be concluded that this phosphatase is not responsible for the inhibition of JNK by LXR. Dexamethasone, a synthetic ligand of GR, was used as a positive control. In accordance with the knowledge of MKP-1 being responsible for the negative regulation of JNK activity

by GR, the inhibitory effect of dexamethasone on JNK activity observed in wild-type cells was completely abolished in MKP-1 KO cells (Figure 15).

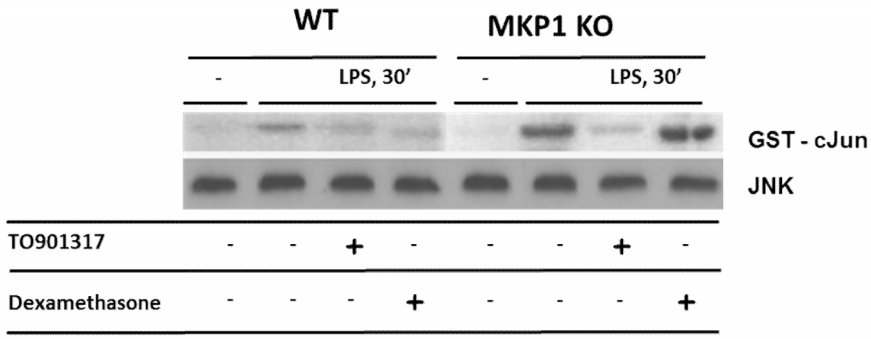

Figure 15. In contrast to GR, MKP-1 is not mediating the inhibition of JNK pathway by LXR. Upper panel shows JNK immunocomplex assay of extracts from BMMs treated as indicated whereas lower panel shows immunoblot analysis of total JNK protein from the same extracts. TO901317 treatment was 2µM, O.N. whereas dexamethasone was 1 µM, 1hour. LPS (100ng/ml) was added for the last 30 min. as indicated. This figure is a representative of three different experiments.

Effect of the LXR ligand on JNK activity is not immediate.

In order to gain more insight into the nature of the negative crosstalk, a time course treatment with the ligand followed by LPS induction was performed in wild-type BMMs. Results showed that more than 6 hours of ligand treatment was required to achieve inhibition of JNK activity and the inhibition got stronger after 12 hours (Figure 16).

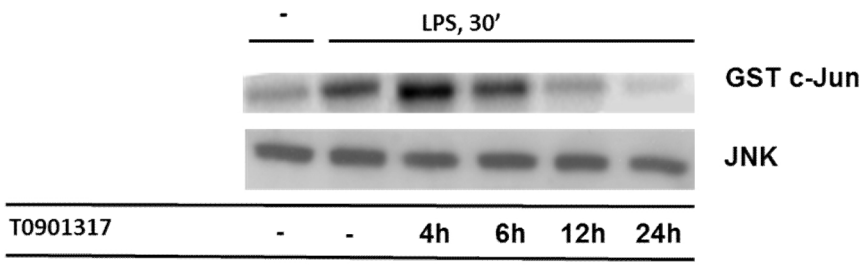

Figure 16. Effect of the LXR ligand TO901317 is not immediate. Upper panel shows JNK immunocomplex assay of extracts from BMMs treated as indicated whereas lower panel shows immunoblot analysis of total JNK protein from the same extracts. BMMs were treated with DMSO (-) or 2µM TO901317 for different periods of time (h=hour) and with 100ng/ml LPS for the last 30 min. as indicated.

PP5 as a potential candidate to mediate the negative regulation of the SAPK pathways by LXR.

PP5 is a member of the phosphoprotein phosphatase family that has been shown to directly bind and inhibit Ask1 and as a consequence inhibit JNK activity (Morita *et al*, 2001; Zhou *et al*, 2004). This information combined with a transcriptomic study that showed in some tissues of mice (e.g. lung tissue) PP5 mRNA levels increase in response to LXR ligand TO901317 treatment (Steffensen *et al*., 2004), supported the notion of PP5 as a candidate for the mediator of this negative regulation.

LXR ligand treatment increases both mRNA and protein levels of PP5.

In order to test this hypothesis, first the effect of ligand treatment on PP5 expression in BMMs was analysed. A time course treatment of the LXR ligand TO901317 was carried out in BMMs derived from wild-type and LXR DKO animals and the PP5 mRNA levels were analysed with quantitative real time PCR.

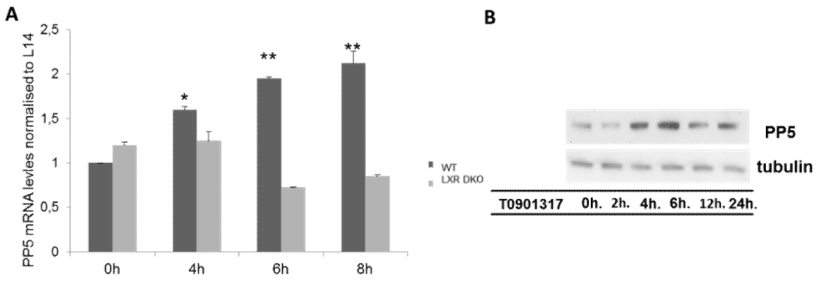

Figure 17. LXR ligand TO901317 increases PP5 expression. A: PP5 mRNA levels in TO901317 treated BMMs determined by quantitative real time PCR. BMMs were treated with 2µM TO901317 for different periods of time (h=hour) as indicated. Data are expressed as the mean ± SEM and are representative of three independent experiments. In each case, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ compared to 0 t.p. **B:** PP5 protein levels in TO901317 treated BMMs determined by immunoblot analysis. Immunoblot analysis of PP5 (upper panel) and tubulin (lower panel) from extracts of BMMs treated with 2µM TO901317 for different periods of time (h=hour) as indicated. Tubulin level was used as loading control. Western blot shown is a representative of two independent experiments.

LXR ligand TO901317 treatment induced a significant increase in mRNA levels of PP5 in wild-type BMMs and this increase was dependent on the receptor itself because it was negligible in BMMs lacking LXRα and LXRβ (Figure 17A). Moreover, LXR ligand TO901317 treatment increased protein levels of PP5 in wild-type BMMs (Figure 17B).

These results show that LXR activated by its ligand is able to increase both mRNA and protein levels of PP5, suggesting that PP5 might be a transcriptional target of LXR.

Effect of the LXR ligand on PP5 mRNA level does not depend on *de novo* protein synthesis.

In order to test whether the effect of the ligand on PP5 level was a direct effect or a mediator was required, protein synthesis in BMMs was inhibited by cycloheximide treatment and cells were treated with TO901317 for 4hours.

LXR ligand TO901317 treatment led to a significant increase in PP5 mRNA level regardless the presence of cycloheximide (Figure 18A). The mRNA levels of a well-studied direct target of LXR (SREBP-1) were also assessed and similar to PP5, the increase in the mRNA levels by LXR ligand TO901317 treatment was not affected by cycloheximide treatment (Figure 18B).

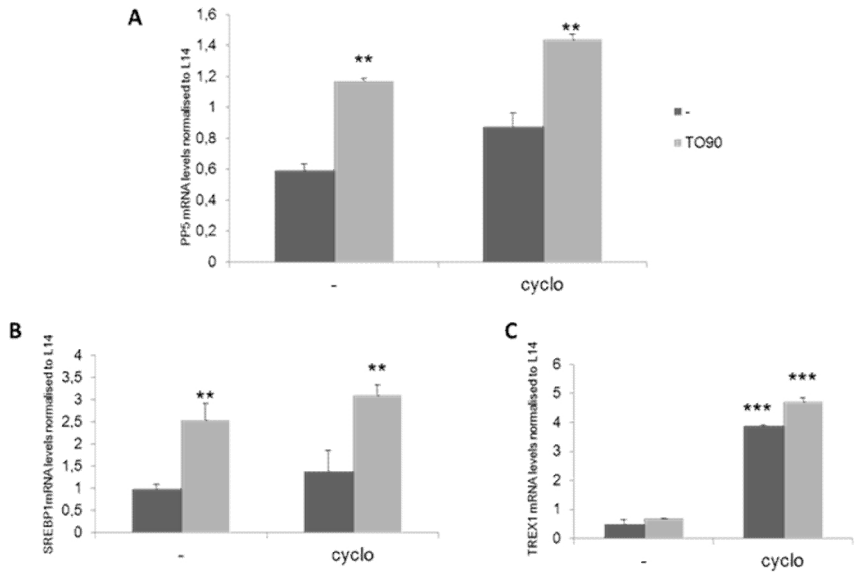

Figure 18. Induction of PP5 mRNA expression by LXR ligand TO901317 does not depend on de novo protein synthesis. PP5 (**A**), SREBP-1 (**B**) and TREX-1 (**C**) mRNA levels determined by quantitative real time PCR analysis. BMMs from wild-type mice were treated with TO901317 (TO90) (2µM) and cycloheximide (cyclo)(10µg/ml) simultaneously and the samples were collected after 4 hours. Data are expressed as the mean ± SEM and are representative of three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ compared to control without ligand treatment in A&B and to control without cycloheximide treatment in C.

To assess the effectiveness of cycloheximide treatment, levels of TREX-1 mRNA were analysed. It was shown that TREX-1 mRNA level increases due to cycloheximide treatment (Serra *et al*., 2011). TREX-1 mRNA level increased significantly in the cycloheximide treated BMMs (Figure 18C) indicating that cycloheximide treatment was successful. Based on these results, it can be concluded that the effect of the ligand on PP5 mRNA levels does not depend on de novo protein synthesis suggesting it is a direct effect on PP5 mRNA synthesis or stability.

A putative LXRE is located on the downstream region of *Ppp5c* gene.

Since our results suggest that PP5 might be a direct target of LXR, it was necessary to show that LXR directly binds to PP5 gene. To test that, first *in silico* analysis of *Ppp5c* gene was done to locate any possible LXREs where the binding can occur. An LXRE has the consensus sequence of AGGTCANNNNAGGTCA. In the downstream region of PP5, a putative LXRE with high similarity to the consensus was located (Figure 19, A).

The binding of LXR/RXR heterodimer to this putative LXRE was analysed by electrophoretic mobility shift assay (EMSA). Results show that the heterodimer does not bind to this region. As a control ABCG1, a known target of LXR, was used and a shift was observed when the LXRα and RXRα were expressed. No shift was observed when the probes for the putative LXRE in the PP5 gene was used (Figure 19B).

Analysis of the available ChIP-Seq data in public data bases also failed to show any binding of LXR or RXR to this putative LXRE that was located on the downstream region of *Ppp5c* gene.

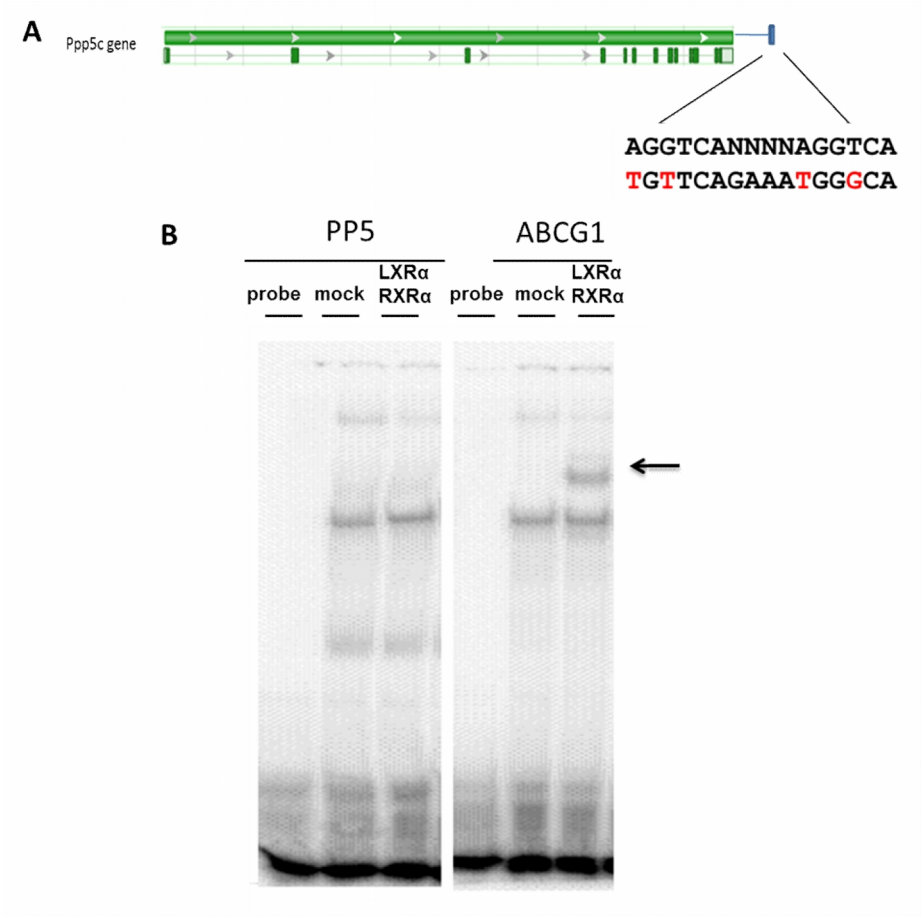

Figure 19. LXR does not bind to the putative LXRE located downstream of PP5 gene. A. Diagram of the location of a putative LXRE on the downstream region of PP5 gene. The diagram is prepared in direct proportion to the real distance, putative LXRE is located 3 kb downstream of *Ppp5c*. In green, *Ppp5c* gene can be seen with the exons located in the lower row. In blue is the location of the putative LXRE. Sequences of consensus (upper) and the putative (lower) LXREs are shown with the mismatches labeled in red. **B.** Electrophoretic mobility shift assay (EMSA) to test the binding of LXR/RXR heterodimer to putative LXRE. LXR and RXR were transcribed and translated *in vitro*. Probes were radioactively labeled with [γ-^{32}P]dATP. Probe for the LXRE on *Abcg1* gene was used as positive control. The arrow points out the shift in the control.

LXR/RXR heterodimer binds to an intragenic region of PP5.

Analysis of the available ChIP-Seq data in public data base revealed binding of LXR and RXR to an intragenic region located on Ppp5c gene (Figure 20A). The available data showed high LXR and mild RXR binding to this intragenic region. ChIP analysis of this region from BMMs treated with a combination of LXR and RXR ligand showed LXR and RXR binding (Figure 20B). Interestingly, untreated BMMs did not show specific binding of LXR or RXR to this region suggesting the requirement of ligand-induced activation of the receptors for the binding to this target region. In parallel, mRNA level of PP5 was analysed in BMMs treated with the same combination of ligands and a significant increase in the level of PP5 was observed similar to the results obtained with LXR ligand TO901317. These results suggested PP5 as a transcriptional target of LXR.

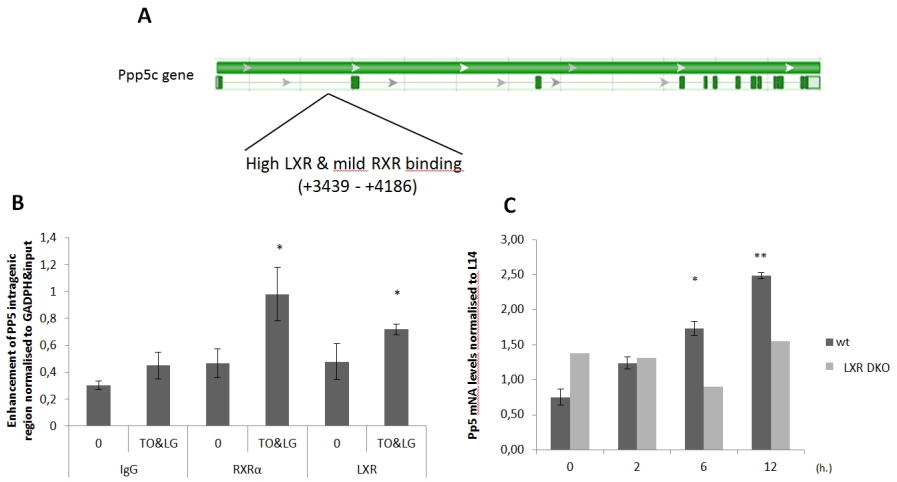

Figure 20. LXR/RXR heterodimer binds to an intragenic region on *Ppp5c* gene. A. Diagram of the location of LXR/RXR binding determined by the analysis of available genomic mapping data on public databases. The diagram is prepared in direct proportion to the real distance and the binding region is located in the first intron (+3439 - +4186). In green, *Ppp5c* gene can be seen with the exons located in the lower row. **B.** The graph shows the ChIP analysis of BMM macrophages treated with a combination of LXR ligand TO9011317, 1μM and RXR ligand LG100268, 1μM (TO&LG) for 90 min. ChIP was carried out using IgG, anti-RXRα or anti-LXR antibodies. Q-RT analysis following ChIP with primers spanning a 119b.p. region in the binding region shown in A. was carried out to analyse the enrichment of the binding. Data are expressed as the mean ± SEM and are representative of four independent experiments. In each case, $^{*}P \leq 0.05$, compared to TO&LG treated IgG immunoprecipated samples.This experiment was performed by Dr. A.F. Valledor and is shown with her permission. **C.** PP5 mRNA levels in TO&LG treated wild-type and LXR DKO BMMs determined by quantitative real time PCR. BMMs were treated with a combination of 1μM TO901317 and 1μM RXR ligand LG100268 for different periods of time (h=hour) as indicated. Data are expressed as the mean ± SEM and are representative of two independent experiments for wild-type cells whereas in LXR DKO it was done only once. In each case, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ compared to 0 t.p.

An activator of PP5, arachidonic acid also inhibits SAPK activity.

Arachidonic acid is an activator of PP5 that binds the auto-inhibitory tetratricopeptide domain at its N-terminus and releases the auto-inhibiton thereby increasing the PP5 activity (Shi, 2009). If PP5 is the mediator of the cross-talk between LXR and SAPK pathways, arachidonic acid should be able to exert a similar effect as the LXR ligand in the SAPK activity.

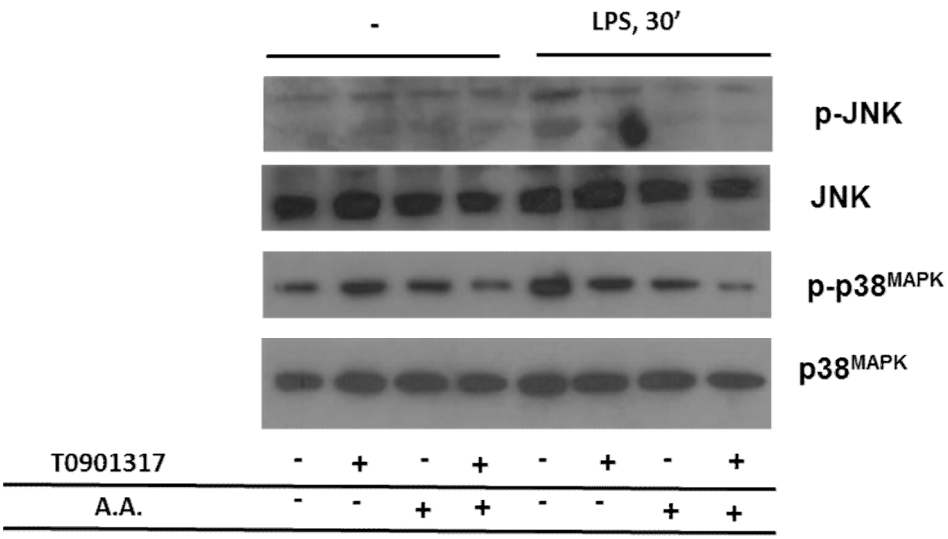

Figure 21. Arachidonic acid inhbits LPS-induced SAPK activation. The immunoblot analysis of phospho JNK and total JNK, phospho $p38^{MAPK}$ and total $p38^{MAPK}$from extracts of wild-type BMMs treated O.N. with 2µM TO901317 or/and 1 hour, 10 µM arachidonic acid (A.A.) and with 100ng/ml LPS for the last 30 min. as indicated. This figure is representative of two independent experiments.

Arachidonic acid treatment was able to inhibit LPS-induced SAPK activity (Figure 21). Moreover, when the cells were treated with both LXR ligand and arachidonic acid, the inhibition of SAPK activity was

stronger pointing to a synergic effect of the two agents (Figure 21). Since the effect of the LXR ligand on PP5 is on the expression level and arachidonic acid acts on the level of activity, this synergic effect is not surprising and is in the favour of the hypothesis that PP5 mediates the negative regulation of SAPK pathways by LXR.

Overexpression of PP5 leads to a decrease in ASK1 activity.

In order to gain more insight into the nature of the action of PP5 on SAPK activity, overexpression studies were carried out in the HEK293 cell line.

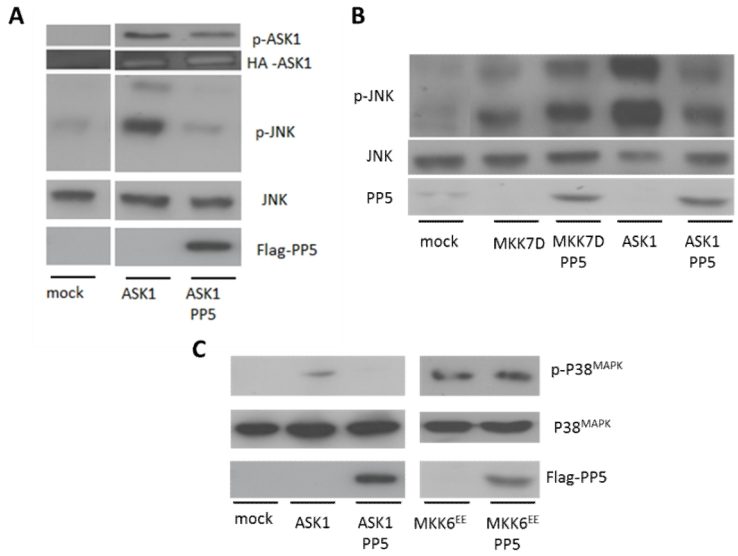

Figure 22. PP5 overexpression leads to inhibition of ASK1 and SAPK activity. A: Immunoblot analysis of phospho- and total ASK1, phospho- and total JNK and PP5 from extracts of mock, ASK1 and ASK1&PP5 transfected HEK293 cells. **B:** Immunoblot analysis of phospho- and total JNK from extracts of mock, MKK7D, MKK7D&PP5 and ASK1, ASK1&PP5 transfected HEK293 cells. **C:** Immunoblot analysis of phospho-and total $p38^{MAPK}$ and PP5 from extracts of mock, ASK1, ASK1&PP5, $MKK6^{EE}$ and $MKK6^{EE}$ &PP5 transfected HEK293 cells.

Overexpression of full-length ASK1 was able to activate the JNK pathway. When PP5 was co-expressed with ASK1, it led to a decrease in the phosphorylation levels of both ASK1 and JNK (Figure 22A). Moreover, we also showed that when the JNK pathway was activated at the level of MKK7, a MAP2K that activates JNK, through over-expression of constitutively active form of this kinase (MKK7D), PP5 overexpression was not able to inhibit JNK activity. Overexpression of both MKK7D and ASK1 proteins led to a higher JNK activity but only in the case of ASK1 overexpression, PP5 co-expression was able to inhibit this activation (Figure 22B).

Effect of PP5 overexpression on $p38^{MAPK}$ was also analysed in the same manner (Figure 22C). Overexpression of ASK1 led to an increase in the phosphorylation level of $p38^{MAPK}$ and therefore $p38^{MAPK}$ activity and co-expression of PP5 was able to inhibit $p38^{MAPK}$. When the $p38^{MAPK}$ pathway was activated by overexpression of a constitutively active form of MKK6 ($MKK6^{EE}$), a MAP2K that activates $p38^{MAPK}$, the effect of PP5 co-expression was abbreviated (Figure 22C).

To summarise, overexpression of PP5 was able to inhibit SAPK pathways when the activation occurs at or upstream of ASK1. Moreover when these pathways were activated downstream through constitutively active forms of the corresponding MAP2Ks, the inhibitory effect of PP5 was abrogated, stating clearly that PP5 does not act directly on JNK or $p38^{MAPK}$ but upstream on the pathway. This finding is in accordance with the data in the literature showing PP5 directly binds and inhibits ASK1 (Morita *et al*., 2001; Zhou *et al.*, 2004).

Overexpression of PP5 decreases AP-1 transcriptional activity and cJun phosphorylation.

In order to assess the role of PP5 on the activity of the JNK pathway, the effect of PP5 overexpression on the transcriptional activity of AP-1 was studied. As it is well defined, JNK phosphorylation leads to phosphorylation of cJun that forms the part of the transcriptional regulator AP-1. To determine the effect of PP5 on the transcriptional activity of this complex, gene reporter assay was used. PP5 overexpression leads to a significant decrease on the ASK1-induced AP-1 dependent transcriptional activity (Figure 23A).

A significant increase in the AP-1 transcriptional activity was observed as a result of ASK1 overexpression. Expression of PP5 alone did not result in any change in the basal activity. Co-expression of PP5 was able to decrease the ASK1-induced AP-1 dependent transcriptional activity on a concentration dependent manner (Figure 23A).

In parallel to the transcriptional activity assays, the phosphorylation levels of ASK1, JNK and the JNK target cJun, were also studied and expectedly co-expression of PP5 led to a decrease in the phosphorylation levels of the named proteins (Figure 23B). The phosphorylation level of cJun was analysed using a specific phospho-antibody (Ser63) demonstrating the JNK dependency cJun phosphorylation.

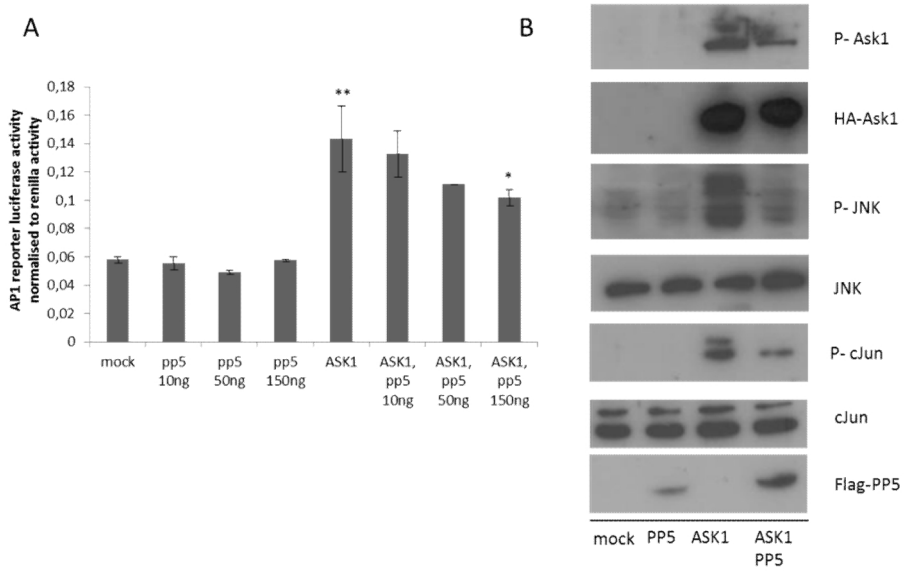

Figure 21. Overexpression of PP5 decreases AP-1 transcriptional activity and cJun phosphorylation. A: Gene reporter assay from HeLa cells transfected with either mock, PP5, ASK1 and PP5&ASK1 in different concentrations as indicated. To assess AP-1 transcriptional activity, a construct with -73b.p. AP-1 binding region of MMP13 gene was used. Transfections were done by PEI method and the dual-luciferase assay was applied after 48hours. Activity of luciferase and renilla were read by an illuminometer and renilla signals were used for normalization. Data are represented as mean ± SEM of triplicate measurements and it is a representative of two independent experiments. $^{*}P \leq 0.05$ compared with ASK1 only and $^{**}P \leq 0.01$ compared with mock. **B:** Immunoblot analysis of phospho- and total ASK1, JNK and cJun as well as PP5 from extracts of HeLa cells transfected by either mock, PP5, ASK1 and ASK1&PP5. Transfections were done by PEI method for 48hours. In order to detect cJun, nuclear extract was isolated through a sucrose cushion followed by immunoblotting. This experiment is a representative of two independent experiments.

PP5 is required for inhibition of SAPK activity by LXR.

In order to assess if PP5 mediates the negative regulation of SAPK pathways (JNK and $p38^{MAPK}$) by LXR, BMMs derived from PP5 KO animals were studied in the context of the ligand's ability to inhibit the activity of the named pathways. First, the effect of the ligand on JNK pathway was analysed.

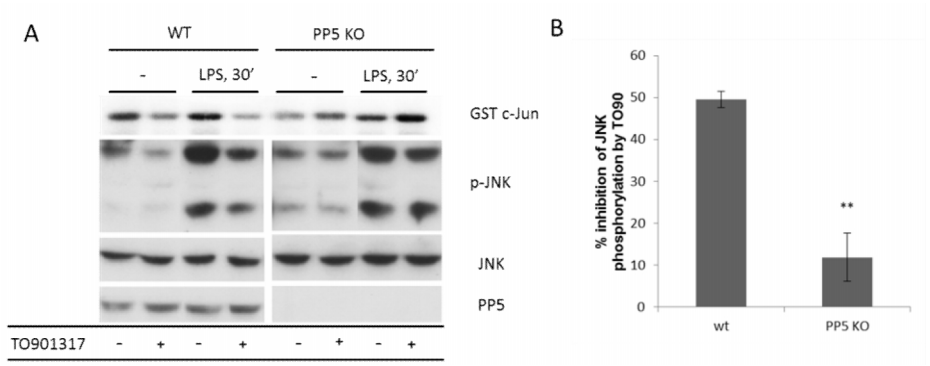

Figure 24. Inhibition of JNK activity by LXR is dependent on PP5. A: JNK immunocomplex assay (upper panel) and immunoblot analysis of phospho- and total JNK as well as PP5 (lower panels) from extracts of wild type (WT) and PP5 knockout (PP5 DKO) BMMs treated O.N. with DMSO (-) or 2µM TO901317 and with 100ng/ml LPS for the last 30 min. as indicated. This figure is representative of three independent experiments. **B:** Percentage inhibition of JNK activity by TO901317 in wild-type and PP5 DKO cells. Quantification of experiments mentioned in A. Phosphorylation levels were calculated as the sum of p46 and p54 isoforms and the phosphorylation levels were normalized according to total JNK protein levels. LPS-induced JNK phosphorylation level was assigned as 100% in each experiment and percentage of JNK inhibition was calculated. Data are expressed as the mean ± SEM and $^{**}P \leq 0.01$ compared to wild-type.

In wild type cells, the inhibition by the LXR ligand TO901317 is clear and efficient whereas this inhibition is almost abrogated in the cells derived from PP5 KO cells (Figure 24A). Quantification of the experiments shows a clear impairment of the inhibitory ability of LXR ligand TO901317 in cells deprived of PP5 compared (11.8%) to wild-type cells (49.6%) (Figure 24B).

In the same manner, the ability of the ligand inhibiting LPS-induced $p38^{MAPK}$ activity was also analysed and the results were similar to what was obtained with JNK activity.

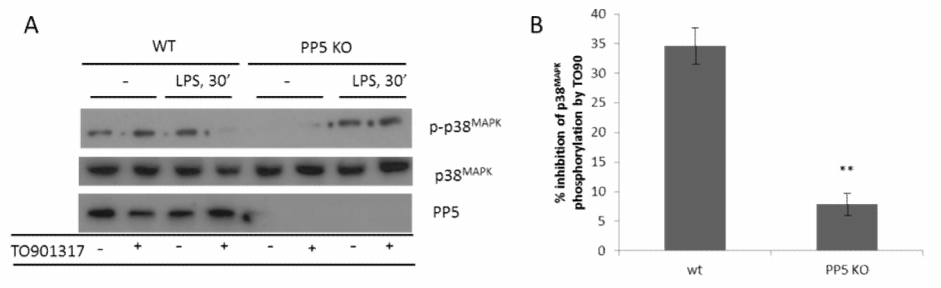

Figure 25. Inhibition of $p38^{MAPK}$ activity by LXR is dependent on PP5. A: The immunoblot analysis of phospho- $p38^{MAPK}$ and total $p38^{MAPK}$ as well as PP5 from extracts of wild type (WT) and PP5 knockout (PP5 KO) BMMs treated O.N. with DMSO (-) or 2µM TO901317 and with 100ng/ml LPS for the last 30 min. This figure is representative of two independent experiments. **B:** Percentage inhibition of $p38^{MAPK}$ activity by TO901317 in wild-type and PP5 KO cells. Quantification of immunblots mentioned in A. Phosphorylation levels of $p38^{MAPK}$ were normalised according to total $p38^{MAPK}$ protein levels. LPS-induced $p38^{MAPK}$ phosphorylation level was assigned as 100% in each experiment and percentage of $p38^{MAPK}$ inhibition was calculated. Data are expressed as the mean ± SEM and $^{**}P \leq 0.01$ compared to wild-type.

LXR ligand TO901317 was able to decrease the phosphorylation levels of both protein kinases efficiently in wild-type cells whereas this ability was diminished in the cells lacking PP5 (Figure 25A). When the results for $p38^{MAPK}$ phosphorylation levels were quantified, it was observed that the inhibition by ligand is highly if not completely impaired in PP5 KO cells (8%) when compared to the inhibition in wild-type cells (36%) (Figure 25B).

In addition to the studies with PP5 knockout mice, ability of LXR ligand to inhibit JNK activity was also tested when PP5 was depleted by RNAi technique in wild-type BMMs. Figure 26 demonstrates that PP5 protein level was efficiently depleted and the inhibitory effect of the LXR ligand was abolished by PP5 siRNA.

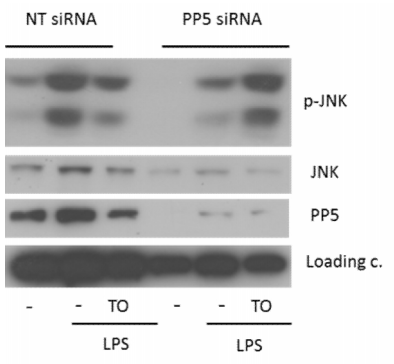

Figure 26. Inhibition of JNK activity by LXR is dependent on PP5. Immunoblot analysis of phospho- and total JNK as well as PP5 (lower panels) protein levels from extracts of wild type BMMs transfected with non-targeting (NT) or PP5 specific siRNA for 48h. and treated O.N. with DMSO (-) or 2µM TO901317 and with 100ng/ml LPS for the last 30 min.

Since the inhibition of LPS-induced SAPK activity by ligand activated LXR is dependent on PP5, it can be concluded that PP5 mediates the negative regulation of SAPK pathways by LXR.

Analysis of the physiological relevance of the LXR-SAPK pathway crosstalk

Role of PP5 on transrepression of proinflammatory genes by LXR

In order to test if PP5 is important for the regulation of the repression of inflammatory signaling by LXR, the BMMS from both wild-type and PP5 KO were treated with LPS to activate inflammatory signaling and in parallel treated with the LXR ligand to repress the expression of the activated genes. Several proinflammatory genes were analysed and here four of them that show activation by LPS and transrepression by ligand are represented.

PP5 does not have an effect on transrepression of the LPS induction of TNFα, IL1-β and IL-6 by LXR.

TNFα expression increases by the addition of LPS, reaches its peak at two hours after the treatment and starts decreasing after that time point (Figure 27A). A similar pattern was achieved in the BMMs from PP5 KO animals (Figure 27B). The repression by the ligand is observed in every time point in both wild-type and knockout cells.

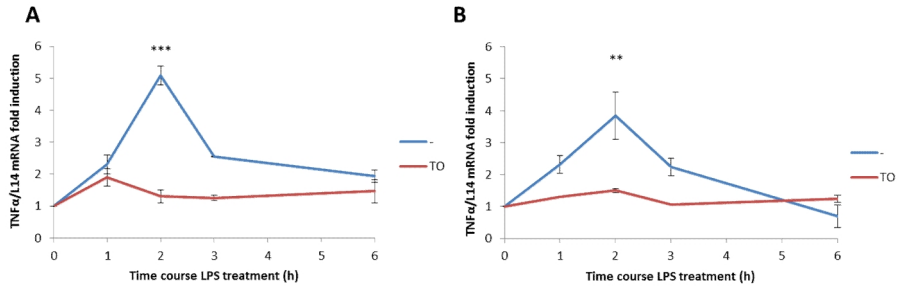

Figure 27. Transrepression of TNFα by LXR ligand in wild-type and PP5 KO cells. Quantitative real time PCR analysis of TNFα gene expression in BMMs derived from wild-type (**A**) and PP5 KO (**B**) mice. Time course LPS (100ng/ml) treatment was done in BMMs that were pretreated with LXR ligand TO901317 (TO) (2μM) or DMSO (-) as indicated. Results were normalised according to the 0 time point in each case. Data are represented as mean of three independent experiments ± SEM and in each case, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, compared to 2h. time point ligand pre-treatment (TO).

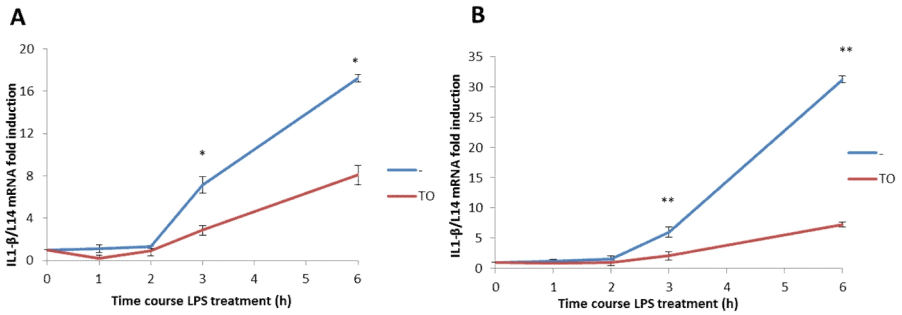

Figure 28. Transrepression of IL1-β by LXR ligand in wild-type and PP5 KO cells. Quantitative real time PCR analysis of IL1-β gene expression in BMMs derived from wild-type (**A**) and PP5 KO (**B**) mice. Time course LPS (100ng/ml) treatment was done in BMMs that were pretreated with LXR ligand TO901317 (TO) (2μM) or DMSO (-) as indicated. Results were normalised according to the 0 time point in each case. Data are expressed as the mean ± SEM and the experiment is a representative of two independent experiments. In each case, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to same time point with ligand pre-treatment (TO).

IL 1-β and IL-6 were also analysed and the expression levels went up by LPS treatment (Figure 28, 29). Six hours after LPS treatment, cells still showed a higher expression of both genes and the ligand treatment as in the case of TNF-α, led to a repression. In both wild-type and PP5 KO cells, ligand treatment was able to repress expression of genes IL 1-β and IL-6 (Figure 28, 29).

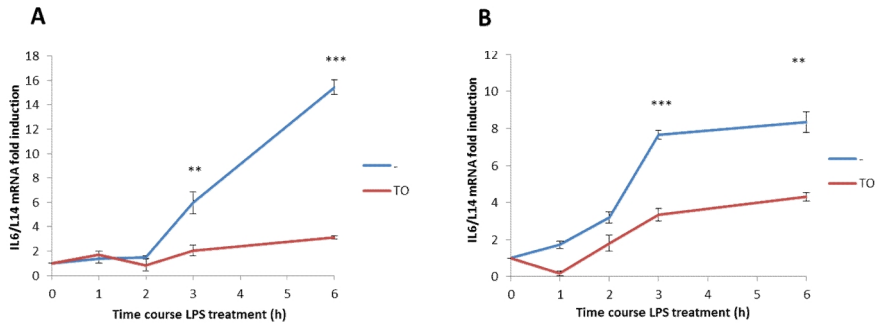

Figure 29. Transrepression of IL6 by LXR ligand in wild-type and PP5 KO cells. Quantitative real time PCR analysis of IL6 gene expression in BMMs derived from wild-type (**A**) and PP5 KO (**B**) mice. Time course LPS (100ng/ml) treatment was done in BMMs that were pretreated with LXR ligand TO901317 (TO) (2μM) or DMSO (-) as indicated. Results were normalised according to the 0 time point in each case. Data are expressed as the mean ± SEM and the experiment is a representative of two independent experiments. In each case, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$ compared to same time point with ligand pre-treatment (TO).

Based on these results, it can be concluded that repression of genes; TNFα, IL1-β and IL-6 by LXR does not depend on PP5.

PP5 is required for successful repression of LPS-induced MMP13 gene expression by LXR ligand.

When the same analysis was carried out with MMP13, it was observed that in the absence of PP5, repression of the LPS-induced MMP13 gene expression was diminished (Figure 30B).

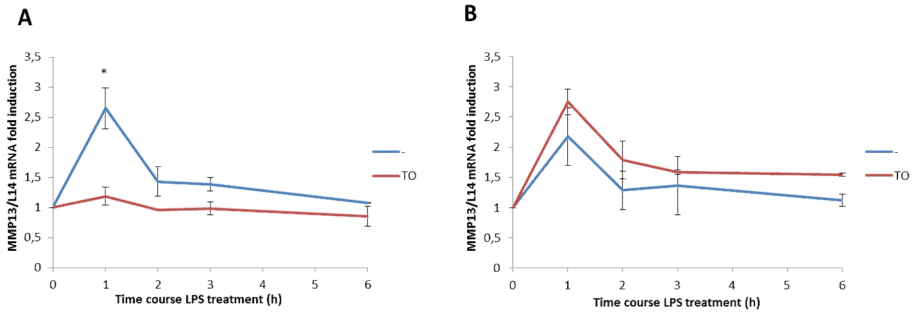

Figure 30. Transrepression of MMP13 by LXR ligand in wild-type and PP5 KO cells. Quantitative real time PCR analysis of MMP13 gene expression in BMMs derived from wild-type (**A**) and PP5 KO (**B**) mice. Time course LPS (100ng/ml) treatment was done in BMMs that were pretreated with LXR ligand TO901317 (TO) (2μM) or DMSO (-) as indicated. Results were normalised according to the 0 time point in each case. Data are represented as mean of two independent experiments ± SEM and $^{*}P \leq 0.05$ when 1h. time point ligand treatment(1h, T) compared to no ligand treatment (1h, -).

The expression of MMP13 peaks at one hour after LPS treatment in PP5 KO cells as well as wild-type cells. In the presence of ligand, the expression levels drop and the peak observed at one hour time point is not observed in wild-type cells. In the PP5 KO cells, ligand is not able to repress the expression of the gene and the peak at one hour is still observed (Figure 30). In conclusion, PP5 is required for the repression of LPS-induced MMP13 expression by LXR.

LXR ligand TO901317 protects thioglycollate peritoneal macrophages from AC-LDL mediated foam cell formation.

Opposite roles for LXR and JNK in the progress of atherosclerosis were defined previously. Activation of LXR by its ligands shows beneficial effects whereas depletion of JNK is beneficial as shown in JNK2 knockout animal studies (Joseph *et al*., 2002; Ricci *et al*., 2004). Therefore, the crosstalk between JNK and LXR pathways might be relevant in atherosclerosis. In order to test this hypothesis, the effect of LXR ligand treatment on Ac-LDL induced foam cell formation was studied. Primary cultures of thioglycollate-elicited peritoneal macrophages derived from wild-type animals were subjected to Ac-LDL for 24 hours to get lipid loaded foam cells. LXR ligand TO901317 treatment was started four hours before Ac-LDL treatment and was repeated 12 hours after Ac-LDL treatment. Oil-red staining of the Ac-LDL loaded cells shows the lipid accumulation in the foam cells (Figure 31A).

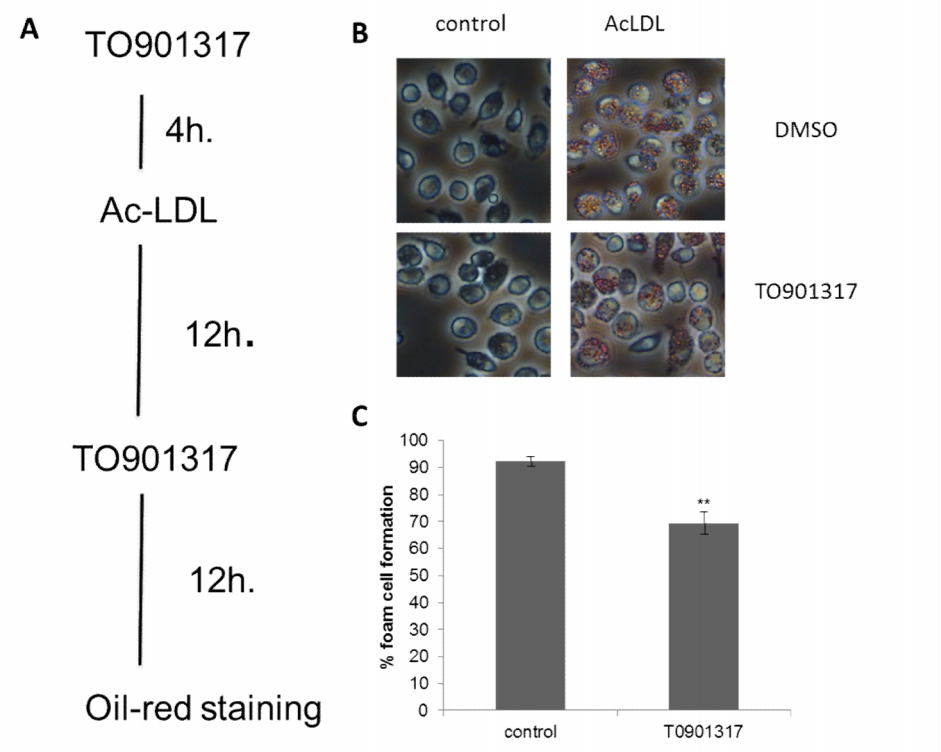

Figure 31. LXR ligand treatment decreases Ac-LDL mediated foam cell formation. A. Flow chart of the foam cell formation assay. Primary cultures of peritoneal macrophages derived from wild-type animals were subjected to Ac-LDL for 24 hours to get lipid loaded foam cells. LXR ligand TO901317 treatment was started four hours before Ac-LDL treatment and was repeated 12 hours after Ac-LDL treatment. **B.** Oil red staining of PMs. Light microscopy, 20X. **C.** Percentage of cells that are lipid loaded foam cells. Cells stained red were counted as positive. More than 300 cells were counted for each condition. Control cells are Ac-LDL treated cells and TO901317 are treated by Ac-LDL and TO901317. Data are represented as mean of triplicates ± SEM and $^{**}P \leq 0.01$ compared to control.

The accumulation of red lipid droplets in AcLDL treated cells is clearly observed (Figure 31B). When the stained cells were counted and the percentage of foam cell formation in each condition was determined, it was observed that the Ac-LDL treatment resulted in an efficient foam cell formation (92%) and LXR ligand treatment led to a significant decrease (69%), (Figure 31C). In other words LXR ligand treatment decreased the foam cell formation by Ac-LDL by 25%.

These results showed that LXR ligand treatment protects macrophages from Ac-LDL mediated foam cell formation and this particular method could be used as a model to study the effects of LXR ligand on foam cell formation.

The effect of LXR ligand on foam cell formation does not depend on PP5.

In order to determine the significance of PP5 action in macrophage protection from foam cell formation by LXR ligand, PMs from PP5 KO animals as well as wild-type animals were studied as described above.

LXR ligand was able to protect from foam cell formation in PP5 KO cells similarly as in wild-type cells (Figure 32). In the absence of PP5, Ac-LDL treatment resulted in high ratio of foam cell formation (94.6%) that is not significantly different from wild-type cells (93.8%), (Figure 32B). In the presence of the LXR ligand TO901317, the ratio of foam cells dropped significantly (74% and 76% in wild-type and PP5 KO cells, respectively) (Figure 32B). In other words, LXR ligand TO901317 treatment was able to protect against foam cell formation by 21% in

wild-type cells and 19% in PP5 KO cells. Although there is a slight difference between wild-type and PP5 KO cells, this difference failed to be statistically significant as the result of three independent experiments that were also carried out in triplicates.

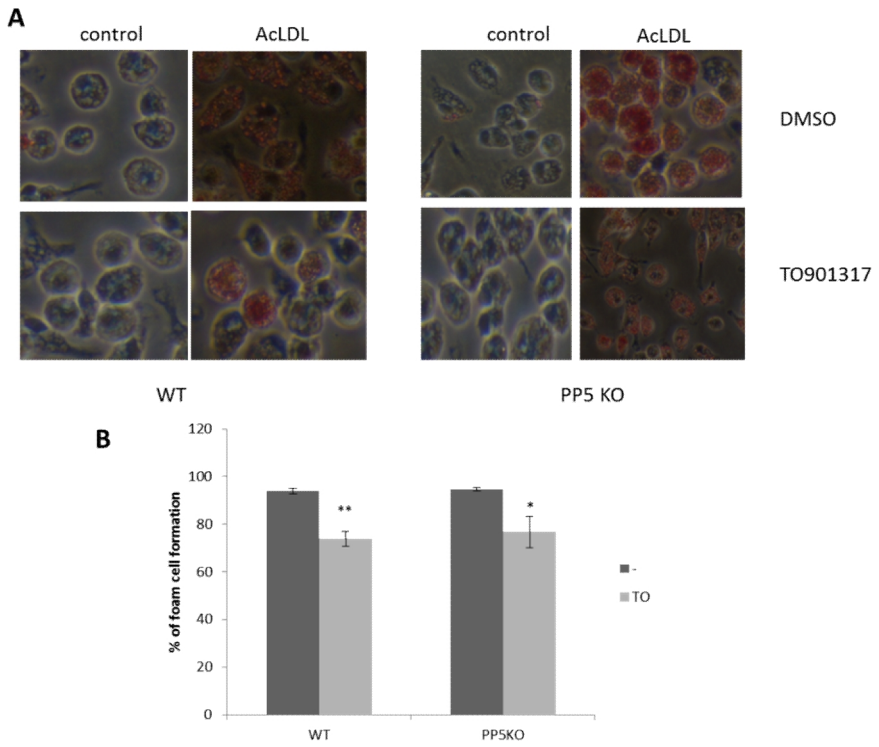

Figure 32. LXR ligand treatment decreases Ac-LDL mediated foam cell formation in wild-type and PP5 KO cells to a similar extend. A. Oil red O staining of PMs derived from wild-type (WT) and PP5 knockout (PP5 KO) mice treated as described in Figure 27, A. Light microscopy, 20X. **B.** Percentage of cells that are lipid loaded foam cells in wild-type (WT) and PP5 knockout (PP5 KO) cells. Cells stained red were counted as positive. More than 300 cells were counted for each condition. Control cells (-) are Ac-LDL treated cells and TO are treated by Ac-LDL and TO901317. Data are represented as mean of three independent experiments ± SEM and $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to control (-).

ABC-transporters; ABCA1 and ABCG1 show similar expression patterns in wild-type and PP5 KO cells.

ABCA1 and ABCG1 are both LXR targets and play essential role in the protection of macrophages from foam cell formation by ligand treatment. ABCA1 mediates the transport of cholesterol and phospholipids from cells to lipid-poor apolipoprotein A-I (apoA-I), whereas ABCG1 mediates the transport of cholesterol from cells to lipidated lipoproteins (Valledor & Ricote, 2004). In parallel to the foam cell formation assay described above, mRNA levels of these transporters were analysed in both wild-type and PP5 KO cells. The ligand treatment led to an increase in the levels of both transporters as expected with a small increase due to AcLDL treatment. Moreover, no difference was observed between wild-type and PP5 KO cells in the expression patterns of the transporters (Figure 33).

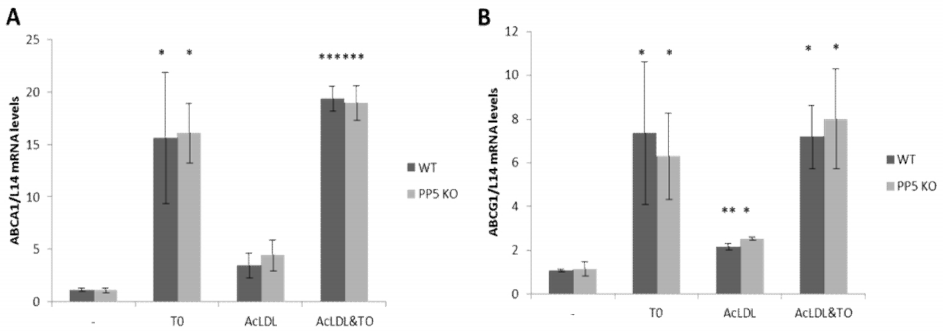

Figure 33. ABCA1 and ABCG1 have similar gene expression patterns in wild-type and PP5 KO PMs. Quantitative real time PCR analysis of genes ABCA1 (**A**) and ABCG1 (**B**) in PMs derived from wild-type (WT) and PP5 knockout (PP5 KO) cells in foam cell formation assay conditions as described in Figure 27, A. Data are represented as mean of three independent experiments ± SEM and $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to control (-).

Scavenger receptors CD36 and SRA show similar expression patterns in wild-type and PP5 KO cells.

The expression levels of scavenger receptors were also analysed in the samples above and the effect of the ligand and Ac-LDL treatments were assessed.

The ligand did not have an effect on the mRNA levels of CD36 and SRA in wild-type as well as PP5 KO cells (Figure 34). On the other hand AcLDL treatment led to a significant increase in the expression level of CD36 and SRA but no difference was observed between wild-type and knockout cells (Figure 34).

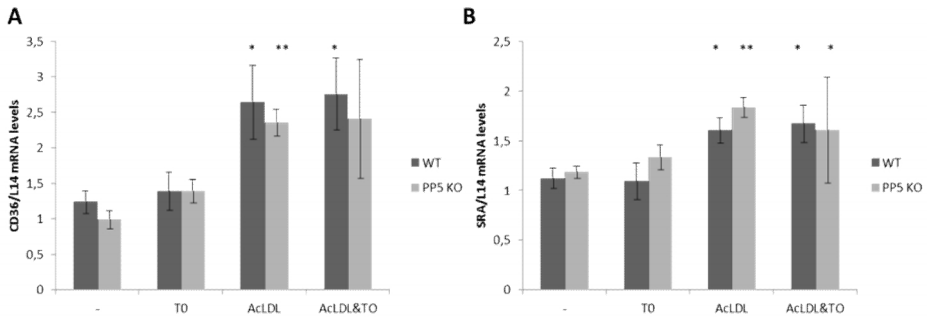

Figure 34. CD36 and SRA have similar gene expression patterns in wild-type and PP5 KO PMs. Quantitative real time PCR analysis of genes CD36 (**A**) and SRA (**B**) in PMs derived from wild-type (WT) and PP5 knockout (PP5 KO) cells in foam cell formation assay conditions as described in Figure 27, A. Data are represented as mean of three independent experiments ± SEM and $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to control (-).

Possible role for PP5 in immune response

LPS treatment increases PP5 mRNA levels.

In previous experiments LPS was used as a tool to activate JNK, $p38^{MAPK}$ pathways and other pro-inflammatory pathways. The effect of LPS alone on PP5 was not assessed. Here we show that LPS treatment leads a significant increase in PP5 mRNA levels (Figure 35).

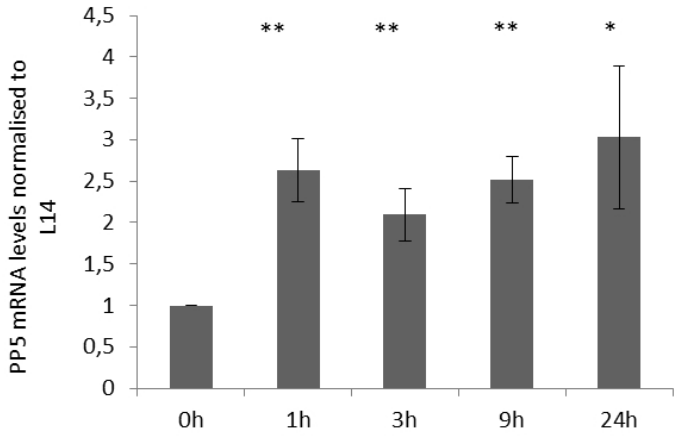

Figure 35. PP5 mRNA levels increase in response to LPS treatment. Quantitative real time PCR analysis of PP5 gene expression in BMMs treated with LPS for different periods of time (h=hour) as indicated. Data are represented as mean of three independent experiments ± SEM and $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to 0 time point (0h).

Cytokines such as M-CSF, IFNγ and IL-4 also increase PP5 mRNA levels.

Since PP5 had an effect on regulation of one of the inflammatory genes tested (MMP13), the question of PP5 being involved in immune response was raised. As a preliminary experiment in the

understanding of PP5's possible role in immune response, the effects of different cytokines on PP5 mRNA levels were analysed. Wild-type BMMs were treated with cytokines such as; Macrophage colony-stimulating factor (M-CSF), Interferon-gamma (IFNγ) and Interleukin 4 (IL-4) and PP5 mRNA level was determined (Figure 36).

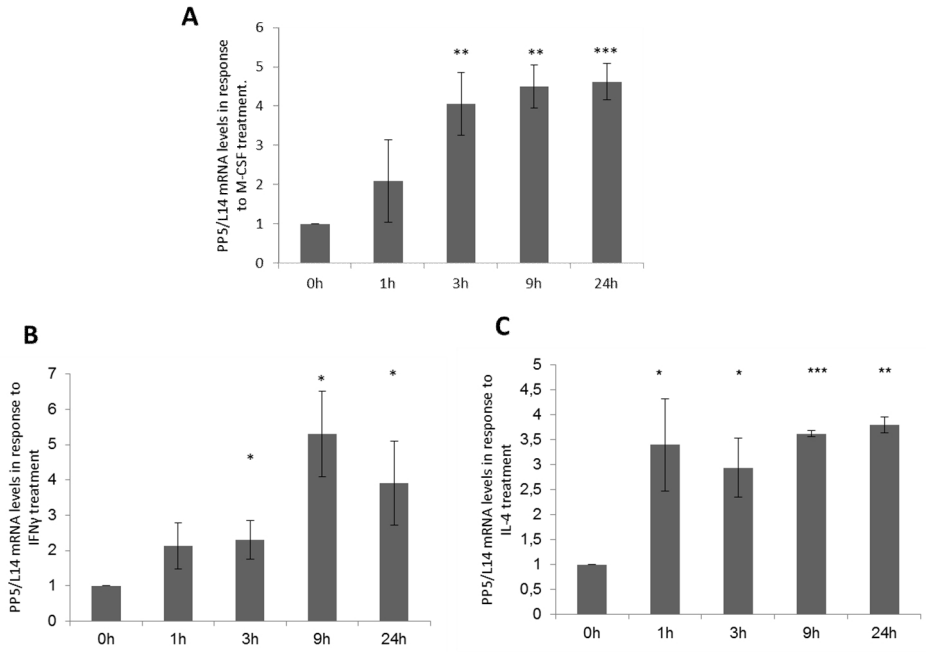

Figure 36. PP5 mRNA levels increase in response to cytokine treatment. Quantitative real time PCR analysis of PP5 gene expression in BMMs treated with M-CSF (**A**), IFNγ (**B**) and IL-4 (**C**) for different periods of time (h=hour) as indicated. Data are represented as mean of three independent experiments ± SEM and $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ compared to 0 time point (0h).

Treatment of BMMs with cytokines such as M-SCF, IFNγ and IL-4 (Figure 36A, B and C, respectively) led to an increase in PP5 mRNA levels suggesting a possible role for PP5 in immune response and macrophage biology.

DISCUSSION

Inflammation is an exceedingly complex process with a crucial role in mammalian physiology. Although it is extremely beneficial for the organism's defense mechanism, the pathogenic capacity of the inflammatory response cannot be underestimated. Many serious, pervasive metabolic diseases such as atherosclerosis and type 2 diabetes have low-grade chronic inflammatory components. Thus, understanding this inflammatory component and targeting it therapeutically proves to be an essential challenge (Tabas & Glass, 2013).

Moreover, a considerable amount of data states that metabolism and inflammation are closely linked and the crosstalk between these processes is fundamental to the pathogenesis of the diseases; atherosclerosis and type 2 diabetes (Tabas & Glass, 2013). SAPKs and LXRs are essential regulators of these two closely linked processes and this study is focused on the crosstalk between these aforementioned pathways.

In our group, it has been shown that JNK pathway is inhibited by TZDs which are synthetic PPARγ ligands used as insulin-sensitizing agents in medicine for treatment of type 2 diabetes. In fact, inhibition of JNK pathway by TZDs is mediating the hypoglycemic action of these drugs *in vivo* (Díaz-Delfín *et al*, 2007). Moreover, as mentioned earlier, there are other examples to the crosstalk between NRs and SAPKs (Caelles *et al.*,1997; Lasa *et al*., 2001; Bruna, *et al.*, 2003; Lu *et al*., 2008; Lanuza-Masdeu *et al*., 2013).

This study extends this to the case of LXR and shows that LXR ligand inhibits LPS induced SAPK activation. LXR DKO cells showed that the inhibitory effect of the ligand was not an out off-target effect and was dependent on the LXR receptor. It was essential to demonstrate this because a study with the LXR ligand TO901317 showed that this synthetic ligand might act as an agonist to another NR; PXR (Mitro *et al*., 2007). Remarkably, so far all the NRs that have shown the ability to inhibit SAPK pathway activation share in common anti-inflammatory properties; therefore, it may be proposed that the inhibition of SAPK cascades is a general anti-inflammatory mechanism used by these NRs.

Time course studies with the LXR ligand showed that the acquisition of the inhibitory capacity required relatively long incubations compared to other NR ligands such as GR ligand dexamethasone, which effectively inhibits JNK activation in 10 minutes after its addition to the culture media. In the case of LXR ligand, the inhibition was detected after a 12 hour incubation time and was still effective after 24 hours. This difference in the timing suggested that the mechanisms of inhibition by GR and LXR might be different. In this regard, GR may inhibit JNK activation by a direct protein-protein interaction, which is mediated by a JNK-docking site located at the N-terminus of the GR LBD (Bruna *et al*. 2003). Despite the high degree of conservation in the amino acid sequence between the LBD of NRs, the amino-acid sequence of this JNK-docking site in the GR is not conserved in any of the other NRs, including LXR, a circumstance that supports the notion that the inhibition of JNK by LXR is likely not to be mediated by direct

LXR-JNK interaction. GR is also able to inhibit SAPK pathway activation by transcriptional-dependent mechanisms, in particular by the induction of MKP-1 gene expression (Kassel *et al*., 2001). In this regard, studies with the MKP1 KO cells showed that, in contrast to GR, MKP1 was not responsible for the inhibition of LPS-induced SAPK activation by LXR.

Since SAPKs are highly regulated by dephosphorylation, another phosphatase, PP5, shown to be inhibiting hypoxia-induced JNK activation (Zhou *et al.*, 2004) was considered as a possible candidate. Moreover, the fact that the PP5 gene expression was increased in different tissues of mice treated by LXR ligand (Steffensen *et al*., 2004) further favored this hypothesis. PP5 was shown to act on the level of ASK1 in regulating JNK pathway (Morita *et al*, 2001; Zhou *et al*, 2004). Our results showed that SAPK activation by overexpression of ASK1 was inhibited effectively by coexpression of PP5. Moreover, ASK-1 phosphorylation was decreased with PP5 co-expression. In contrast to the study showing that PP5 inhibited hypoxia induced JNK activity but not $p38^{MAPK}$ (Zhou *et al*., 2004), in our experimental settings inhibition of $p38^{MAPK}$ was observed by PP5 co-expression. Since PP5 was shown to act on ASK1 level, and ASK1 regulates both SAPKs, the observation of PP5 affecting both SAPKs is the most consistent. Moreover, in the overexpression studies, when SAPKs; JNK and $p38^{MAPK}$ were activated with overexpression of constitutively active forms of MAP2Ks; MKK7 and MKK6, respectively, PP5 co-expression failed to inhibit those activities. This is in line with the findings in the literature that PP5 acts on the level of ASK1 and not on the MAPK itself.

After demonstrating that PP5 overexpression had an inhibitory effect on the phosphorylation thus the activity of the SAPKs, the effect of PP5 on AP-1 activity was tested. ASK1 overexpression led to an increase in transcriptional activity of an AP-1 dependent reporter construct and co-expression of PP5 inhibited this activation. Moreover, the effect of PP5 overexpression was extended to JNK specific phosphorylation of c-Jun that is related to its transactivation activity. Together these results showed that PP5 inhibited the SAPK pathway and their target cJun/AP-1.

The observation that quite long time periods were needed for the inhibitory effect of the LXR ligand suggested that the mechanism underlying this crosstalk might require a transcriptionally controlled mediator. Initial evidence of PP5 as a potential LXR target gene came from the transcriptomic study performed in mice that showed that PP5 mRNA was increased in lung and heart tissues in mice treated with LXR ligand. Moreover, this effect was LXR dependent since it was abolished in LXR DKO mice (Steffensen *et al*., 2004). Our results showed that activation of LXR by the ligand increases both mRNA and protein levels of PP5. Moreover, inhibition of protein synthesis did not affect the increase of PP5 mRNA level in response to LXR activation suggesting a transcriptionally-dependent effect. These findings together with ChIP study showing that LXR/RXR heterodimer binds to an intragenic region of *Ppp5c* gene, strongly suggest PP5 as a novel target of LXR. Binding of the LXR and RXR to PP5 gene seemed to require ligand activation of the receptors and was not observed in the basal state. This was quite an unexpected result because LXRs were

shown to be bind to target sequences even in the absence of ligand activation in a complex with corepressors. Although these results seemed to be unexpected, it should also be pointed out that PP5 is not the only LXR target gene that behaves this way in ChIP studies. It was shown that another defined transcriptional target of LXR also requires activation of the LXR/RXR heterodimer by the combination of the ligands for each of the NRs (personal communication, Dr. A.F. Valledor; University of Barcelona).

The *in silico* analysis of the region that the heterodimer was shown to bind failed to detect any LXRE consensus sequence. Moreover, this region is not on the classically identified promoter region. Genome wide studies have showed that NRs tend to control gene transcription by binding to distal enhancer regions in combination with other transcription factors (Carroll *et al*., 2005; Lefterova *et al*., 2008; Lupien *et al*., 2008; Nielsen *et al*., 2008). Moreover, in the intragenic region that showed LXR/RXR binding, an AP-1 binding motif with the sequence TGACTCA (+3847 - +3853) was detected by *in silico* analysis. This finding was in line with Chip-on-chip study that showed AP-1 and LXR/RXR binding sites tend to be in close vicinity to each other. Same study also showed direct protein-protein interaction between LXR and cJun/cFos proteins suggesting that AP-1 and LXR/RXR heterodimers might be interacting with each other in order to target gene expression (Shen *et al*., 2011). Although this study was done in keratinocytes, another study also showed enhanced that AP-1 motifs were co-enriched in LXR bound regions in primary macrophages (Heinz *et al*., 2010).

Since our results suggest PP5 as a transcriptional target of LXR and showed that it inhibited SAPK activity as well as AP-1 transcriptional activity, the essential point was to test the role of PP5 in LXR mediated negative regulation of SAPK activity. The studies with PP5 KO BMMs showed that negative regulation of LPS-induced JNK and $p38^{MAPK}$ activation by LXR was dependent on PP5. Similar results regarding LXR-inhibition of LPS-induced JNK activity were obtained when PP5 was transiently silenced by RNAi technique, therefore corroborating the previous conclusion.

Last but not least, activation of PP5 by arachidonic acid treatment alone was sufficient to inhibit LPS induced JNK and $p38^{MAPK}$ activation. The effect of the LXR ligand and arachidonic acid were synergic which was expected since LXR ligand is a transcriptional activator whereas arachidonic acts at a post-translational level. Nevertheless, a potential connection between these two regulatory mechanisms was brought into question; a possible role of LXR in increasing the cellular arachidonic acid levels. In collaboration with Dr. M.A. Balboa (University of Valladoid) mRNA expression levels of several phospholipases in response to LXR ligand were analysed. LXR ligand induced an increase of mRNA expression of group IV cytosolic phospholipase A2 (cPLA2) enzyme that is the main phospholipase responsible of arachidonic acid release (data not shown and provided by Dr. M.A. Balboa, University of Valladoid). This finding gave rise to the hypothesis that LXR plays a dual role in regulation of PP5; increasing its expression by transcriptional activation and increasing its activity through induction of cPLA2 enzyme, and thereby, cellular

arachidonic acid levels. The further study of the subject would be interesting in revealing LXR-dependent regulatory mechanism of PP5 activity.

PP5 being controlled by lipid regulatory mechanisms such as LXR activation and arachidonic acid, and regulating JNK and $p38^{MAPK}$ is another piece of evidence that points towards the tight link between metabolism and inflammation.

Based on these results, it can be concluded that LXR inhibits LPS induced JNK and $p38^{MAPK}$ activity through its transcriptional target PP5. After establishing the negative crosstalk between LXR and SAPK pathways and identifying PP5 as one of its mediators, the focus of the project was turned to studying the physiological relevance of this crosstalk.

As discussed earlier in the Introduction section, LXRs inhibit expression of many proinflammatory genes that are targets of NFκB and AP-1 transcriptional regulators. Since SAPKs are regulators of AP-1 activity and our results show that PP5 can inhibit AP-1 activity, the negative crosstalk between LXR and SAPKs might be essential for the regulation of inflammatory pathways by the LXRs.

In order to test this possibility, BMMs from wild-type and PP5 KO cells were treated with LPS to induce inflammatory signaling in the presence or absence of the LXR ligand. The expression levels of *Tnfa*, *Il-1b*, *Il-6* and *Mmmp13* were monitored at different time points up to 6 hours after LPS treatment. *Tnfa* showed a peak at 2 hours and after

that the expression went down in wild-type and PP5 KO in the absence of the ligand. The peak at 2 hours was completely diminished with the LXR pre-treatment showing that LXR repressed the LPS induced activation of *Tnfa.* The inhibitory effect of the LXR ligand was observed in wild-type and PP5 KO cells and to a similar extend, suggesting that PP5 was not essential for the negative regulation of *Tnfa* gene by LXR. Similarly, LPS-induced expression of genes *Il-1b* and *Il-6* were repressed effectively by LXR ligand in wild-type cells as well as PP5 KO cells. The results showed that PP5 did not have a role in the transrepression of these defined NFκB target genes; *Tnfa* (Shakhov *et al*., 1990; Collart *et al*., 1990)*, Il-1b* (Hiscott *et al*., 1993)*, Il-6* (Libermann & Baltimore, 1990; Son *et al*., 2008).

Nevertheless, when the effect of LXR ligand in LPS induced Mmp13 gene expression was analysed, it was observed that the inhibitory effect of LXR ligand was abrogated in PP5 KO cells. *Mmp13* gene expression showed a peak after an hour of LPS treatment and this peak was not observed in the presence of the ligand in wild-type cells. In contrast, in the absence of PP5 the ligand did not show the inhibitory effect on the expression of *Mmp13*. Mmp13 is one of the "pure" AP-1 target genes subject to negative regulation by LXR. It was shown that NCoR is essential for transrepression of *Mmp13* along with other AP-1 targets. Moreover, cJun was shown to be responsible for the recruitment of NCoR to *Mmp13* promoter region (Ghisletti *et al*., 2009). Furthermore, c-Jun N-terminal phosphorylation (Ser63/73) was shown to have an essential role in promoting the events (recruitment of the proteolytic machinery) leading to NCoR dismissal from the

promoter region that is required for the activation of the AP-1 target genes in macrophages (Ogawa *et al*., 2004). Taking into consideration the published data together with the results of this work, it can be proposed that through transcriptional activation by LXR, PP5 inhibits SAPK activity and, thereby, prevents cJun N-terminal phosphorylation which in turn diminishes the LPS induced removal of NCoR from a subset of AP-1 target genes represented by *Mmp13* thereby keeping them in a repressed state.

Since LXR ligands have been shown to be beneficial in atherosclerosis mouse models, the potential role of PP5 as a negative regulator of SAPK activity in the progress of the disease was tested. Mice deficient of JNK2 and ApoE, developed smaller atherosclerotic plaques compared to Apo KO mice (Ricci *et al*., 2004). This finding demonstrated a role for JNK2 in the progress of atherosclerosis and increased the possibility that JNK and LXR crosstalk, therefore PP5, having a role in the disease progress as well.

In this work, foam cell formation in primary macrophages loaded with modified LDL was used to study the role of PP5. AcLDL treatment of PMs from wild-type mice showed lipid accumulation and LXR ligand treatment led to significant decrease in the percentage of foam cells, proving it to be a suitable model for the work. Unfortunately, when the same model was applied to the PP5 KO, no statistically significant difference was detected in the protective effect of the LXR ligand on foam cell formation with respect to the wild-type. When JNK2 KO cells were used, as expected they were protected from foam cell formation

compared to wild-type. Interestingly, same experiment showed that the LXR ligand had a significant protective effect producing a significantly smaller percentage of foam cells (data not shown). Although, this experiment was carried out in cells derived from one single animal in triplicates and should be repeated, it suggested that LXR-JNK crosstalk might not be essential for foam cell formation and they might act independently through distinct mechanisms in the progress of atherosclerosis supporting the results obtained with the PP5 KO cells.

In order to demonstrate the physiological relevance of the negative crosstalk between SAPK and LXR pathways, other animal models of inflammation need to be considered.

Since PP5 proved to be essential for the regulation of *Mmp13* gene expression by LXR, models where MMP13 played an important role would be a reasonable choice for further study. MMP13, also known as collagenase 3 is an enzyme that functions in collagen degradation, as the name implies. During embryonic development, MMP13 is expressed in the skeleton as required for restructuring the collagen matrix for bone mineralization. In pathological situations such as human carcinomas and in rheumatoid arthritis and osteoarthritis, it is overexpressed (Johansson *et al*., 2000). Rheumatoid arthritis is a chronic inflammatory disease that is marked by synovial inflammation and destruction of articular extracellular matrix. Activated cells of the synovium produce pro-inflammatory and matrix-degrading effector molecules, which maintain the inflammation and lead to the

destruction of the involved joints (Ospelt *et al*., 2004). JNK was shown to be important in an animal model of adjuvant arthritis. Inhibition of JNK activity by subcutaneous treatment of animals with SP600125 resulted in decreased swelling in the paws of the animals. Moreover, radiographic analysis showed a marked decrease in bone and cartilage damage associated with decreased AP-1 activity and MMP13 expression in the JNK inhibitor treated animals (Han *et al*., 2001). Therefore, it could be interesting to study the effects of the LXR ligand in this inflammation model in wild-type and PP5 KO animals.

Last but not least, gene expression of PP5 in response to different stimuli was analysed in BMMs. Treatment with LPS, as well as cytokines such as M-SCF, IFNγ and IL-4 led to an increase in PP5 mRNA levels in BMMs. As stated above, LPS activates ASK1 activity through TLR4 signaling so LPS activation of PP5 that can inhibit ASK1 activity might be yet another mechanism that adds to the complex regulation of inflammatory signaling in an attempt to avoid inflammation from reaching pathological levels. Since PP5 seems to be subject to regulation by cytokines as well, it might be a general regulator of immune response requiring further study of the subject.

In this work, PP5 is suggested as a novel target of LXR that negatively regulates activation of SAPK pathways; $p38^{MAPK}$ and JNK. Also, it is proposed that through the inhibition of SAPK activity, and thereby cJun/AP-1 activity, PP5 is mediating negative regulation of LPS-induced *Mmp13* gene expression by LXR in primary macrophages (Figure 37).

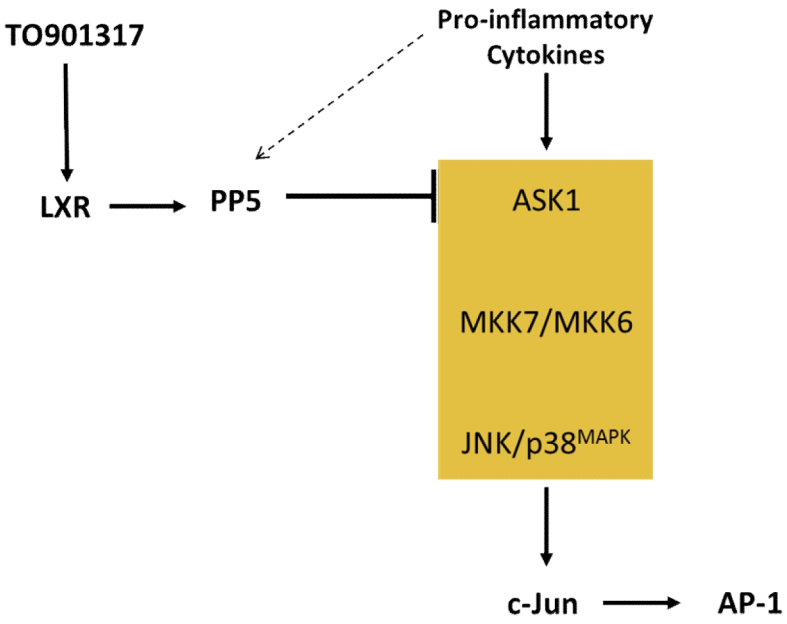

Figure 37. PP5 dependent regulation of SAPK pathways by LXR. PP5, a potential novel target of LXR, inhibits ASK1 activity by direct interaction and dephosphorylation. This leads to a decrease in the activities of $p38^{MAPK}$ and JNK, resulting in a decrease in c-Jun phosphorylation and finally less AP-1 transcriptional activity represented by the target gene MMP13. The dashed line represents the increase in PP5 mRNA in macrophages in response to cytokines as well as LPS.

CONCLUSIONS

1- Ligand activated LXR inhibits LPS-induced JNK and $p38^{MAPK}$ activation in murine primary macrophages.

2- MKP-1 does not mediate the negative regulation of LPS-induced JNK and $p38^{MAPK}$ activation by LXR.

3- LXR ligand treatment increases both PP5 mRNA and protein levels in murine primary macrophages.

4- Inhibition of protein synthesis did not affect the increase of PP5 mRNA level in response to LXR activation suggesting a transcriptionally-dependent effect.

5- LXR and RXR binding to an intragenic region (+3439 - +4186) on Ppp5c gene was demonstrated with ChIP studies suggesting PP5 as a novel transcriptional target of LXR.

6- PP5 overexpression resulted in a decrease in ASK1-induced JNK and $p38^{MAPK}$ activation as well as cJun phosphorylation and AP-1 transcriptional activity.

7- Negative regulation of LPS-induced JNK and $p38^{MAPK}$ activation by LXR is dependent on PP5.

8- LXR ligand treatment protects murine macrophages against AcLDL-induced foam cell formation in wild-type and PP5 KO cells to a similar extend.

9- PP5 is required for transrepression of LPS-induced gene expression by LXR in the case of Mmmp13 but not in Tnfα, Il-1β, Il-6.

10- PP5 mRNA levels increase in response to LPS, M-CSF, IL-4 and IFN-γ suggesting a role for PP5 in immune response and macrophage biology.

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ABBREVIATIONS

ABC	ATP binding casette
AF1	Activation function 1
AP-1	Activator protein 1
APC	Anaphase-promoting complex
ApoE	Apolipoprotein E
ASK1	Apoptosis signal-regulating kinase 1
AT1	AngII receptor type 1
ATF	Activating transcription factor
ATM	aAtaxia telangiectasia mutated kinase
ATR	ATM and Rad3 related kinase
BMMs	Bone marrow macrophages
cAMP	Cyclic adenosine monophosphate
ChREBP	Carbohydrate response-element binding protein
COX	Cyclooxygenase
CRE	cAMP response Elements
DAMP	Damage-associated molecular pattern
DBD	DNA binding domain
DUSP	Dual specificity phosphatase
Elk-1	ETS domain containing protein 1
ER	Endoplismic reticulum
ER	Estrogen receptor
ERK	extracellular signal-regulated kinase
FCP	TFIIF-associating component of RNA polymerase II CTD phosphatase
FXR	Farnesoid recepor
GPCR	G-protein-coupled receptor
GR	Glucocorticoid receptor
HDM	Histone demethylase
HFD	High fat diet
HIF-1	Hypoxia inducible factor-1
HMT	Histone methytransferase
Hsp27	Heat shock protein 27
IFN	Interferon
IKK	Inhibitor of kappa B kinase
IL	Interleukin
IRF3	IFN regulatory factor 3
JNK	c-Jun amino-terminal kinase
LBD	Ligand binding domain
LDLR	Low-density lipoprotein receptor
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog
MAPK	Mitogen-activated protein kinases

MCP-1	Monocyte chemotactic protein–1
MK2	MAPK-activated protein kinase 2
MKP	MAPK phosphatase
NCoR	Nuclear receptor co-repressor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLK	Nemo-like kinase
NLR	Nod-like receptor
NR	Nuclear receptor
NSAID	Nonsteroidal anti-inflammatory drugs
$p38^{MAPK}$	p38 mitogen activated protein kinase
PAMP	Pathogen-associated molecular pattern
PKA	cAMP dependent protein kinase
PP5	Protein serine/threonine phosphatase 5
PPAR	Peroxisome proliferator-activated receptors
PPM	Metal dependent protein phosphatases
PPP	Phosphoprotein phosphatases
PSP	Protein serine/threonine phosphatases
RAR	Retinoic acid receptor
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SAPK	Stress Activated Protein Kinase
SCP	Small CTD phosphatase
SGK	Activating serum and glucocorticoid induced kinase
SMRT	Silencing mediator of retinoic acid and thyroid receptor
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
TPR	Tetratricopeptide repeat
TR	Thyroid hormone receptor
TRAF	TNFR-associated factor
TRE	TPA response elements
Trx	Thioredoxin
TZD	Thiazolidinediones
VDR	Vitamin D receptor