Selective roles of the Nuclear Receptors LXR in the transcriptional control of classical and alternative macrophage activation

Efectos selectivos de los receptores nucleares LXR en el control transcripcional de la activación clásica y alternativa de macrófagos

Theresa Elizabeth León Moreno

Programa de doctorado de Biomedicina

Departamento de Fisiología e Inmunología. Facultad de Biología

SELECTIVE ROLES OF THE NUCLEAR RECEPTOR LXR IN THE TRANSCRIPTIONAL CONTROL OF CLASSICAL AND ALTERNATIVE MACROPHAGE ACTIVATION

EFECTOS SELECTIVOS DEL RECEPTOR NUCLEAR LXR EN EL CONTROL TRANSCRIPCIONAL DE LA ACTIVACIÓN CLÁSICA Y ALTERNATIVA DE MACRÓFAGOS

Memoria presentada por:

Theresa Elizabeth León Moreno

Para optar al grado de:

Doctora por la Universidad de Barcelona

Directora:

Dra. Annabel Valledor

Doctorando:

Theresa Elizabeth León Moreno

Barcelona, Julio 2013

A toda mi familia.

A mi guapo JAB.

The development of this doctoral thesis has been possible thanks to the following financial sources:

Project: 080930

Projects: SAF2007-63543, SAF2010-14989, SAF2011-23402

Table of Contents

List of abbreviations

The following table describes the significance of various abbreviations and acronyms used throughout the thesis.

Abbreviation	Meaning
ABC	ATP-binding cassette transporters
acLDL	Acetylated-low density lipoprotein
Aim	Apoptosis inhibitor of macrophage
AML1	Acute myeloid leukemia 1
APC	Antigen-presenting cell
Apo	Apolipoprotein
AT	Apoptotic thymocytes
BAL	Bronchoalveolar lavage
BMDM	Bone marrow derived macrophage
C/EBP	CCAAT/enhancer-binding protein
CBP	CREB-binding protein
CCL	Chemokine (C-C motif) ligand
CIITA	Class II transactivator
CORO2A	Coronin, actin binding protein, 2A
CSF-1	Colony stimulating factor 1
CTL	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand
ChREBP	Carbohydrate responsive element-binding protein
DAMP	Damage-associated molecular pattern molecule
DBD	DNA-binding domain
DC	Dendritic cell
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FcγR	Receptor for the Fc (constant) region of immunoglobulin G
GAS	Interferon-gamma activated sequence
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPS2	G-protein pathway suppressor 2
HDAC	Histone deacetylase
HDM	House dust mite

Idol	Inducible Degrader of the LDL receptor
IFN	Interferon
Ig	Immunoglobulin
IKK	IjB kinase
IL	Interleukine
IL-4Rα	IL-4 receptor α chain
IRAK	Interleukin-1 receptor-associated kinase
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon response factor
IRS	Insulin receptor substrate
Itgal	Integrin alpha L
IκB	Inhibitor of NFκB
JAK	Janus kinase
JNK	c-jun N-terminal kinase
LBD	Ligand binding domain
LBP	LPS-binding protein
LBP	LPS binding protein
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPS	Lipopolysaccharide
LXR	Liver X receptor
LXRE	LXR response element
Mal	MyD88 adapter like
MAPK	Mitogen-activated protein kinase
M-CFU	Macrophage colony- forming unit
Mcpip1	MCP-1 (monocyte chemotactic protein-1)-induced protein
M-CSF	Macrophage colony stimulating factor
MD-2	Myeloid differentiation factor 2
MDC	Macrophage-derived chemokine
Mertk	c-Mer proto-oncogene tyrosine kinase
Mgl1	Macrophage galactose-type C-type lectin
MHC	Major histocompatibility complex
Mrc1	Mannose receptor, C type 1

MyD88	Myeloid differentiation marker
NCoR	Nuclear receptor corepressor
NFκB	Nuclear factor kappa B
NF-κB	Nuclear factor-κ B
NK	Natural killer
NKT	Natural killer T
NO	Nitric oxide
NOS2	Nitric oxide synthase 2
NR	Nuclear Receptor
oxLDL	Oxidized LDL
PAMP	Pathogen-associated molecular pattern
Pdpn	Podoplanin
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide-3-kinase
PIAS	Protein inhibitors of activated STAT
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
Ptgs	Prostaglandin-endoperoxide synthase 2 and cyclooxygenase
PU.1	Transcription factor PU.1
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SEM	Standard error of the mean
Serpinb2	Serine peptidase inhibitor clade B, member 2
SH2	Src-homology type 2 domains
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
SOCS	Suppressor of cytokine signaling proteins
SRC	steroid receptor coactivator-1
SREBP1c	Sterol regulatory element binding protein-1c
STAT	Signal Transducer and Activator of Transcription
SUMO	Small ubiquitin-like modifier
TAB2	TGF-beta activated kinase 1
TAK	Transforming growth factor-b-activated kinase

TARC	Thymus and activation regulated chemokine
TGF-β	Transforming growth factor beta
Th	T helper
TIR	Toll/interleukin-1 receptor
TLR	Toll- like receptor
TNF	Tumor necrosis factor
TRAF6	tumour necrosis factor receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-containing adapter molecule
UBC9	Ubiquitin carrier protein 9
UBCH5	Ubiquitin-conjugating enzyme E2D 1

Introduction

I. Immune system. General definitions

The immune system is a complex network of cells and molecules that act together in a dynamic way to protect the body from infection. The immune system has evolved to detect a wide variety of dangerous agents, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissues. Functionally, the immune system has been divided into two related components: the innate and the adaptive (or acquired) immune system.

The INNATE IMMUNE SYSTEM is the less specific component of the immune system, it provides the first line of defence against infection, and can be seen to comprise four types of host defences or barriers. *Anatomic barriers* include the skin and mucosal surfaces that provide mechanical barriers against infection. *Physiologic barriers* of the innate system are body temperature, low pH and chemical mediators (e.g. lysozymes, cytokines and the complement system). *Cellular barriers* of the innate immune system include the group of professional phagocytic cells comprised of neutrophils, monocytes, macrophages, dendritic cells and mast cells, which internalize (phagocytose) and digest pathogens; and include other type of cells such as basophils, eosinophils and natural killer cells that display different functions to help attacking invading microbes. The *inflammatory response*, considered one of the first responses of the immune system to infection, is a complex sequence of events that function to eliminate harmful agents and initiate repair of the tissue damaged (Kindt et al., 2007).

The ADAPTIVE IMMUNE SYSTEM is activated by the innate immune system and it has the ability to recognize and 'remember' specific pathogens, and to mount stronger and faster attacks each time the pathogen is encountered. It is 'adaptive' in the sense that it prepares the immune system for future challenges by the same or highly related pathogen. The cells of the adaptive immune system are the lymphocytes. B cells and T cells are the major types of lymphocytes; B cells are involved in the *humoral immune response*, whereas T cells are involved in the *cell-mediated immune response*. Both B cells and T cells carry receptor molecules that recognize specific targets. The B cell antigen-specific receptor is an antibody (immunoglobulin) molecule on the B cell surface that recognizes whole pathogens without any need for antigen processing.

Once a B cell encounters its specific antigen, and receives additional signals from a helper T cell, it further differentiates into an effector cell, known as a *plasma cell*, which secretes antibodies. These antibodies bind to antigens, making them easier targets for phagocytes, and trigger the complement system cascade (Janeway et al., 2001). Therefore, plasma cells connect this branch of the adaptive immune system, the humoral response, with components of the innate immune system in order to produce an effective defense against the infection.

T cells recognize non-self targets, such as pathogens, only after antigens have been processed and presented in combination with a self receptor called major histocompatibility complex (MHC). There are two major subtypes of T cells: the *cytotoxic T cell* and the *helper T cell*. Cytotoxic T cells only recognize antigens coupled to Class I MHC molecules and are able to kill infected cells, while helper T (Th) cells only recognize antigens coupled to Class II MHC molecules in the surface of antigen presenting-cells (APC) and are specialized in producing cytokines to orchestrate the immune response. Professional APCs include macrophages, B lymphocytes and dendritic cells. The type of T cell activated, and the type of response generated depends, in part, on the context in which the APC first encountered the antigen. Proliferating helper T cells that develop into effector T cells differentiate into two major subtypes of cells known as Th1 and Th2 cells, and another novel T helper subtype, the Th17 cells, defined by the cytokines they produce when stimulated. This polarization depends on the signals they receive in the moment of antigen recognition, the nature of the pathogen (intracellular or extracellular) and the cytokines of the microenvironment. In brief, Th1 cells are key in the host response against intracellular bacteria and protozoa and its effector cytokine is interferon gamma (IFN-γ); Th2 cells in turn, are important in responses triggered by interleukin (IL)-4, and fighting against multicellular helminths; and Th17 are IL-17 producing cells with regulatory functions and also mediate host immune response against extracellular bacteria and fungi (Janeway et al., 2001).

Once B cells and T cells are activated and begin to replicate, some of their offspring become long-lived *memory cells*. Throughout the lifetime of an animal, these memory

cells can recognize each specific pathogen encountered and can mount a strong and faster response if the pathogen is detected again (Janeway et al., 2001).

This thesis will focus on an important cell type of the innate immune system, macrophages, and the regulation of the gene expression profile that these cells display in pro-inflammatory and anti-inflammatory conditions.

II. Macrophages

Macrophages are myeloid cells that are critical effectors and regulators of the innate immune response, playing an important role in inflammation and also in tissue homeostasis. They constantly survey their immediate surroundings for signs of tissue damage or invading organisms and are prepared to stimulate lymphocytes and other immune cells to respond when danger signals are phagocytosed or detected by cell surface receptors. In addition to it, macrophages are involved in the resolution phase of inflammation and in tissue repair (Murray and Wynn, 2011).

In addition to fighting infections, resident tissue macrophages are involved in maintaining healthy tissues by removing dead and dying cells, necrotic and toxic materials, and secreting growth factors. For example, alveolar macrophages facilitate the removal of allergens from the lung, whereas macrophages in liver (Kupffer cells) participate in the clearance of pathogens and toxins from the circulation, and spleen macrophages eliminate senescent red blood cells from the circulation and recycle iron molecules (Murray and Wynn, 2011). Tissue macrophages also suppress inflammation mediated by inflammatory monocytes, thereby ensuring that tissue homeostasis is restored following infection or injury (Loke et al., 2007; Mosser and Edwards, 2008).

Based on the need to perform such different functions, macrophages display striking plasticity, being able to change their phenotype and transcriptional program depending on the external stimuli that they receive. In a general way, activated macrophages have been classified in classically activated (M1), which display a pro-inflammatory phenotype, and alternative activated macrophages (M2), which are involved in tissue repair, although several macrophage subsets with distinct functions

have also been described as will be discussed in a further section (Macrophage activation) (Figure 1).

Macrophages are important as well in cholesterol metabolism, contributing to reverse cholesterol transport, the process by which extrahepatic (peripheral) cholesterol is returned to the liver for excretion in the bile and ultimately in the feces (Cuchel and Rader, 2006). Thus, macrophages not only orchestrate inflammatory responses and host defence against pathogens but are also critical for the clearance of lipoproteins.

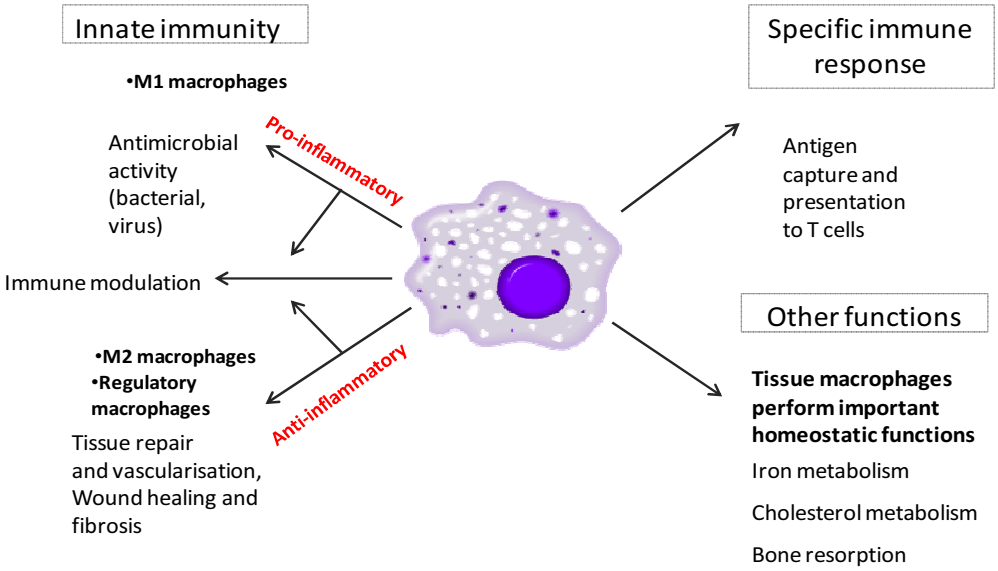

Figure 1. Schematic diagram of macrophage functions.

Macrophage differentiation

All the leukocytes, the white blood cells that mediate the body's immune response, have a common origin in hematopoietic stem cells and develop along distinct differentiation pathways in response to internal and external cues. The process by which all the blood cells are originated and develop implies their survival, proliferation and differentiation from their progenitors and it is called haematopoiesis (Ogawa, 1993).

Within the bone marrow, IL-1, IL-3, and/or IL-6 induce heteromitosis in the hematopoietic stem cells. This division gives rise to a new stem cell and a pluripotent

myeloid cell, also referred to as granulocyte-erythrocyte-megakaryocyte-macrophage colony forming unit (GEMM-CFU). In the presence of IL-1 and/or IL-3, this precursor differentiates into a progenitor of both macrophages and granulocytes (GM-CFU). At this point, the action of IL-3 and granulocyte- macrophage colony-stimulating factor (GM-CSF) induce the proliferation of these myeloid precursors, however, the presence of macrophage colony-stimulating factor (M-CSF) induces their proliferation and differentiation to monocytic precursors, the macrophage colony- forming unit (M-CFU). The M-CFUs can differentiate to monocytes and to the common dendritic cell (DC) precursors. In the presence of M-CSF, the M-CFU gives rise to monoblasts, promonocytes and the subsequent generation of monocytes.

Several transcription factors are involved in the process of monocyte and macrophage differentiation, being important for the selection of specific gene expression programs. For example, the myeloid transcription factor PU.1 is required for the earliest steps of myeloid lineage commitment in hematopoietic stem cells and its absence results in general myeloid lineage deficiencies, especially macrophages (McKercher et al., 1996). The C/EBP (CCAT/Enhancer-binding protein) family of transcription factors and AML1 (acute myeloid leukemia 1) have also been reported to play specific roles during macrophages differentiation (Valledor et al., 1998; Geissmann et al., 2010).

Monocytes exit to the blood and can enter tissues under inflammatory conditions where they give rise to subsets of macrophages and to inflammatory dendritic cells (DC). Monocytes have also been considered the developmental intermediate between bone marrow precursors and different tissue macrophages. Specialized tissue-resident macrophages include osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue) and Kupffer cells (liver). However, more recent studies have demonstrated that many tissue macrophages do not originate from monocytes in a steady state. For example, neither microglia (tissue macrophages of the central nervous system) nor Langerhans cells (skin) are dependent on the bone marrow for their renewal in the steady state and possibly during inflammation (Merad et al., 2002; Mildner et al., 2007).

Macrophage activation

Macrophages are cells susceptible to many different signals. Following injury or infection, macrophages can respond to endogenous cytokines that are rapidly generated by innate immune cells (e.g. NK cells) and also by antigen-specific adaptive immune cells (e.g. T cells). These signals can exert a marked effect on the physiology of macrophages, and also macrophages themselves can produce several factors that influence their own fate. Thus, depending on the cytokines that are induced in the microenvironment, macrophages will display a specific phenotype.

According to these phenotypes, macrophage activation was initially classified in classical or alternative, although the diversity of stimuli and the differences in their physiology and function generated a big controversy and the need for further expansion of this classification. The phenotypes described include the *classically activated* macrophages (M1), induced by microbial products such as the bacterial lipopolysaccharide (LPS), and by the Th1 cytokine IFN-γ; the *alternatively activated* macrophages (M2a), that are induced by T helper 2 (Th2) cytokines such as interleukin-4 (IL4) and interleukin-13 (IL-13); and the *deactivated* macrophages, or *regulatory* macrophages, induced by phagocytosis of apoptotic cells and Fc-receptor (FcR) cross-linking (M2b); and by interleukin-10 (IL-10) and tumor growth factor beta (TGF-β) (M2c) (Gordon, 2003; Mantovani et al., 2004; Mosser and Edwards, 2008).

Classical activation of macrophages

The classical activation of macrophages, or the acquisition of the M1 phenotype, is linked to the development of an inflammatory response and antimicrobial activity. Macrophages express a broad range of pathogen recognition receptors (PRRs) by which they recognize pathogen-associated molecular patterns (PAMPs) on the surface of foreign and potentially dangerous invaders. An important group of PRRs are the toll-like receptors (TLRs) (reviewed in Moresco et al. 2011) that perceive several microbe associated structures such as cell wall components (i.e. Peptidoglycan), sequences of bacterial and viral DNA (i.e. unmethylated CpG dinucleotides) (Bauer et al., 1999) and

double-stranded RNA (Alexopoulou et al., 2001). So far, 13 paralogous TLRs have been identified in mice and humans combined, 10 in humans and 12 in mice. One of the most studied activating molecules of macrophages is the Gram negative cell wall component LPS, which is recognized by TLR4. Intracellular signalling in response to the activation of many TLRs, and specially TLR4, leads to classical activation of macrophages.

There are some endogenous signals that can also induce the classical activation of macrophages, these are the damage/danger-associated molecular pattern molecules (DAMPs). They are commonly cytosolic or nuclear proteins, (i.e. heat-shock proteins), which can initiate and perpetuate immune responses in a non-infectious inflammatory setting (Foell et al., 2007).

The classical activation of macrophages is characterized by an increased phagocytic activity, the production of reactive oxygen species (ROS) through the initiation of the *respiratory burst* or *oxidative burst* process, the induction of the inducible nitric oxide synthase (NOS2) enzyme that produces nitric oxide (NO) – products that all together exert a powerful microbicidal activity – and by the secretion of pro-inflammatory cytokines and an increase in the antigen presentation function to T lymphocytes through the MHC class II.

The inflammatory response is normally initiated by the resident cells present in the tissue of injury or infection, including tissue macrophages. These cells produce pro-inflammatory cytokines that activate the vascular endothelium and increase its permeability, through the induction of adhesion molecules on endothelial cell surfaces, and secrete chemokines (small size chemotactic cytokines), which act as a chemoattractant recruiting monocytes, neutrophils and other effector cells from the blood to the site of infection or tissue damage. Monocytes quickly differentiate into macrophages and DCs at the site of infection, which continue to secrete pro-inflammatory mediators such as the cytokines tumour necrosis factor (TNFα), IL-1β and IL-6, and produce NO which participate in the activation of the oxidative processes that contribute to the killing of invading organisms.

a. Lipopolysaccharide (LPS)

LPS is a PAMP present in the outer cell membrane of Gram negative bacteria, recognized by the receptor TLR4 of immune cells during infection. Stimulation of macrophages with LPS elicits a variety of different signalling events that culminate in the activation of macrophage effector functions, including the production of cytokines, chemokines and other communication signals important for the coordination of the inflammatory response (table 1). This macrophage response results from the integration of a complex intracellular signalling network, which include signals directly elicited by the activated TLR4 receptor complex and signals that are part of autocrine feedback loops (Bode et al., 2012).

The activation of macrophages by LPS has to be tightly controlled to prevent the detrimental effects of offensive macrophage activation. A moderate inflammatory response induced by LPS is beneficial in fighting against infections of Gram negative bacteria. However, when this response is highly intense with an excessive secretion of TNFα to the blood, it can lead to an endotoxic shock (or *septic shock*) that can cause the death of the individual (Cauwels and Brouckaert, 2007). In this context, one of the most critical anti-inflammatory feedback mechanisms of macrophages is the release of IL-10, at a late stage after activation by LPS, and subsequent induction of sustained activation of signal-transducer and activator of transcription (STAT)-3. The activation of STAT-3 has been correlated with the dampening of the inflammatory response, through negative control of the production of TNFα and other cytokines (Williams et al., 2004).

Table 1. Inflammatory mediators and other products induced by LPS in macrophages

MEDIATOR	DESCRIPTION AND EFFECTOR FUNCTIONS	REF.
TNF-α	Pro-inflammatory cytokine. Tumor toxicity; Induction of apoptosis and necrosis; Circulatory shock; Induction of cytokine production (via NFκB)	(Wajant et al., 2003)
IL1-β	Pro-inflammatory cytokine. Induce production of IL-1, IL-6, IL-8, TNFα, GM-CSF and PGE_2; T cells activation (IL-2); B cells proliferation/maturation; NK cytotoxicity; Chemokine production; ICAM and VCAM expression; Fever; Induction of acute phase proteins	(Eder, 2009; Dinarello, 2009)
IL-6	Pro-inflammatory cytokine. Differentiation of myeloid stem cells and B cells into plasma cells; T cells proliferation; Differentiation of Mϕ; Regulate production of acute phase proteins and the induction of fever; Enhance release of anti-inflammatory mediators	(Naka et al., 2002; Neurath and Finotto, 2011)
IL-10	Anti-inflammatory cytokine. Suppress antigen presentation, immune mediator release (including IL-1β and TNFα) and phagocytosis of monocytes and Mϕ	(Fiorentino et al., 1991; Sabat, 2010)
IL-12	Important factor in polarization of T cells towards Th1 phenotype. Also induces interferon gamma production by T cells and NK cells and enhances NK cell activity.	(Lamont and Adorini, 1996)
IFN-α/β	Both interferons induce resistance to viruses and inhibit cell proliferation. Regulates expression of class I MHC.	(Pestka et al., 2004)
TGF-β	Inhibits growth of a number of cells types; affects tissue remodelling, wound repair, development and haematopoiesis. Suppressive effects on the expansion of certain immune-cell populations; switch factor for IgA.	(Li et al., 2006)
NOS-2	Nitric oxide synthase 2, is inducible by a combination of LPS and certain cytokines. Produces nitric oxide (NO) that is a reactive free radical which acts as a biologic mediator in several processes, including antimicrobial, antitumoral and neurotransmission activities.	(Reviewed in Coleman 2001)
Prostaglandins e.g. PGE_2	Metabolites of arachidonic acid. Stimulate vasodilatation, attraction of neutrophils, Mo, DC to the inflammatory site. Induce IL-10, suppress production of pro-inflammatory cytokines. Promote DC activation and differentiation	(Kalinski, 2012; Rossi et al., 2005)
PAF	Platelet-activating factor; lipid mediator eliciting responses in a wide variety of cell types. Involved in platelet activation, airway constriction, and hypotension.	(Shindou et al., 2005)
IL-8/ CXCL-8	Chemokine (C-X-C motif) ligand (CXCL)-8. Mediates chemotaxis and activation of neutrophils	(Remick, 2005)
CCL-2/ MCP-1	Chemokine (C-C motif) ligand (CCL)-2. Mediates chemotaxis of monocytes, memory T cells and DC to site of infection. Induce migration of endothelial cells in the process of angiogenesis	(Yadav et al., 2010)
MIP-1α/β (CCL-3 & CCL-4)	Chemokines. Mediate chemotaxis of T-cells, Mo, DC and NK. Upregulation of arachidonic acid, histamine and modulate Th-differentiation	(Maurer and von Stebut, 2004)

Mϕ, macrophage; DC, dendritic cell; Mo, monocyte; NK, natural killer cell.

Signal transduction of LPS

Molecular recognition of LPS implies the action of TLR4, together with other components of the LPS-sensing machinery, that involves the plasma-derived LPS-binding protein (LBP), the cell membrane associated receptor CD14 and the TLR4-interacting factor MD-2 (Wright et al., 1990, 1989; Chow et al., 1999; Shimazu et al., 1999) (Figure 2).

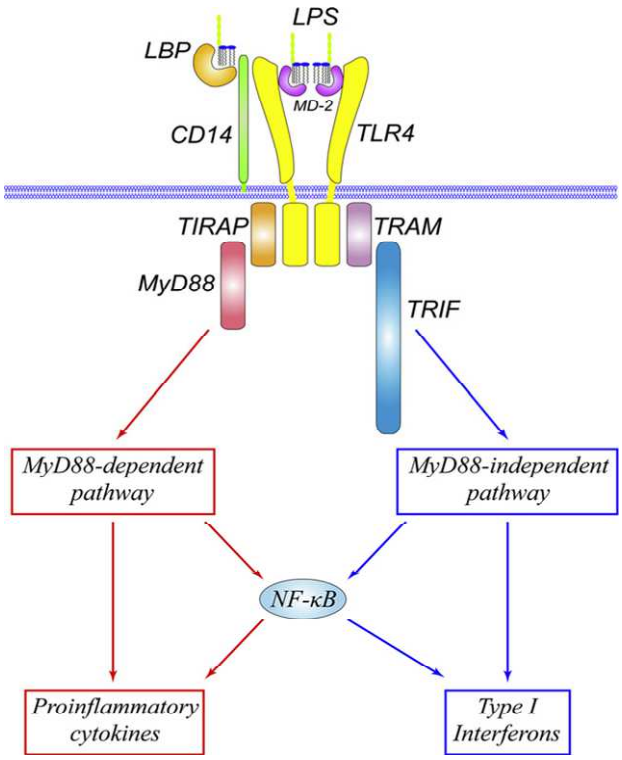

Figure 2. Overview of LPS/TLR4 signalling (Lu et al., 2008). LPS recognition is mediated by LBP, CD14, and the TLR4/MD-2 receptor complex. LPS/ TLR4 signalling can be separated into MyD88-dependent and MyD88-independent pathways, which mediate the activation of pro-inflammatory cytokine and Type I interferon genes.

TLR4 is a type I transmembrane receptor protein comprising a conserved cytosolic region, termed the Toll-IL-1R (TIR) domain, being characteristic for the IL-1 receptor/TLR superfamily and belongs to a subgroup that is, like all the other TLRs, further characterized by a large leucine rich repeat within its extracellular domain. MD-2 is associated with the extracellular domain of TLR4 and is essentially required for cell surface expression and LPS recognition of TLR4, as well as for ligand-induced receptor clustering (Nagai et al., 2002). The interaction between LPS and the TLR4/MD-2

receptor complex triggers the oligomerization of TLR4 and subsequent activation of signalling via the cytoplasmic TIR domain of TLR4 (Pålsson-McDermott and O'Neill, 2004).

Subsequent signals activated by TLR4 can be subdivided into those dependent on the myeloid differentiation marker MyD88 (and MyD88 adapter like, MAL, also known as TIRAP), which occur early and drive the induction of inflammatory cytokines, and those that are TRIF-dependent, which occur later and use the adapters TIR-containing adapter molecule (TRIF) and TRIF-related adapter molecule (TRAM), and are responsible for the induction of type I interferon (IFN-α/β) as well as inflammatory cytokines (Kawai and Akira, 2010) (Figures 2 and 3).

TLR4 initially recruits the adaptor protein TIRAP and MyD88. MyD88 then recruits and activates the protein kinase IRAK (IL-1 receptor-associated kinase). IRAK is autophosphorylated and dissociates from the receptor to interact with TRAF6 (TNF receptor-associated factor 6), and the TAK1 (TGF-β activated kinase-1) complex through the adaptor protein TAB2, leading to early-phase activation of NF-κB. TAK1 is implicated in the activation of NF-κB through the phosphorylation of the inhibitor of NF-κB (IκB). TAK1 phosphorylates IKKb, activating the IKK complex to phosphorylate IkB leading to IkB degradation and release of NF-kB. TAK1 also activates the mitogen-activated protein (MAP) kinase cascades that lead to activation of the transcription factors AP-1 and cyclic AMP (cAMP) response element-binding protein (CREB). CREB and AP-1 transcription factors cooperate with NF-kB to induce transcription of proinflammatory cytokines, such as TNFα and IL-1 (Kawai and Akira, 2010; Moresco et al., 2011) (Figure 3).

All the three groups of MAPkinases, the p38 MAPK, the extracellular signal-regulated kinases (Erk1/2) and the c-jun N-terminal kinase (JNK), have been demonstrated to be involved in regulation of the inflammatory macrophage response towards LPS (Valledor et al., 2000b), but in particular the p38MAPKα pathway is critical for normal immune and inflammatory response and plays a major role in regulation of inflammatory mediator release in macrophages in vivo (Cuadrado and Nebreda, 2010).

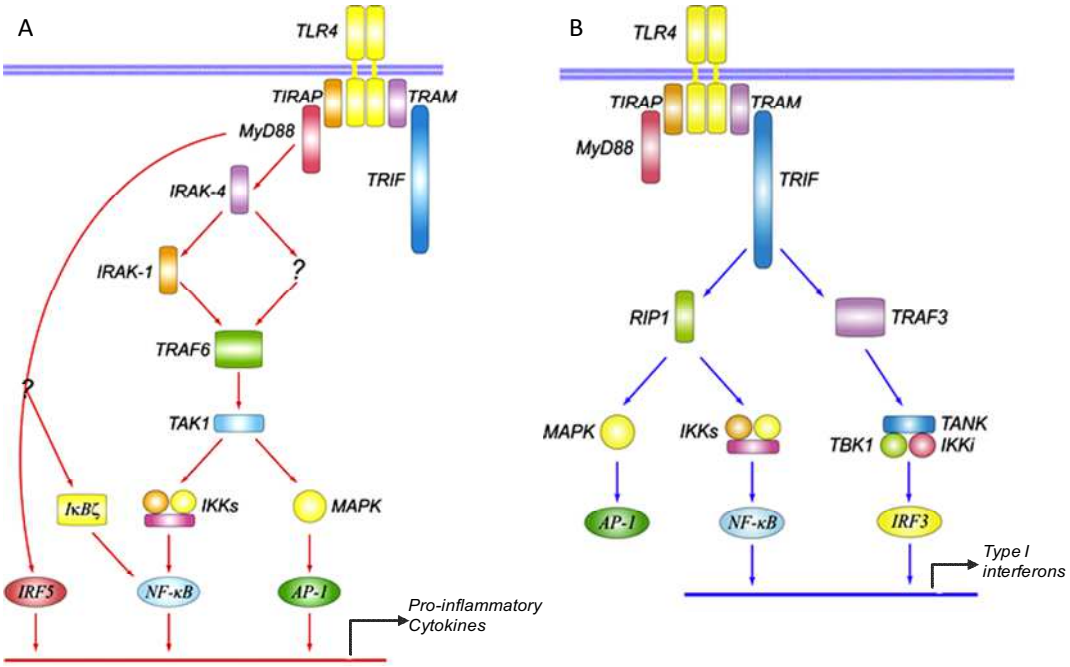

Figure 3. TLR4 induced signalling. A) The MyD88-dependent pathway. TLR4 initially transmits signals for the early-phase activation of NF-κB by recruiting TIRAP and MyD88. MyD88 activates IRAKs/ TRAF6 as well as the transcription factors NF-κB, AP-1 and IRF-5 further downstream. These transcription factors induce expression of pro-inflammatory cytokine genes. B) The MyD88-independent pathway (TRIF dependent pathway). TLR4 is then transported into Rab11a-positive phagosomes that contain bacteria, where it recruits TRAM and TRIF and activates TRAF3-TBK1-IRF3 axis as well as late-phase NF-κB activation for the induction of type I IFN. Both early- and late-phase activation of NF-κB is required for the induction of inflammatory cytokines. Adapted from Kawai and Akira 2011 and Y.-C. Lu et al. 2008.

TLR4 is also endocytosed and delivered to intracellular vesicles to form a complex with TRAM and TRIF, to initiate the TRIF-dependent pathway (Figure 3). This complex TLR4-TRAM-TRIF recruits TRAF3 and the protein kinases TBK1 and IKKi, which catalyze the phosphorylation of IRF3, leading to the expression of type I IFN. TRAM-TRIF also recruits TRAF6 and TAK1 to mediate late-phase activation of NF-kB and MAP kinases (Barton and Kagan, 2009).

Whereas activation of the TRIF-dependent pathway is sufficient for type I IFN induction, activation of both the MyD88- and TRIF-dependent pathways is required to drive robust NF-kB and MAP kinase activation and the subsequent induction of inflammatory cytokines. TAG, a splice variant of TRAM, binds intracellular TLR4 to

disrupt the TRAM-TRIF interaction, thus terminating activation of the TRIF-dependent pathway (Pålsson-McDermott and O'Neill, 2004).

b. Interferon gamma (IFNγ)

Interferons (IFNs) are a family of structurally related cytokines that play a crucial role in the resistance of vertebrates to pathogen infections, being initially discovered by their hallmark function of antiviral activity (Isaacs and Lindenmann, 1957), however they have also been related to antitumour activity and immunomodulatory effects (Reviewed in Ikeda et al. 2002; Rosenzweig and Holland 2005).

There are three types of IFNs: type I, type II and IFN-like cytokines (Pestka et al., 2004). The type I IFNs are represented by IFN-α and IFN-β, that are induced in virally infected cells to confer an antiviral state on uninfected cells. IFN-γ is the only type II interferon and it is mainly secreted by natural killer (NK), natural killer T (NKT) cells and by activated $CD8^+$ T cells and $CD4^+$ Th1 effector cells (Perussia 1991; Sad et al. 1995; Schroder et al. 2004). The IFN-γ secretion by NK cells is considered to be important in early host defense against infection, acting locally and playing a role in cell self-activation and activation of nearby cells; whereas T lymphocytes seem to be the major source of IFN-γ during the adaptive immune response.

IFN-γ signalling

The binding of IFN-γ to its receptor activates the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway which modulates the transcriptional activation of hundreds of genes and mediates diverse biological responses. The IFN-γ receptor (IFN-GR) consists of two ligand-binding receptor chains (IFNGR1) associated to another two additional chains (IFNGR2). After engagement of the IFNGR with IFN-γ, the intracellular receptor domains suffer a conformational change that allows the interaction with members of the JAK family, the protein tyrosine kinases JAK-1 and -2, which are non-receptor protein tyrosine kinases. The intracellular domain of IFNGR1 is involved in binding to JAK-1 while IFNGR2 contains JAK-2 binding motives. Binding of the ligand induces the auto-phosphorylation and activation of JAK-2, which then

phosphorylates JAK-1 (Igarashi et al., 1994). Activated JAK-1 phosphorylates the Tyr440 residues in the IFNGR1 chains, which generates in the receptor a binding site for STAT-1 via its Src homology 2 (SH2) domain (Heim et al., 1995). Phosphorylation of STAT-1 on Tyr701 results in the formation of STAT-1 homodimers, known as Gamma-Activated Factor, which translocate to the nucleus. Besides, phosphorylation of STAT-1 at Ser727 is essential for maximal ability to activate transcription of target genes (Wen et al., 1995). Once in the nucleus, STAT-1 homodimers bind to response elements in the DNA called GAS (gamma interferon activated sequence) and enhance transcriptional activation by recruiting several transcriptional coactivators.

The IFN-γ modulation of genes occurs in two waves of gene transcription. Primary responsive genes are induced early due to the binding of STAT-1 homodimers to GAS elements present in their promoters. Examples of these genes are the interferon regulatory factor (*Irf*)*1*, *Cxcl9* (MIG1) and *Cxcl10* (IP-10). Some of the genes that are upregulated in this early phase, between the first 15-30 min of IFN-γ treatment, code for transcription factors that participate in the secondary response and induction of their expression does not require the synthesis of new proteins or factors. During the secondary wave of transcription, STAT1 form a complex with the transcription factor IRF1, induced in the early phase of response, and then binds to interferon-sensitive response elements (ISRE) located in the promoters of many IFN-γ target genes such as *Nos2*, *Casp1*, *Ptgs (Cox2)*, *Ciita*, *Tap1*, etc. (reviewed in Saha et al. 2010) (Figure 4).

Although homodimeric phosphorylated STAT-1 molecules are well studied and are the most exemplified in the IFN-γ signalling, other active complexes such as STAT-1 heterodimers (e.g., STAT-1:STAT-2) and heterotrimers (e.g., STAT-1:STAT-1:IRF-9, STAT-1:STAT-2:IRF-9) form during this signalling and are also known to bind to GAS elements (Darnell et al., 1994; Stark et al., 1998; Matsumoto et al., 1999). Moreover, it has been reported that often STAT-3 and sometimes STAT-5 are also activated by IFN-γ (Durbin et al., 1996; Eilers and Decker, 1995).

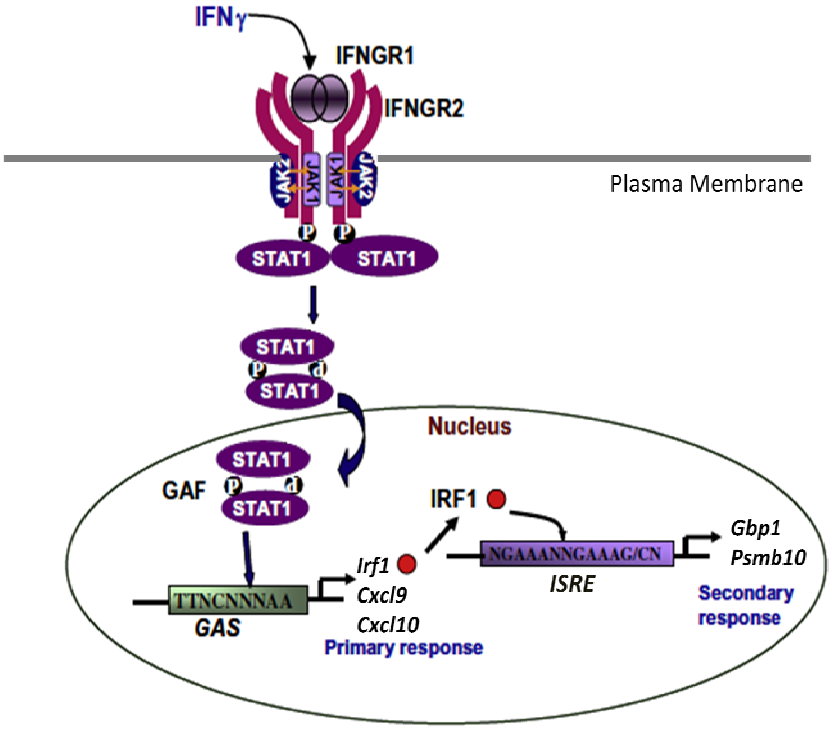

Figure 4. IFN-γ signalling pathway and modulation of genes. Binding of IFN-γ results in receptor oligomerisation followed by trans-phosphorylation and activation of JAK1 and JAK2. Activated JAKs phosphorylate IFNGR1 resulting in docking of STAT1. Phosphorylated STAT1 homodimerizes to form Gamma-Activated Factor which binds to GAS located in the promoters of several primary response genes and increases transcription by recruiting several coactivators. Upon induction, the transcription factor IRF1 binds to ISRE and enhances transcription of several secondary response genes. Figure adapted from Saha et al. 2010.

IFN-γ induced signalling needs to be tightly regulated to avoid detrimental consequences of excessive stimulation. In this context, it is known that the activation of STAT1 by IFN-γ is not continuous and is inhibited after some time. One of the best studied negative regulators of the JAK-STAT pathway are the suppressor of cytokine signalling (SOCS) proteins which inhibit JAK activity. SOCS proteins inhibit cytokine signalling by various mechanisms. Studies have shown that SOCS1 directly interacts and inhibits the catalytic activities of JAK-1, JAK-2, JAK-3, and TYK2. SOCS3 also inhibits JAK-2 activity, and other inhibitory mechanisms include competing with STAT to bind to the receptor, degrading signalling molecules, etc (Krebs and Hilton, 2001; Croker et al., 2008). Protein inhibitors of activated STAT (PIAS) are also key repressors of IFN-γ activity. They act as small ubiquitin-related modifier (SUMO)-ligases, as well as decoy receptors. PIAS SUMOylate and inactivate STAT dimers in the nucleus (Rakesh and

Agrawal, 2005). In addition, the SH2 domain-containing tyrosine phosphatase (SHP2) has been implicated as a negative regulator of IFN-γ signalling.

IFN-γ biological roles

IFN-γ plays a predominant pro-inflammatory role. It mainly functions by stimulating the development and actions of cells of innate immune system, via activation of NK cells, macrophages, and cytotoxic T cells (Boehm et al., 1997; Schroder et al., 2004). The coordinated actions of IFN-γ are associated with the protection against viral infections, intracellular and extracellular bacteria and small protozoa (Sher and Coffman, 1992; Reiner and Locksley, 1995). However, recent evidence indicates a protective role for IFN-γ in Th1-associated autoimmune diseases from reports revealing an increasing number of pathways by which IFN-γ can counteract harmful inflammation (reviewed in Kelchtermans et al. 2008) (Figure 5).

As it was mentioned before, macrophage stimulation with IFN-γ induces an M1 or classical activated phenotype. Activated macrophages, among many other properties, show greatly enhanced microbicidal activity for many intracellular and phagocytosed organisms, e.g. Mycobacteria, Toxoplasma, Trypanosoma, and Leishmania. IFN-γ regulates the transcription of several genes that co-ordinately achieve this effector state in macrophages, increasing the respiratory burst and the synthesis of NOS2, and consequently increasing the production of oxygen and nitrogen reactive species, and increasing antigen processing and presentation through the MHC class I and II.

In addition, IFN-γ enhances the macrophage response to other inflammatory stimuli, like TLR ligands, tumour necrosis factor (TNF) and type I IFNs, a phenomenon that has been called “priming”. The priming of TLR responses by IFN-γ consists in the increased expression of TLR-induced inflammatory mediators, including cytokines and chemokines through a mechanism that implies the activation of NF-κB. On the other hand, the priming of macrophages in response to type I IFNs is characterised by an increased activation of STAT-1 (Hu and Ivashkiv, 2009).

IFN-γ also regulates leukocyte attraction and directs growth, maturation, and differentiation of many other cell types (Boehm et al., 1997), in addition to regulating B

cell functions such as immunoglobulin (Ig) production and class switching towards Ig subtypes that are efficient in opsonisation and promotion of phagocytosis (Finkelman et al., 1988). The biological activities of IFN-γ are summarized in Table 2.

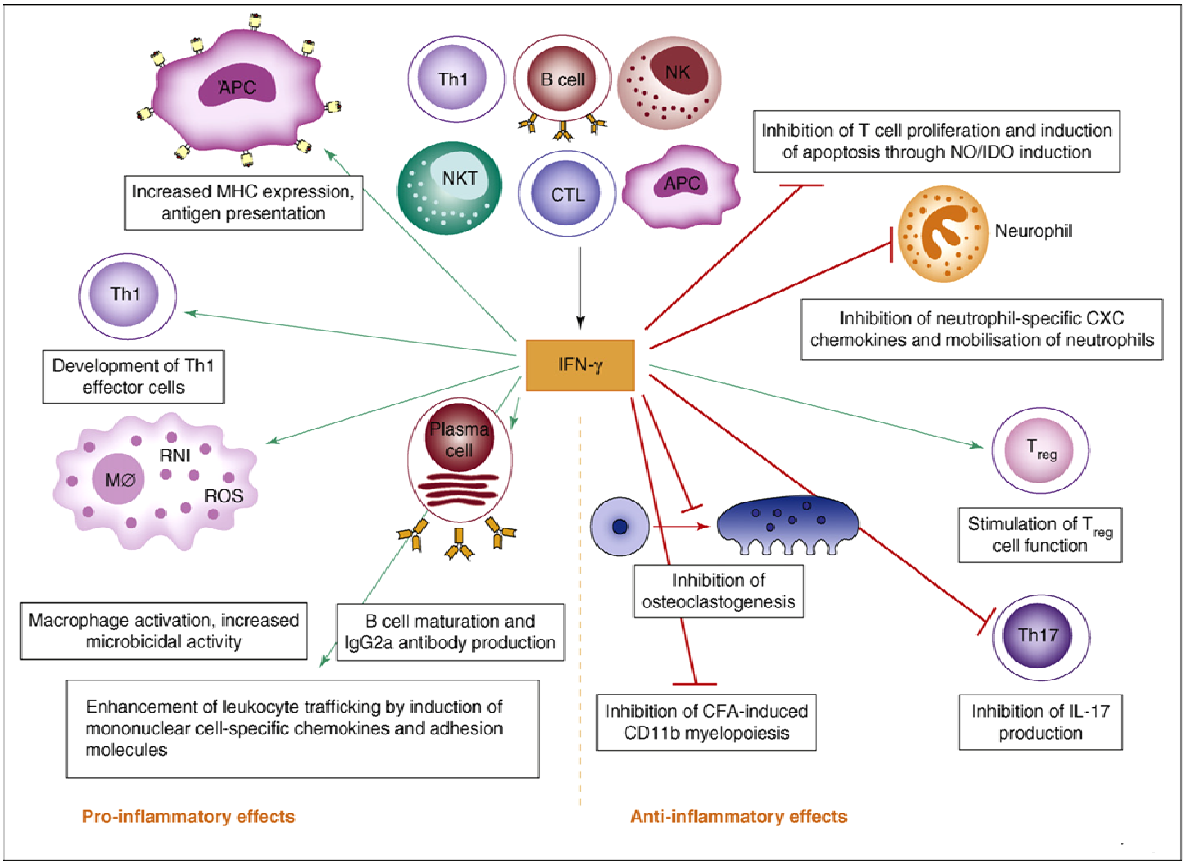

Figure 5. Overview of IFN-γ functions in the immune system (Kelchtermans et al., 2008)**.** This Figure summarizes how IFN-γ can function both as an inducer and a regulator of immune responses. Red and green arrows represent, respectively, inhibitory and stimulatory actions of IFN-γ. IFN-γ can be produced (black narrow) by Th1 and B cells, NK and NKT cells, CTL and APCs. RNI, reactive nitrogen intermediates; ROS, reactive oxygen species.

IFN-γ facilitates Th1 differentiation by enhancing the synthesis of IL-12 in antigen-presenting cells. IL-12 is one of the main mediators of cellular immunity. It is initially secreted by dendritic cells, neutrophils, and mainly by macrophages in response to LPS and other pathogenic microorganism derived products (Trinchieri, 1995). IFN-γ potentiates IL-12 transcription in response to these stimuli, and IFN-γ itself, is capable of inducing IL-12 expression in macrophages (Yoshida et al., 1994). IL-12 activates NK

cells and it is the main mediator of naive T lymphocyte differentiation to the Th1 phenotype, thus it acts as a connector between innate immunity and the antigen-specific adaptive response. IL-12 induces the secretion of IFN-γ by the CD4+ T cells and NK cells. In consequence, a positive feedback system is established that favours Th1 cell differentiation and high IFN-γ production (Trinchieri, 1995).

IFN-γ blocks the development of Th2 cells by inhibiting the production of IL-4 and blocking the IL-4-STAT6 pathway, which is required for Th2 formation (Szabo et al., 1995), and by preventing Th2-cell proliferation (reviewed in George R Stark et al. 1998).

In B lymphocytes, IFN-γ is able to direct immunoglobulin (Ig) class switching towards certain of IgG with high opsonising capacity, mainly the IgG2a, that are recognized by phagocyte cells through receptors for the IgG constant region (FcγR) (Snapper et al., 1988; Boehm et al., 1997). This activity favours clearance of pathogens by phagocytic cells.

IFN-γ inhibits the differentiation of Th17 lymphocytes, as demonstrated *in vivo* and *in vitro*. Th17 lymphocytes are important in the establishment of aggressive host defence against extracellular bacteria and yeast, through elevated neutrophil infiltration and extensive tissue damage. Differentiation of Th17 cells is produced by the action of cytokines IL-6, IL-1, TGF-β, IL-21 and IL-23. In contrast, IFN-γ is able to promote the differentiation of regulatory T lymphocytes (T reg). This population of lymphocytes acts to restrict the activation of effector T lymphocytes and maintain homeostasis (reviewed in Hu and Ivashkiv 2009).

Table 2. Summary of IFN-γ biological activities.

BIOLOGICAL EFFECT	MEDIATOR	REF.
Antimicrobial activity. IFN-γ increases the expression of inflammatory mediators and immune effectors including cytokines and chemokines. IFN-γ amplifies TLR signalling by increasing the transcription of several TLRs and components of the pathway. IFN-γ also suppresses TLR-induced IL-10 expression, blocking the IL-10-STAT3 axis; it interrupts a feedback inhibition loop and results in increased production of inflammatory cytokines and enhanced macrophage activation. IFN-γ highly activates two important anti-microbial molecules, the immunity-related p47 GTPases (p47 IRG) important in fighting against Listeria, Salmonella, and mycobacteria infections; and p65 guanylate-binding proteins (p65 GBP), that induce antibacterial responses involving phagocytic oxidases, autophagic effectors, and inflammasome. IFN-γ induces the respiratory burst, which leads to production of reactive oxygen species (ROS). ROS are highly reactive molecules that include superoxide anions, hydrogen peroxide and hydroxyl radical. These molecules have strong bactericidal activity. IFN-γ also upregulates the expression of NOS2 in antigen presenting cells (APC). This enzyme converts L-arginine into L-citrulline and NO. NO and nitrogen reactive products can easily penetrate bacterial walls and produce toxic effects in bacteria. IFN-γ upregulates as well the FcγR, which increases the phagocytic activity of opsonised material.	Macrophages, neutrophils, monocytes and eosinophils.	(Schroder et al., 2006; Hu et al., 2008, 2006; MacMicking, 2004; Yang et al., 2007; MacMicking et al., 1997; Yamamoto et al., 2012)
Modulation of antigen presentation. IFN-γ enhances the activity of antigen presentation through MHC-I, by upregulating the expression of *Tap2*, *Psmb8* and *Psmb10*, and therefore increasing the quality and diversity of peptides that are presented in the cell surface. IFN-γ also upregulates *Tapasin* and cell-surface MHC-class I expression, which is important for host response to intracellular pathogens, increasing the possibility for cytotoxic T lymphocytes to recognize infected cells. IFN-γ upregulates the class II antigen presentation pathway in APCs, inducing not only the expression of MHC-II, but also inducing molecules that participate in the process of MHC-II loading with peptide (the Ii chain, DM and cathepsins B, H, and L, which are lysosomal proteases involved in generating MHC- binding peptides). Most importantly, IFN-γ increases also the expression of CIITA, the master regulator of MHC-II. These effects lead to increased activation of CD4+ T cells by APCs.	MHC-I pathway in most nucleated cells, and MHC-II in APCs.	(Gobin et al., 2003, 1999; Wright et al., 1995; LeibundGut-Landmann et al., 2004)
Antiviral activity. IFN-γ induces an antiviral state enhancing the	Virally infected	(Haller et al.,

production of antiviral enzymes including: the double stranded RNA-activated protein kinase (PKR), 2'-5'-oligoadenylate synthase (OAS), Ribonuclease L, double-stranded RNA-specific adenosine deaminase (ADAR) and the Mx GTPase family antiviral proteins.	mammalian cells	2007; Feng et al., 1992; Justesen et al., 2000; Patterson et al., 1995)
Regulation of leukocyte trafficking. IFN-γ upregulates leukocyte extravasation to places of inflammation through enhancing the expression of certain adhesion molecules such as ICAM1 and VCAM1. As part of its critical activity in regulating leukocyte-endothelium interactions, certain chemokines such as IP-10, MIP-1α and 1-β, RANTES (CCL5), and MCP-1 (CCL2) are strongly and consistently induced by IFN-γ.	Lymphocytes, macrophages, endothelial cells, fibroblasts, keratinocytes and others.	(Boehm et al., 1997)
Anti-tumour immunity, growth suppression and cell death. IFN-γ directly reduces cell growth and survival in a number of cells through mechanisms that involve repression of proteins involved in signalling, cell cycle progression, survival and angiogenesis and induction of cell cycle dependent kinase inhibitors (CDKIs) and pro-apoptotic proteins, e.g. caspases. IFN-γ can act as an anti-apoptotic agent in macrophages (mediated by p21), but it can also be pro-apoptotic through the induction of IRF1, which is involved in induction of apoptosis by signals such as genetic damage.	Macrophages and other cells.	(Mandal et al., 1998; Chawla-Sarkar et al., 2003; Street et al., 2002; Xaus et al., 1999)

Alternative activation of macrophages

Macrophage alternative activation is a complex process that renders highly heterogenic subpopulations of macrophages and a large diversity of Th2 phenotypes. Classification of the alternatively activated macrophages (M2) contemplates additional subtypes, M2a macrophages are induced by IL-4 or IL-13, M2b macrophages are induced by immune complexes, and the M2c phenotype is induced by IL-10 and glucocorticoids (Mantovani et al., 2004; Benoit et al., 2008).

The development of an M2a phenotype is induced by IL-4 and IL-13, cytokines that are produced in the context of Th2 type responses. These responses are prominent during allergic reactions, and also serve to direct cellular and humoral activities to parasitic and selected pathogen infections.

Several cell types produce IL-4 and IL-13. Basophils and mast cells are important early sources of innate IL-4 production (Brandt et al., 2000). These cells produce IL-4 in response to injury and to chitin, a structural biopolymer that is found in some fungi and parasites (Reese et al., 2007). IL-4 is also produced by conventional CD4+ Th2, CD8+ T cells (Paliard et al., 1988; Seder et al., 1992) and NKT cells (Yoshimoto et al., 1995), which indicates that alternative activation can be of both innate and acquired origin.

IL-4 signalling pathway

IL-4 is initially recognized by the IL-4 receptor α chain (IL-4Rα), a member of the hematopoietin receptor superfamily. Binding of IL-4 to IL-4Rα induces the heterodimerization of IL-4Rα with a second chain. The gamma common chain (γc) appears to be the preferred chain for heterodimerization. On the other hand, IL-4Rα chain can also function as a component of the IL-13 receptor (Nelms et al., 1999; Miloux et al., 1997; Murata et al., 1998).

IL-4R engagement activates the members of the JAK family of proteins JAK-1 and JAK-3, inducing the phosphorylation of specific tyrosine residues in the cytoplasmic domain of the receptor. STAT-6 then binds to the phosphorylated domain of the receptor and is phosphorylated at a C-terminal tyrosine residue by the activated kinases (Darnell, 1997; Velazquez et al., 1992). Phosphorylated STAT-6 leaves the receptor and forms homodimers with another STAT6 molecule through a C-terminal phospho-tyrosine residue. This complex translocates to the nucleus and binds to the promoter of IL-4 responsive genes like *MHC class II*, *Il4Rα*, *FcRε* (Delphin and Stavnezer, 1995; Kaplan et al., 1996; Reichel et al., 1997; Ryan et al., 1996; Shimoda et al., 1996) (Figure 6). IL-4 is also able to generate a negative feedback mechanism by inducing the regulatory proteins suppressors of cytokine signaling (SOCS) (Dickensheets et al., 2007).

IL-4 also mediates proliferative responses by activating another pathway of signal transduction mediated by insulin receptor substrate (IRS)-1/2. IRS-1/2 binds to one of the phosphorylated motifs of the IL-4Rα chain; this interaction induces IRS1/2 phosphorylation via the action of receptor associated kinases, like the JAK kinases. Phosphorylated IRS-1/2 interacts with downstream signalling molecules such as the

regulatory subunit of phosphoinositide-3-kinase (PI3K) and the adapter molecule Grb-2. These interactions induce activation of the PI3K and Ras-MAPK signalling pathway (Varin and Gordon, 2009).

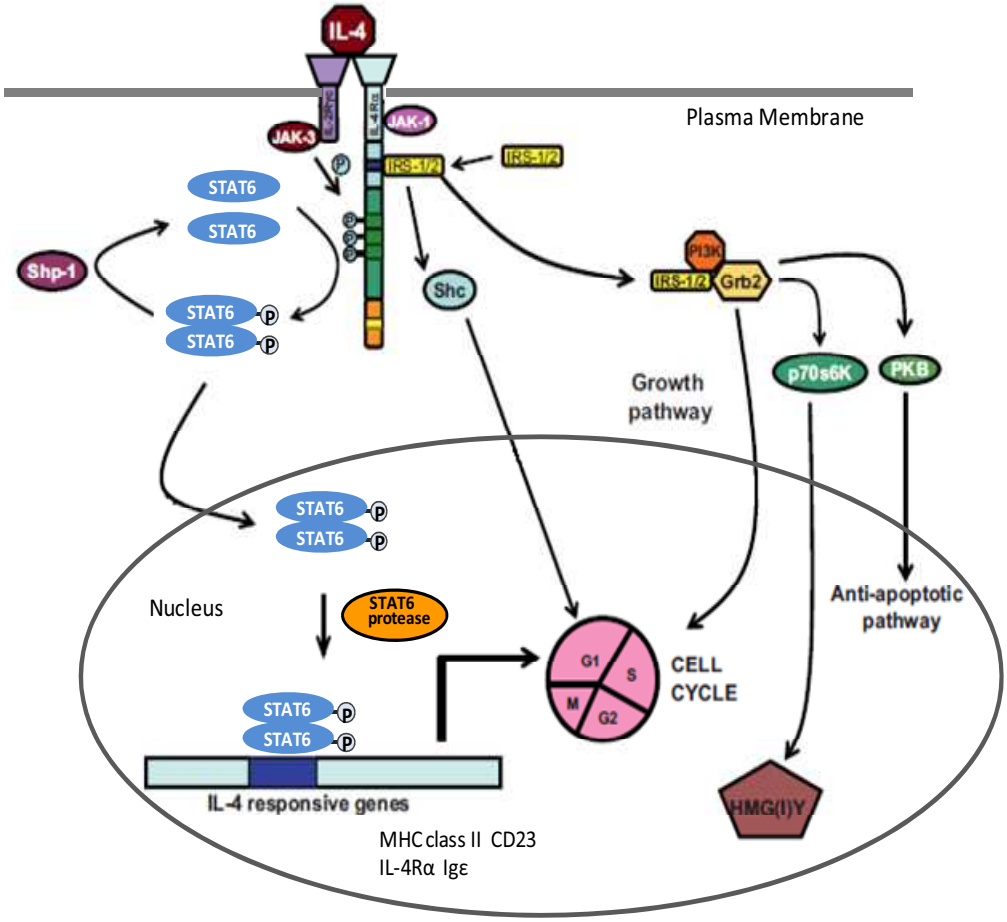

Figure 6. IL-4 receptor signalling. IL-4 mediates the heterodimerisation of the IL-4Rα with IL-2Rγ chain and activates members of the JAK kinase family inducing the phosphorylation of the cytoplasmic domain of the IL-4R. The phosphorylated tyrosines are the site of interaction with signalling molecules inducing different pathways like the Jak/STAT-6 pathway. STAT-6 binds to the phosphorylated domain of the IL-4R, is phosphorylated and leaves the receptor to form a homodimer with a second molecule of STAT-6. The complex translocates to the nucleus and binds to promoters of IL-4 responsive genes. Another pathway is mediated by IRS-1/2, inducing proliferative responses via the PI3K and Ras-MAPK signalling pathways (Figure adapted from Varin and Gordon 2009).

IL-4 biological roles in macrophages

IL-4 and IL-13 cytokines have similar effects in macrophages. Stimulation of macrophages with IL-4 rapidly converts them into a population of cells that produce

molecules that promote wound healing, and thus they are also called wound-healing macrophages or alternative activated macrophages (M2) (Mosser and Edwards, 2008). For example, IL-4 stimulates arginase activity in macrophages, allowing them to convert L-arginine to L-ornithine, a precursor of polyamines and collagen, thereby contributing to the production of extracellular matrix components. Human and murine alternative macrophages also secrete a number of fibrogenic factors such as fibronectin 1 (FN1) and matrix associated protein βIG-H3 (Gratchev et al., 2001; Song et al., 2000), and the coagulation factor XIII (F13A1) (Töröcsik et al., 2005), which bears transglutaminase activity promoting blood coagulation (Figure 7).

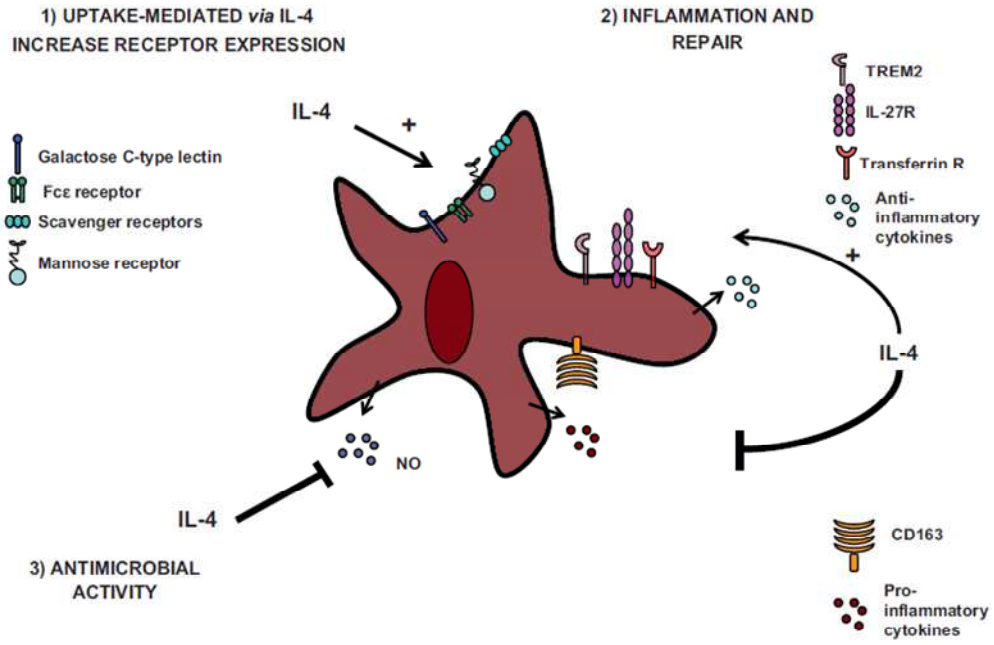

Figure 7. Effects of IL-4 on macrophage cell biology (Varin and Gordon, 2009)**.** Alternatively activated macrophages (M2) are characterized by modification of the receptor expression profile, which in turn affects endocytosis and phagocytosis **(1)**. Moreover, M2 macrophages have anti-inflammatory and repair properties associated with the stimulation of anti-inflammatory cytokines and inhibition of pro-inflammatory cytokines **(2)**. In addition to reducing inflammation, M2 macrophages down-regulate host protection against different pathogens via reduced NO production **(3)**.

In peritoneal macrophages, IL-4 was demonstrated to induce an increase in the expression of MHC-class II molecules, and inhibited the production of pro-inflammatory cytokines, like TNF-α, and of NO. In addition, IL-4 increased expression of

the mannose receptor, which favours endocytosis of mannosylated ligands (Stein et al., 1992). In general, macrophages treated with IL-4 and/or IL-13, produce minimal amounts of pro-inflammatory cytokines and are less efficient than classically activated macrophages at producing toxic oxygen and nitrogen radicals, and at killing intra cellular pathogens (Reviewed in J. P. Edwards et al. 2006; Martinez et al. 2009).

Alternatively activated macrophages play an important role in the protection of the host by decreasing inflammation and promoting tissue repair. In addition, there are several studies that have suggested that one of the goals of M2 macrophages is the development of Th2-dependent immunity to promote the elimination and control of infection by extracellular pathogens such as helminths and fungi (Reviewed in Martinez et al. 2009). However, alternative activation of macrophages can also diminish host protection against selected pathogens. For example, IL-4-induced M2 macrophages promote Leishmania infection by inhibiting Th1 and humoral responses of the host, and by suppressing macrophage killing activity via reduced NO production (Hölscher et al., 2006).

M2a macrophages are characterized by high levels of non-opsonic receptors, like mannose receptor (MRC1) and galactose C-type lectin receptor (Mgl1) (Raes et al., 2005) and express Th2 cell chemokines, including the CCR4 ligands, e.g. CCL2 (MCP-1), CCL4 (MIP-1), CCL5 (Rantes), CCL17 (TARC) and CCL22 (MDC). The M2b and M2c populations produce high levels of CCL1 and CCL18 respectively (reviewed in Martinez et al. 2009). A summary of some of the main alternatively activated macrophage markers induced by IL-4/IL-13 is shown in table 3.

Table 3. Principal markers of alternative macrophage activation.

MARKER	DESCRIPTION AND FUNCTIONS	REF.
Chemokines		
TARC (CCL17)	Thymus- and activation-regulated chemokine. Attracts T cells and macrophages. Expression controlled by IL-4/IFN-γ in an antagonistic manner by a combined STAT6/STAT1-binding element.	(Wirnsberger et al., 2006)
AMAC1 (CCL18)	MIP1α like, alternative macrophage activation-associated CC-chemokine (AMAC)-1. Attracts lymphocytes, immature DCs and monocytes. Induced by IL-4	(Kodelja et al., 1998)
MDC (CCL22)	Monocyte-derived chemokine. Attracts Th2 cells and other CCR4-expressing cells. Induced by IL-4. Downregulated by IFN-γ.	(Mantovani et al., 2002)
Cytokine receptors		
IL-27Rα	Inhibits pro-inflammatory cytokine production. Upregulated by IL-4.	(Rückerl et al., 2006)
Metabolic factors		
Arginase 1	Counteracts NOS2. Expression induced by IFN-γ/LPS. Can be induced by the STAT6 or STAT3 pathways.	(Munder et al., 1999)
12,15-lipoxygenase	Mediates peroxisome proliferator-activated receptor (PPAR)γ upregulation by IL-4. Expression inhibited by IFN-γ.	(Conrad et al., 1992)
Receptors		
Stabilin 1 (STAB1)	Endocytic receptor that may be involved in lysosomal sorting. Induced by IL-4.	(Goerdt and Orfanos, 1999)
Mannose receptor (*Mrc1*)	Endocytosis of specific ligand and secretion of soluble receptor increased. Selective downregulation by IFN-γ. Linked with M2 macrophages but widely expressed on many macrophages subsets.	(Stein et al., 1992; Doyle et al., 1994; Montaner et al., 1999; Martínez-Pomares and Gordon, 1999)
Mgl1 (CLEC10A)	Macrophage galactose-type C-type lectin receptor.	(Raes et al., 2005)
DCIR	C-type lectin containing an ITIM motif. Induced by IL-4	(Martinez et al., 2006)
Products		
Ym1	Chitinase-like protein that can bind to extracellular matrix. Expression controlled by IL-4/IFN-γ in an antagonistic manner. Strongly induced by IL-4.	(Raes et al., 2002)
FIZZ1 (RELMα)	Resistin-like secreted protein. Can promote deposition of extracellular matrix. Expression controlled by IL-4/ IFN-γ in an antagonistic manner. Strongly induced by IL-4. Not expressed in humans.	(Raes et al., 2002)
TGM2	Transglutaminase 2 (C polypeptide, protein-	(Martinez et al.,

	glutamine-gamma-glutamyltransferase). Upregulated by IL-4.	2013)
Collagenase/MMP1	Induced by TH 2-cell membrane factors and IL-4 in GM-CSF- differentiated human monocytes.	(Chizzolini et al., 2000)
IGF1	Macrophage-derived insulin like growth factor. Stimulates fibroblast proliferation and survival. Induced by IL-4.	(Wynes and Riches, 2003)

The role of M2 macrophages in allergy and asthma

Asthma is a complex airway inflammatory disease involving several cell types, including eosinophils and CD4+ Th2 cells; the latter have been shown to be an important source of IL-4, IL-13 and also IL-5, which are important drivers of responses to allergens. Recent studies using animal models and human monocytes have suggested a role for M2 macrophages in allergic lung inflammation and human asthma (Kurowska-Stolarska et al., 2009; Staples et al., 2012). However, the specific role of macrophages in these contexts remains controversial, even though they are the most prevalent immune cell type in the lungs.

E. Y. Kim et al. 2008 reported that M2 macrophages are required for the development of airway disease following infection with Sendai virus, which is a mouse para influenza virus. The authors found that M2 macrophages secrete IL-13 and that their depletion significantly attenuated Th2-driven inflammation in the lung. M2 macrophages induced during rhinovirus infection have also been shown to exacerbate eosinophilic airway inflammation by producing the chemokine CCL11 (also known as eotaxin 1), which recruits eosinophils (Nagarkar et al., 2010). Other chemokines produced by alternative macrophages have been related to allergic asthma, namely the Th2 chemokines CCL17 (TARC) and CCL22 (MDC) (Panina-Bordignon et al., 2001; Bisset and Schmid-Grendelmeier, 2005). However, the specific contribution of M2 macrophages and the proteins they express to airway inflammation are not fully understood, as the expression of many of these proteins is not exclusive to Th2 cytokine-stimulated macrophages.

Indeed, some studies have questioned the importance of macrophages in the development of allergic airway disease and instead support a role for another type of mononuclear phagocyte, CD11c+ DCs, in the development of eosinophilic inflammation and Th2-associated cytokine production in the lung (van Rijt et al., 2005). Additional reports have also identified a suppressive role for M2 macrophages in allergy and asthma. For example, M2 macrophages have been shown to inhibit asthma symptoms associated with chronic fungal infections by facilitating the uptake and removal of fungal conidia (Bhatia et al., 2011).

Macrophages and lipid homeostasis

In addition to their role as effector immune cells, macrophages also play important metabolic functions related to their exceptional phagocytic capacity and dynamic lipid metabolism. Macrophages are critical for the clearance of apoptotic cells and oxidized lipoproteins, and they exert a fundamental role in lipid homeostasis. In normal conditions, macrophages develop an important scavenger activity, internalizing low density lipoproteins (LDL) through LDL receptors. After their internalization, LDL particles are degraded in the lysosomal compartment, where hydrolytic enzymes generate cholesterol and fatty acids from cholesteryl esters. The cholesterol liberated from the modified lipoproteins by lysosomal hydrolase needs to be reesterified into cholesteryl esters for intracellular storage, otherwise the free cholesterol can be toxic to the cell. There is a dynamic balance between the amount of free cholesterol and cholesteryl esters within the cell, which is regulated by two enzymes located in the endoplasmic reticulum: Acyl-coenzyme A:cholesterol acyltransferase-1 (ACAT1) and neutral cholesterol ester hydrolases (nCEH) (Reviewed in A. C. Li and Palinski 2006). Free cholesterol and phospholipids are mobilized towards the plasmatic membrane through the action of ABC family of transporters (ATP-binding cassette), including ABCA1 and ABCG1. Then, those lipids are transferred to extra-cellular acceptor apolipoproteins, such as apolipoprotein A-I (apoA-I), that belong to high density lipoproteins (HDL). This process is known as cholesterol efflux and constitutes the first step in reverse cholesterol transport (RCT) from peripheral tissues to the liver

(Reviewed in Ikonen 2008) (See as well the section 'LXR and regulation of metabolic functions').

All the LDL lipid components, including cholesteryl esters, phospholipids, sterols and triglycerides can suffer oxidation and then generate oxidized-LDL particles (oxLDL). Macrophages can become very efficient in the internalization of these particles, especially in conditions that provoke the accumulation of oxLDL and other modified forms of LDL, such as acetylated LDLs, through the expression of scavenger receptors in their surface. These scavenger receptors, namely CD36, scavenger receptor (SR)-A and SR-B1, are molecules that have evolved as molecular pattern receptors that mediate the phagocytosis of pathogens and apoptotic cells (reviewed in Hazen 2008). Accumulation of LDL derivates in the macrophages inhibits the expression of the classic LDL-receptor, but not the expression of scavenger receptors (Brown and Goldstein, 1986), which allows macrophages to keep accumulating high quantities of lipids and become foam cells (lipid laden macrophages) (Li and Glass, 2002; von Eckardstein et al., 2001). Foam cells are present and participate in all the stages of the formation and progression of atherosclerosis, a cardiovascular disease with an important inflammatory component. In atherosclerosis, it is thought that macrophages lodge in the intima and subintima of arteries, are loaded of cholesterol and eventually lead to the formation of obstructive atherosclerotic plaques that are prone to rupture, leading to thrombosis with consequences such as myocardial infarction or stroke (reviewed in Woollard and Geissmann 2010).

III. Nuclear Receptors

Nuclear receptors (NR) represent a large family of intracellular receptors operating as transcription factors in a ligand-dependent manner. NRs bind to specific response elements on target genes as homo- or hetero-dimers and regulate gene expression by interacting with various transcription co-regulators including co-activators and co-repressors (Reviewed in Hsia et al. 2010). Because of their ability to regulate gene expression in a ligand-dependent manner, the NRs can serve as direct communicators

between the extracellular environment and the genome. They modulate essential processes such as development, cell differentiation, metabolism, and proliferation through the positive and negative regulation of their target genes at the transcriptional level. Typically, NR ligands are biologically active molecules such as steroids, thyroid hormone, vitamin D, retinols and products of lipid metabolism (Reviewed in Aranda and A. Pascual 2001); however, the NR family also includes orphan NRs whose ligands are not yet identified (Kliewer et al., 1999).

In addition to their role in development and homeostasis, many NRs also function as modulators of innate and adaptive immune responses. One NR that has been thoroughly studied is the glucocorticoid receptor (GR). GR mediates anti-inflammatory and immune-suppressive effects in addition to regulating genes involved in development and metabolism. Other nuclear receptors, including retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator-activated receptor (PPAR) and the liver X receptor (LXR), whose ligands are vitamin A (retinol), vitamin D, fatty acid and cholesterol metabolites respectively, are also important in the regulation of inflammatory events, (Nagy et al., 2012; Glass and Saijo, 2010; Austenaa et al., 2009), and the effects of LXR in particular will be discussed in detail in this work.

The mechanisms by which some nuclear receptors negatively regulate the expression of inflammatory genes are collectively known as transrepression, and they imply the interaction of nuclear receptors with other molecules that are bound to the promoter of transrepressed genes rather than a direct interaction with response elements in the DNA (Glass and Ogawa, 2006).

In general, the NR superfamily share a common structure that consists of a relatively less conserved N-terminal activation domain (AF1), a highly conserved central DNA-binding domain (DBD), a hinge region and a conserved C-terminal ligand binding domain (LBD) that contains a second and very strong activation domain (AF2) (Mangelsdorf et al., 1995; Aranda and Pascual, 2001). The LBD confers the ligand specificity of each receptor, and also contributes to the homo- or heterodimerization of the receptor. The AF-2 is responsible for the interaction between nuclear receptors and co-regulatory molecules (Figure 8).

Figure 8. Schematic representation of the nuclear receptor structure. The variable N-terminal region contains the ligand-independent AF-1 activation domain and the conserved DNA-binding domain (DBD), responsible for the recognition of specific DNA sequences. A variable hinge region connects the DBD to the conserved ligand-binding domain (LBD) as well as the dimerization surface. The ligand-dependent AF-2 core activation domain is within the C-terminal portion of the LBD. (Adapted from Aranda and Pascual 2001)

Liver X Receptor

The liver X receptors (LXRs) are members of the nuclear receptor superfamily and function as important regulators of genes involved in cholesterol, fatty acid and glucose metabolism, as well as in inflammation and cell proliferation (Reviewed in Pascual-García and Annabel F Valledor 2012). The receptors were first considered as orphan receptors, but it has now been established that various endogenous cholesterol metabolites and oxidized forms of cholesterol (oxysterols), as well as the plant sterol β-sitosterol function as natural ligands of the LXRs (Janowski et al., 1996; Lehmann et al., 1997). In addition, potent synthetic agonists such as T0901317 (T1317) and GW3965 have been developed (Schultz et al., 2000; Collins et al., 2002) and are widely used in the study of the biological functions of LXR.

Two isoforms of LXR have been identified, LXRα (NR1H3) and LXRβ (NR1H2), codified by different genes with more than 75% of homology in their DBD and LBD sequences. LXRα is predominantly expressed in macrophages and in certain specific tissues including liver, adipose tissue, intestine, lung, kidney and spleen, while LXRβ has a ubiquitous pattern of expression (Svensson et al., 2004; Repa and Mangelsdorf, 2000). Interestingly, LXRα expression in macrophages is induced by its own ligands, as well as by ligands of PPARγ (Laffitte et al., 2001; Li et al., 2002; Whitney et al., 2001) and the naturally occurring polyphenol resveratrol (Sevov et al., 2006).

In addition, several studies have demonstrated diverse post-transcriptional modifications in LXRs, including acetylation, ubiquitination (Li et al., 2007; Kim et al., 2009), phosphorylation (Chen et al., 2006; Yamamoto et al., 2007; Torra et al., 2008), O-Glc-NAcylation (Anthonisen et al., 2010) and sumoylation (Ghisletti et al., 2007; Venteclef et al., 2010).

LXRs form permissive heterodimers with RXR that can be activated by ligands of either partner to induce the transcription of their target genes. The LXR/RXR heterodimer binds to LXR-response elements (LXRE) in the promoter region of target genes; generally these LXREs are directly repetitions separated by four nucleotides that have a *consensus* sequence: (G/T/A)G(G/T)T(C/T)Annnn(C/A/T)G(G/T)(T/G)CA (Edwards et al., 2002). In the absence of ligand, the heterodimer is believed to form a complex with co-repressors such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Chen and Evans, 1995) and nuclear receptor co-repressor (N-CoR) (Hörlein et al., 1995), to keep the transcriptional activity of their target genes repressed. Binding of ligand to LXR results in a conformational change that facilitates dissociation of associated co-repressors and recruitment of co-activators in order to allow gene transcription to occur (Reviewed in C K Glass and M G Rosenfeld 2000; B. L. Wagner et al. 2003) (Figure 9).

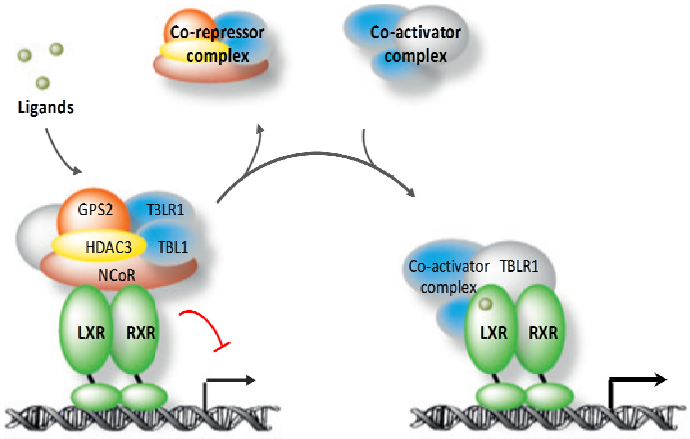

Figure 9. Molecular mechanism of LXR activation of target gene transcription. LXRs bind to a specific DNA sequence, called the LXR response element (LXRE). LXRs form a transcriptional unit as permissive heterodimers with retinoid X receptors (RXRs), whereupon ligand activation leads to dissociation of the co-repressor complex and recruitment of the co-activator complex. Figure adapted from Jakobsson et al. 2012.

LXRs and regulation of metabolic functions

LXRs play a crucial role in the regulation of lipid metabolism, through the control of several genes that mediate cholesterol homeostasis in different tissues (Reviewed in A-González and Castrillo 2011). Activation of LXRs induce the expression of various sterol transporters from the ATP-binding cassette (ABC) family with important functions in reverse cholesterol transport, e.g. ABCA1 and ABCG1 (Repa et al., 2000b; Venkateswaran et al., 2000), sterol excretion into bile and feces, and limiting sterol absorption in the intestine, e.g. ABCG5 and ABCG8 (Baldán et al., 2009; Lee et al., 2001; Lu et al., 2001; Repa et al., 2002) (Table 4). In addition to promoting reverse cholesterol transport, LXRs also reduce cellular uptake of cholesterol by inducing the expression of inducible degrader of LDL receptor (Idol, previously named Mylip), an E3 ubiquitin ligase that targets several members of the LDL receptor family for degradation (Zelcer et al., 2009; Hong et al., 2010). Therefore, LXRs are considered "sterol sensors" that coordinately orchestrate the expression of key molecules involved in cholesterol storage, efflux and elimination (table 4).

Table 4. LXR regulated genes implicated in metabolism and inflammation.

GEN	FUNCTION	RESPONSE TO LXR ACTIVATION*
Metabolism		
Abca1	Cholesterol and phospholipid transporter. Mediates cholesterol efflux.	Up-regulated
Abcg1	Cholesterol transporter. Mediates cholesterol efflux and redistribution.	Up-regulated
Abcg8	Limits intestinal absorption and promotes biliary excretion of sterols	Up-regulated
Srebp1c (Sterol regulatory element binding protein 1c)	Transcription factor. Central regulator of genes required for fatty acid and triglyceride synthesis.	Up-regulated
Fasn (Fatty acid synthase)	Enzyme involved in lipogenesis.	Up-regulated
Acetyl-CoA carboxylase	Enzyme involved in lipogenesis.	Up-regulated
Stearoyl-CoA desaturase 1	Enzyme involved in lipogenesis.	Up-regulated
Chrebp (carbohydrate-responsive element-	Transcription factor. Responsible for transmitting signals generated in response to extracellular glucose to the L-type pyruvate kinase (L-PK	Up-regulated

binding protein)	(PKLR)) promoter in hepatocytes and orchestrating the whole lipogenic programme in concert with SREBP1c and LXR	
Apoe (Apolipoprotein -E)	Cholesterol acceptor in high density lipoproteins (HDL); critical ligand for lipoprotein uptake by the LDL receptor.	Up-regulated
Apo C-I/C-IV/C-II gene cluster	Regulation of lipid transport.	Up-regulated
Glucokinase	Enzyme involved in glucose metabolism. Facilitates phosphorylation of glucose to glucose-6-phosphate	Up-regulated
Glut4	Insulin dependent glucose transporter	Up-regulated
Idol	E3 ubiquitin ligase, inducible degrader of LDL-receptors	Up-regulated
Phosphoenolpyruvate carboxykinase	Enzyme involved in gluconeogenesis	Down-regulated
Glucose-6-phosphatase	Enzyme involved in gluconeogenesis	Down-regulated
Inflammation		
Nos2	Inducible nitric oxide synthase. Catalyzes the production of NO from L-arginine	Transrepressed
Ptgs (COX-2)	Induces the formation of proinflammatory mediators including prostaglandins, prostacyclin and thromboxane	Transrepressed
Il6	Pro-inflammatory cytokine	Transrepressed
Il1b	Pro-inflammatory cytokine	Transrepressed
Mcp1 (CCL2)	Inflammatory chemokine	Transrepressed
Mip1b (CCL4)	Inflammatory chemokine	Transrepressed
Cxcl10 (IP10)	Inflammatory chemokine	Transrepressed

(*Reviewed in A-González and Castrillo 2011; Pascual-García and Annabel F Valledor 2012; Zelcer and Peter Tontonoz 2006)

LXRs also regulate lipogenesis, through the induction of sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP), two central regulators of fatty acid synthesis (Repa et al., 2000a; DeBose-Boyd et al., 2001; Schultz et al., 2000; Cha and Repa, 2007), and several other enzymes such as fatty acid synthase (FAS) (Joseph et al., 2002a), acyl-CoA carboxylase (Talukdar and Hillgartner, 2006), and stearoyl-CoA desaturase 1 (Wang et al., 2004).

All these processes outlined above in which LXR activity takes part, regulating the expression of genes involved in reverse cholesterol transport, bile acid metabolism,

and intestinal cholesterol absorption, are considered anti-atherogenic. In fact, LXR agonists have indeed shown therapeutic effectiveness in murine experimental models of atherosclerosis (Joseph et al., 2002b; Terasaka et al., 2003). However, LXRs also promote fatty acid and triglyceride synthesis, which are considered independent risk factors for cardiovascular disease. In this sense, mice treated with LXR agonists suffer a marked increase in plasma triglyceride levels (Schultz et al., 2000), limiting until the moment their potential therapeutic advantages as anti-atherogenic drugs in humans.

LXRs also participate in the regulation of glucose metabolism. In the liver, LXR ligands repress the expression of genes implicated in gluconeogenesis, including the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), the phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase. Ligands also induce the expression of glucokinase in the liver, promoting glucose catabolism in this organ, and the insulin-sensitive glucose transporter 4 (GLUT4) in adipose tissue, that mediates glucose uptake (Laffitte et al., 2003).

LXRs as inflammatory response regulators

In addition to their role in the regulation of certain metabolic functions, LXRs also have an important role in the control of the immune response. LXRs agonists repress the expression of several inflammatory genes through a process known as transrepression, which is also performed by other nuclear receptors to interfere with inflammatory signalling pathways. In the last few years, several studies have demonstrated the anti-inflammatory actions or immune regulatory roles of LXR agonists in different cell types and models of inflammatory disease (reviewed by Pascual-García and Annabel F Valledor 2012).

In macrophages, LXR agonists are able to transrepress subsets of inflammatory genes activated by LPS/TLR4 engagement, IL-1β, TNF-α, polyinosinic:polycytidylic acid (poly I:C/TLR3 engagement) or IFN-γ (Ghisletti et al., 2007; Joseph et al., 2003; Pascual-García et al., 2013). The effects of LXR agonists seem to differ depending on the cell type and activating stimuli, and the exact mechanisms underlying transrepression are not fully understood (Figure 10 and table 4).

Besides to their effects inhibiting the expression of proinflammatory genes in cells of the immune system, LXRs also inhibit the expression of acute phase proteins in the liver (Blaschke et al., 2006) and LXRβ was identified as the major mediator of attenuated hepatic acute-phase response (APR) (Venteclef et al., 2010).

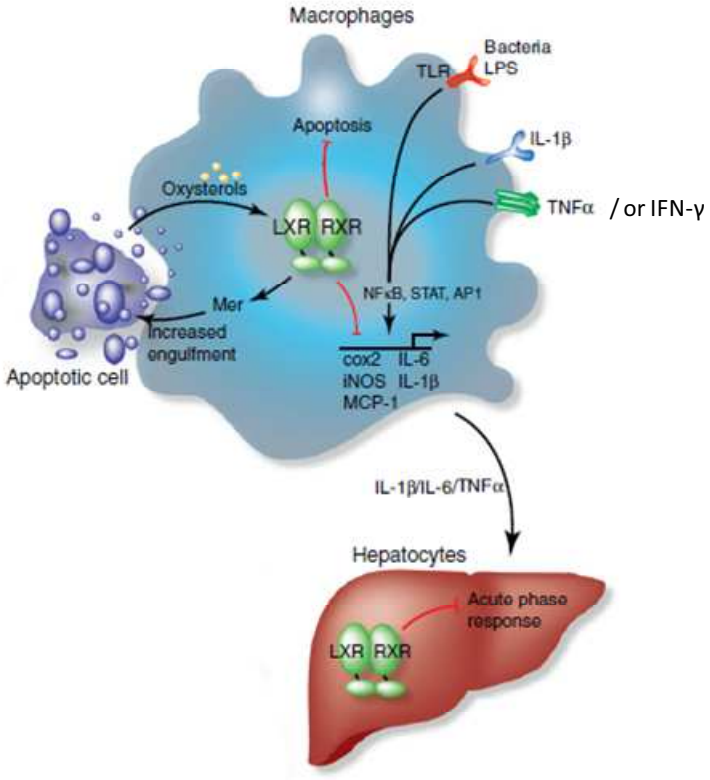

Figure 10. Biological roles of LXRs in the immune system. LXRs exert transrepression of pro-inflammatory signalling in immune cells and hepatocytes. In macrophages specifically, LXRs transrepress pro-inflammatory genes activated by LPS, IL-1β, TNF-α and IFN-γ. In addition to it, the positive role of LXRs in stimulating macrophage-mediated phagocytosis increases clearance of apoptotic cells. Figure adapted from Jakobsson et al. 2012.

In normal conditions and in the absence of pro-inflammatory stimuli, inflammatory genes are repressed by a co-repressor complex that interacts on their promoters (Ogawa et al., 2004). After pro-inflammatory stimulation, the induction of transcription requires an initial step involving clearance of this co-repressor complex from specific gene promoters (Ghisletti et al., 2009). In fact, some of the components of the co-repressor complex are subjected to ubiquitylation and proteasome-dependent degradation (Hoberg et al., 2004). Interference with the release of co-repressor proteins, including NCoR and SMRT, from the inflammatory gene promoters has been

reported as a possible mechanism by which several nuclear receptors, among them LXRs, inhibit the expression of LPS-induced genes in macrophages (Ghisletti et al., 2007) (Figure 11).

Specific post-translational modifications of LXRs have been implicated in the mechanism of transrepression. Studies in macrophages (Ghisletti et al., 2007) and hepatocytes (Venteclef et al., 2010) have indicated that LXR agonists can trigger post-translational modification by members of the small ubiquitin-like modifier (SUMO) family, thereby allowing LXRs to exert transrepression. In mammals, there are three members of the SUMO family: SUMO-1, -2 and -3, that can interact with target proteins. SUMO is activated in an ATP-dependent manner by the heterodimeric E1 SUMO-activating enzyme SAE1/2. Activated SUMO is then transferred to an E2-conjugating enzyme, e.g. UBC9, and is conjugated to specific lysine residues in substrate proteins in cooperation with SUMO E3 ligases. The best characterized E3 ligases are the members of the PIAS family (Palvimo, 2007), although the histone deacetylases HDAC4/5 and HDAC2 have also been implicated in SUMO conjugation and may function as E3 ligases (reviewed in Treuter and Venteclef 2011).

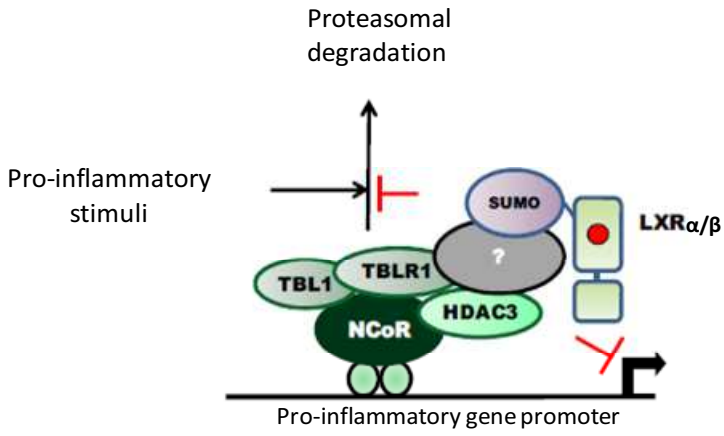

Figure 11. Mechanism of LXR mediated transrepression of pro-inflammatory genes. Pro-inflammatory genes are normally transrepressed in non stimulated cells. The repression of these genes is mediated by co-repressor complexes, typically containing NCoR or SMRT, transducin (beta)-like 1 X-linked (TBL1), TBL1-related protein (TBLR1) and histone deacetylase (HDAC) 3. The transcriptional induction of a gene induced by a pro-inflammatory signal requires an initial step of liberation of the co-repressor complex. In certain inflammatory genes, SUMOylation of LXR prevents co-repressor complex removal from the promoter, inhibiting transactivation of that gene by the pro-inflammatory signal. The molecule that interacts with SUMOylated-LXR seems to be different depending on the cell type (GPS2 in liver, CORO2A in macrophages). Figure adapted from Pascual-García and Annabel F Valledor 2012.

The SUMOylation process for LXR is not completely understood; the proteins involved seem to depend on the cell type and the inflammatory stimuli. In LPS-stimulated macrophages, GW3965-induced LXRβ activation leads to the SUMOylation of LXRβ by SUMO2/3 in a process that requires HDAC4 as an E3 ligase (Ghisletti et al., 2007). In IFN-γ-stimulated astrocytes, the mechanism seems to differ between ligand activated-LXRα and –LXRβ. LXRα is SUMOylated by SUMO2/3 and HDAC4 acting as the E3 ligase, whilst LXRβ is SUMOylated by SUMO-1 and PIAS1 is required as an E3 ligase (Lee et al., 2009). SUMOylated-LXRs form a complex with STAT-1 in astrocytes, which results in inhibition of the recruitment of STAT-1 to the IRF1 promoter. In this study, STAT-1 phosphorylation or its translocation to the nucleus was not affected (Lee et al., 2009). However, another group that worked with the human monocyte cell line THP-1 observed a reduction of STAT-1 phosphorylation on serine and tyrosine residues induced by IFN-γ in response to the LXR ligand 22-(R)-hydroxycholesterol (Li et al., 2011).

SUMOylated LXRs associate to the NCoR–SMRT corepressor complex at the promoters of inflammatory response genes, presumably interacting with the complex subunits G-protein pathway suppressor 2 (GPS2) and/or coronin 2A (CORO2A), depending on the cell type, thereby preventing corepressor complex removal and gene activation. The mechanism operating on the promoter of the C-reactive protein gene in the liver seems to selectively involve the interaction of SUMOylated-LXRβ with GPS2 (Venteclef et al., 2010, 2011), whilst in macrophages the mechanism seems to require the interaction of SUMOylated LXRs with the complex subunit CORO2A (Huang et al., 2011). CORO2A is an actin binding protein that mediates TLR-induced NCoR dissociation from the Nos2 promoter by a mechanism involving interaction with oligomeric nuclear actin (Huang et al., 2011). Huang et al. propose that CORO2A binds to SUMOylated LXR trough a SUMO2/SUMO3-interaction motif blocking the recruitment of actin that is necessary for NCoR removal from inflammatory gene promoters.

In contrast to the well documented requirement for heterodimerization of LXR with RXR, for transcriptional activation of their target genes, a question that still remains

unclear is whether LXR needs to form heterodimers with RXR to transrepress inflammatory genes.

LXR as positive regulators of specific genes of the Immune response

In addition to its anti-inflammatory effects, LXR agonists have shown to positively regulate the expression of some genes important for the immune response. For example, LXR deficient mice were more susceptible to infection by *Mycobacterium tuberculosis* in correlation with the fact that these mice were not capable of mounting an effective early neutrophilic airway response and Th1 and Th17 cell responses were abrogated in the lungs of these animals (Korf et al., 2009). This study showed that LXRs are involved in the host defense in airway infections. In contrast, LXR-deficient mice have been shown to be markedly resistant to systemic infection with *Leishmania chagasi/infantum*. In this context, primary macrophages derived from mice lacking LXRs generated higher levels of NO and IL-1β and were able to kill parasites in the presence of IFN-γ more efficiently than wild-type macrophages (Bruhn et al., 2010). Altogether these studies suggest that the LXR pathway can affect differently the immune response against pathogen infections, and its effects are not just restricted to transrepression of pro-inflammatory genes.

It has also been demonstrated that LXRs protect macrophages from committing apoptosis upon infection by various pathogens, thereby helping to maintain a strong macrophage response in these situations. LXR activation was shown to increase macrophage survival during different bacterial infections, including *Listeria monocytogenes* (Joseph et al., 2004), *Bacillus anthracis*, *Escherichia coli* and *Salmonella typhimurium*, and in response to several forms of cellular stress, such as growth factor deprivation (Valledor et al., 2004). These effects were related with downregulation of the expression of several members of the caspase family and other pro-apoptotic factors, and with induction of the apoptosis inhibitory protein (AIM) secreted by macrophages, which is a specific target of LXRα (Joseph et al., 2004; Valledor et al., 2004). AIM has also been suggested to play a role on preventing macrophage

apoptosis within atherosclerotic lesions, contributing to the formation of plaques (Arai et al., 2005). However, the exact mechanism by which AIM mediates its anti-apoptotic effects remains unclear.

Recently, macrophage-secreted AIM has been shown to be endocytosed by adipocytes via the scavenger receptor CD36. Within adipocytes, AIM bound to and inhibited the activity of cytosolic fatty acid synthase, therefore stimulating the efflux of free fatty acids and glycerol from adipocytes (Kurokawa et al., 2010). Palmitic and stearic acids released upon AIM-dependent adipocyte lipolysis were able to engage TLR4 signalling and stimulate the production of chemokines promoting monocyte/macrophage recruitment to adipose tissue (Kurokawa et al., 2010). The pro-inflammatory actions of AIM contrast with the general role of LXRs as negative regulators of inflammation. It would be interesting to know whether the lipolysis-mediated proinflammatory activities of AIM are also observed under conditions of simultaneous stimulation of LXR-mediated transrepression.

Liver X receptor agonists regulate positively the expression of the anti-inflammatory enzyme arginase II (Marathe et al., 2006). The consumption of L-arginine by arginase activity for polyamine production results in decreased availability of this amino acid for nitric oxide synthesis mediated by NOS2. Based on these effects, LXR agonists have the potential to interfere with nitric oxide production both by inhibiting *Nos2* expression and by upregulating arginase II expression and therefore indirectly affecting NOS activity. Moreover, LXR agonists have been recently shown to exert indirect positive regulation of arginase I expression via upregulation of IRF-8 (Pourcet et al., 2011). The model proposed by Pourcet et al. described the association of IRF-8 with the transcription factor PU.1 on a composite element in the promoter region of the arginase I gene. Occupancy of this element by IRF-8/PU.1 was increased by the LXR agonist TO901317, consistent with the induction of IRF-8 expression in response to LXR activation (Pourcet et al., 2011).

Recent studies have highlighted a role for the LXR pathway in the positive regulation of genes that mediate phagocytosis of apoptotic cells. Several years ago, ABCA1, one of the first target genes identified for LXRs, was reported to participate in the regulation

of phagocytosis, favouring engulfment by inducing local modifications of the membrane composition in phospholipids (Hamon et al., 2000). Recently, c-mer tyrosine kinase (Mertk) has been also shown to be a direct target for LXR (A-Gonzalez et al., 2009). Mertk is a member of the Axl/Mer/Tyro3 receptor tyrosine kinase family, that functions as a receptor for Gas6, a protein that binds to phosphatidylserine exposed as an ‘‘eat-me’’ signal in the external side of the plasma membrane of dying cells (Scott et al., 2001). As a consequence, Mertk collaborates in macrophage-mediated engulfment and clearance of apoptotic cells. On the other hand, phagocytosis of apoptotic cells results in LXR activation (A-Gonzalez et al., 2009). Therefore, apoptotic cells are able to promote their own clearance by providing a mechanism to activate LXR and induce Mertk and ABCA1 in phagocytic cells. Interestingly, functional expression of LXRs is required for inhibition of the expression of several proinflammatory mediators and for transcriptional induction of the deactivating cytokines TGF-β and IL-10 in macrophages that had engulfed apoptotic cells. These observations suggest that the LXR pathway serves an important role also in macrophage deactivation in response to phagocytosis of apoptotic cells (A-Gonzalez et al., 2009).

Objectives

1. To study LXR-mediated transrepression effects on proinflammatory genes in primary macrophages and foam cells.

2. To explore the role of the LXR target gene Idol in transrepression of proinflammatory genes and in promoting the clearance of apoptotic cells.

3. To evaluate the effect of LXR agonists on macrophage alternative activation and on a model of allergic asthma. To study reciprocal effects of macrophage alternative activation signals on the expression of LXR target genes.

Materials and Methods

Reagents

Recombinant murine IFN-γ was purchased from Pierce Biotechnology (Rockford, IL). TO901317 (T1317) was purchased from Cayman Europe (Tallinn, Estonia). GW3965 was obtained from GlaxoSmithKline (Research Triangle Park, North Carolina). Recombinant murine IL-4 and IL-10 were purchased from PeproTech (Rocky Hill, NJ). Recombinant human TGF-β was obtained from eBioscience (San Diego, CA). LPS was purchased from Sigma-Aldrich (St. Louis, MO) and acetylated low density lipoproteins (acLDL) were purchased from Kalen Biomedical (Montgomery Village, MD).

Animals and cell cultures

Bone marrow-derived macrophages (BMDM) were obtained from six to ten-week-old C57BL/6 mice (Harlan, Indianapolis, IN) as described (Valledor et al., 2000a). Bone marrow precursors were cultured in DMEM (PAA Laboratories, Velizy-Villacoublay, France), supplemented with 20% heat inactivated fetal calf serum (FCS) (PAA Laboratories) and 30% L-cell conditioned medium as a source of M-CSF. L-cell conditioned medium was obtained from L929 cells cultured in DMEM supplemented with 10% FCS.

Thioglycollate-elicited macrophages were isolated by peritoneal lavage 3 days following peritoneal injection of 2.5 ml 3% thioglycollate (DIFCO). Cells were washed and plated in RPMI medium 1640 (PAA Laboratories) and 10% FCS; after 5 hr the medium was replaced by fresh medium containing 0.5% FCS.

Raw264.7 macrophages (American Type Culture Collection) were cultured in DMEM supplemented with 10% FCS.

LXR-deficient mice (Repa et al., 2000b; Peet et al., 1998) were donated by Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). STAT6-deficient mice (Charles River Laboratories; Wilmington, MA, USA), were provided by Dr. Antonio Celada (Universitat de Barcelona, Spain) and LDLR-deficient mice (Jackson Laboratories; Bar Harbor, Maine, USA) were provided by Dr. Blanco-Vaca (Hospital de la Santa Creu i Sant Pau, Barcelona, Spain). RXRαβ-deficient mice were provided by Dr. Mercedes Ricote (Centro Nacional de Investigaciones Cardiovasculares, CNIC; Madrid, Spain).

Briefly, RXRα and β-floxed (RXRαβ DKO) mice containing *loxP* sites flanking exon regions in both RXRα and β, were crossed with mice harboring a Cre recombinase under control of the lysozyme M promoter (Lys-cre mice). Peritoneal macrophages obtained from this model proved to be RXRαβ-deficient.

Foam cell formation

Foam cells were generated by incubating BMDM with acLDL (10 μg/ml) for 24 h and the same medium with acLDL was maintained during the time of subsequent experiments. Confirmation of foam cell formation was performed by light microscopy after staining the cells with Oil-Red-O. Briefly, cells were washed with PBS and fixed with 10% formalin solution (Sigma-Aldrich). After three rinses with H_2O-mQ, cells were incubated with Oil-Red-O (Sigma-Aldrich) working solution in 36% TEP during 2h. The plates were washed extensively with H_2O-mQ and cells were analyzed by light microscopy.

RNA extraction and cDNA synthesis

Cells were washed twice in cold PBS and total RNA was extracted using Trizol (Invitrogen, San Diego, CA) as recommended by the manufacturer. Briefly, Trizol was added to lyse the cells and the lysate was passed to a 1.5 ml tube. Chloroform was used to phase separation; samples were incubated 15 min at room temperature and centrifuged at 12.000x *g* for 15min. RNA was isolated from the upper aqueous phase by adding 2-propanol (Sigma-Aldrich) and centrifuged at 12.000x *g* for 10min. RNA pellet was washed with 75% ethanol, centrifuged and final pellet was dissolve in H_2O-mQ. For cDNA synthesis, 1 μg RNA was subjected to reverse transcription using M-MLV Reverse transcriptase RNase H Minus, Point Mutant, oligo$(dT)_{15}$ primer and PCR nucleotide mix (Promega; Madison, WI).

Quantitative real-time PCR analysis.

Quantitative real time (q-RT-PCR) was performed using the Power SYBR Green Reagent Kit (Applied Biosystems, Foster City, CA) following the manufacturer's

recommendations. The sequence of primers used for qRT-PCR analysis are shown in Table 5. Real-time monitoring of PCR amplification was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems) with the following settings: 95°C (10 min) – [95°C (30s) - 60°C (30s) - 72°C (30s)] x 35 cycles. Data were expressed as relative mRNA levels normalized to ribosomal L14 expression.

Table 5. Primers used for q-RT-PCR analysis

Gene	Forward Primer	Reverse Primer
Abca1	5'-GCGAGGGCTCATCGACAT-3'	5'-GAAGCGGTTCTCCCCAAAC-3'
Abcg1	5'-TCACCCAGTTCTGCATCCTCTT-3'	5'-GCAGATGTGTCAGGACCGAGT-3'
Arg1	5'-TTGCCAGACGTAGACCCTGG -3'	5'-CAAAGCTCAGGTGAATCGGC-3'
Aim	5'-GTTGGATCGTGTTTTTCAGA-3'	5'-TCCCACTAGCTGCACTTTGGT-3'
Apoc2	5'-ACCTGTACCAGAAGACATACCC-3'	5'-GTAAAAATGCCTGCGTAAGTGC-3'
Apoe	5'-CTGACAGGATGCCTAGCCG-3'	5'-CGCAGGTAATCCCAGAAGC-3'
Ccl2	5'-AGGTCCCTGTCATGCTTCTG-3'	5'-GCTGCTGGTGATCCTCTTGT-3'
Ccl5	5'-CTGCTGCTTTGCCTACCTCT-3'	5'-TCCTTCGAGTGACAAACACG-3'
Ccl17	5'- TGCTTCTGGGACTTTTCTG-3'	5'-CATCCCTGGAACACTCCACT-3'
Ccl22	5'-CCTTCTTGCTGTGGCAATTCAG-3'	5'-CTCGGTTCTTGACGGTTATCA-3'
Ccr8	5'-AAGAAAGGCTCGCTCAGATAATTG-3'	5'-TCGTGTAATCCATCGAGGCAG-3'
Cxcl10	5'-TCCCTGCGAGCCTATCCTG-3'	5'-TTTTCATCGTGGCAATGATCTC-3'
Cxcl11	5'-AATTTACCCGAGTAACGGCTG-3'	5'-ATTATGAGGCGAGCTTGCTTG-3'
Idol	5'-ATGCTGTGCTATGTGACGAGG-3'	5'-TCGATGATCCCTAGACGCCTG-3'
Il1b	5'-TGGGCCTCAAAGGAAAGAAT-3'	5'-CAGGCTTGTGCTCTGCTTGT-3'
IL4	5'-CACGGATGCGACAAAAATCA -3'	5'- CTCGTTCAAAATGCCGATGA-3'
IL6	5'-CCACAGATACAAAGAAATGATG-3'	5'-ACTCCAGAAGACCAGAGGAAT-3'
Il12b	5'-GGAAGCACGGCAGCAGAATA-3'	5'-AACTTGAGGGAGAAGTAGGAATGG-3'
Itgal	5'-CCAGACTTTTGCTACTGGGAC-3'	5'-GCTTGTTCGGCAGTGATAGAG-3'
L14	5'-TCCCAGGCTGTTAACGCGGT-3'	5'-GCGCTGGCTGAATGCTCTG-3'
Lxra	5'-CCTTCCTCAAGGACTTCAGTTACAA-3'	5'-CATGGCTCTGGAGAACTCAAAGAT-3'
Lxrb	5'-CATTGCGACTCCAGGACAAGA-3'	5'-CCCAGATCTCGGACAGCAAG-3'
Mcpip	5'-AGACCTGTGGTCATCGACG -3'	5'-TAGTTCCCGAAGGATGTGCTG-3'
Mgl1	5'-TGAGAAAGGCTTTAAGAACTGGG-3'	5'-GACCACCTGTAGTGATGTGGG-3'
Mrc1	5'-GGTTCACCTGGAGTGATGGTTC-3'	5'-GTGGATTGTCTTGTGGAGCAGG-3'
Mip1b	5'-TTCCTGCTGTTTCTCTTACACCT-3'	5'-CTGTCTGCCTCTTTTGGTCAG-3'
Nos2	5'-GCCACCAACAATGGCAACA-3'	5'-CGTACCGGATGAGCTGTGAATT-3'
Pdpn	5'-GAGGAACTGTCCACCTCAGC-3'	5'-ATGGCTAACAAGACGCCAAC-3'
Ptgs2	5'-ATTCTTTGCCCAGCACTTCA-3'	5'-GGGATACACCTCTCCACCAA-3'
Rps18a	5'-GCTCGCGGGTTGAGAGAAG-3'	5'-ACTGAGCAACAACACATCCTC-3'
Serpinb2	5'- CCATAGTTCTCCTCGGTGCT -3'	5'- TCTCCTGCTTGTGCCTGTAA -3'
Srebp1c	5'-AGGCCATCGACTACATCCG-3'	5'-ATCCATAGACACATCTGTGCCTC-3'
Tnfa	5'-CCAGACCCTCACACTCAGATC-3'	5'-CACTTGGTGGTTTGCTACGAC-3'
Ym1	5'-TGTTCTGGTGAAGGAAATGCG-3'	5'-CGTCAATGATTCCTGCTCCTGT-3'

Protein extraction and Western blot analysis

Cells were washed twice in cold PBS and lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50mM HEPES, pH 7.5, 250mM NaCl, 1µg/ml aprotinin, 1µg/ml leupeptin, 1µg/ml iodoacetamide, 1mM PMSF, 1mM sodium orthovanadate). Insoluble material was removed by centrifugation at 13,000 x g for 8 min at 4°C. Cell lysates (50–100µg) were boiled at 95°C in Laemli SDS-loading buffer and separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare Europe Gmbh, Munich, Germany). Membranes were blocked in 5% milk in TBS-0.1% Tween 20 (TBS-T) during 1 h and then incubated with a primary antibody overnight at 4°C. To study the phosphorylation of STAT-6 we used an anti-phospho-Stat6 (Tyr641) antibody (Cell Signaling, Beverly, MA). Total Stat6, expression was determined by using an anti-STAT-6 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antibodies to measure Phospho-ERK (Erk1/2), Phospho-p38, total IκBα, and Phospho-IκBα (Ser32/36) expression were from Cell Signaling. Anti total-ERK antibody was obtained from Santa Cruz Biotechnology Inc. To check for protein loading and transfer, we used a monoclonal anti-mouse β-actin antibody (Sigma-Aldrich) or a rabbit polyclonal anti-α-tubulin antibody (Invitrogen). In general, membranes were washed three times in TBS-T and then incubated for 1h with peroxidase-conjugated secondary antibodies. Anti-mouse IgG was obtained from Sigma-Aldrich whereas anti-rabbit IgG was purchased from Jackson ImmunoResearch (Pennsylvania, PA). After three washes of 15min with TBS-T, enhanced chemiluminescence (ECL) detection were performed (GE Healthcare Europe) and the membranes were exposed to X-ray films (Fujifilm, Tokyo, Japan).

Construction of pcDNA3-3XFLAG-Idol and pCDNA3.3XFLAG-empty vectors

The cDNA sample of murine Idol was amplified from BMDM by PCR and cloned into a plasmid containing three copies of the FLAG-tag sequence (Brizzard and Chubet, 2001). The primers used to amplify the coding sequence of mouse Idol (Gene bank accession number NM_181043) were: Forward: 5'-GGAATTCCATGCTGTGCTATGTGACGAGGCCGG-3', including an EcoRI restriction site and one extra nucleotide to obtain Idol sequence in frame with the 3XFLAG sequence from

the vector; and Reverse: 5'-CGCGGATCCTCAGATGACAGTCAGATTGAGGAGACTG-3', which includes a BamHI restriction site. As template for the amplification we used a cDNA sample obtained by reverse transcription of total RNA isolated from T1317-treated macrophages. The PCR products were initially sub-cloned into p3XFLAG-CMV^{TM}-7.1 vector (Invitrogen), after digestion of both the plasmid and the insert with the restriction enzymes EcoRI and BamHI (New England Biolabs; Ipswich, MA). Ligation was performed using the T4 DNA ligase (Promega) overnight at 14°C. p3XFLAG-CMV^{TM}-7.1 is a shuttle vector that contains an enhancer–promoter sequence from the human cytomegalovirus gene for high-level transcription, and has a 3XFLAG tag. The 3XFLAG-Idol sequence from this vector was then amplified by PCR using the Forward primer 5'-GGGGTACCCATGGACTACAAAGACCATGACGG-3', that includes a KpnI restriction site, and the same Reverse primer used for the Idol sequence preparation. The amplified product was purified from an agarose gel with the Gel Extraction Kit QIAquick (Qiagen), digested with KpnI and BamHI (New England Biolabs) and cloned into a pcDNA3 vector (Invitrogen). The new construct will be now on designated as pcDNA3-3XFLAG-Idol. As a control, pcDNA3-3XFLAG-empty vector was generated by cutting out the Idol sequence from the pcDNA3-3XFLAG-Idol with Not I restriction enzyme (New England Biolabs) (Figure 12). The presence of a neomycin resistance gene on the pcDNA3 vector allowed us to generate cell clones stably transfected with these constructs (see next section).

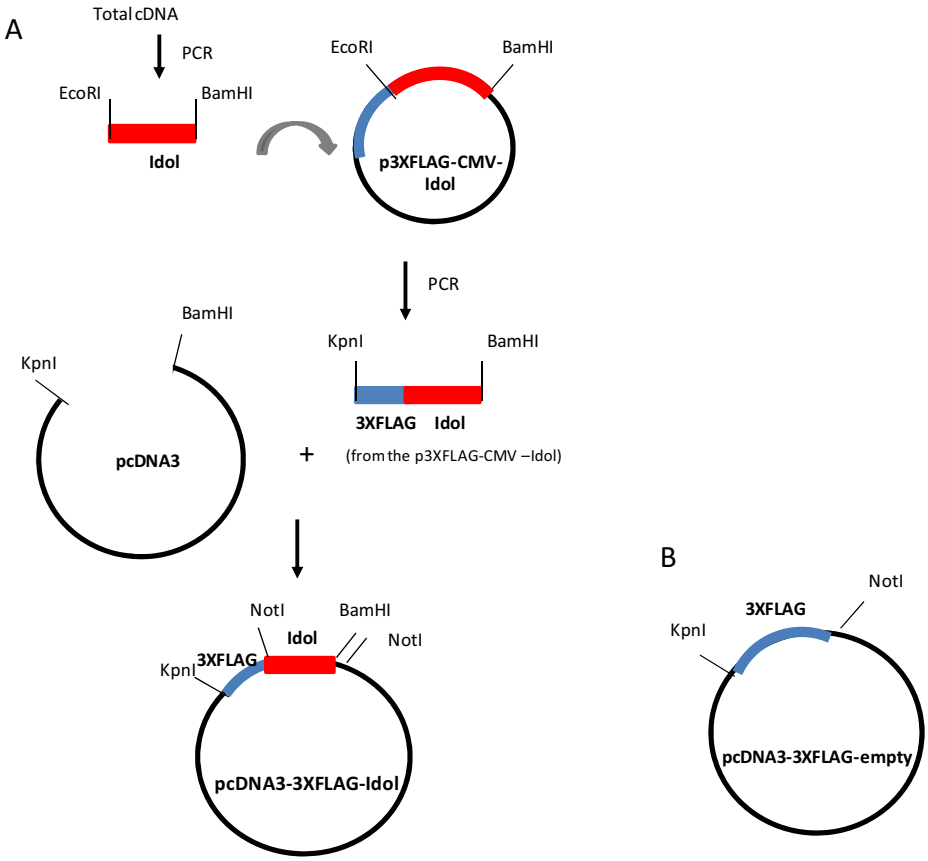

Figure 12. A) Construction scheme of the pcDNA3-3XFLAG-Idol. The 3XFLAG-Idol was amplified by PCR from the p3XFLAG-CMV-Idol construct; it was digested with KpnI and BamHI and cloned into the pcDNA3 (Invitrogen)**. B) Scheme of the pcDNA3-3XFLAG-empty construct.** This vector was obtained by cutting out the Idol sequence from the pcDNA3-3XFLAG-Idol construct with the restriction enzyme NotI.

Stable transfection of Raw264.7 macrophages

Transfection of empty vector (pcDNA3-3XFLAG-empty) or vector bearing murine 3XFLAG-Idol (pcDNA3-3XFLAG-Idol) into Raw264.7 macrophages cells was conducted in 60 mm diameter dishes. Cells at 50% confluence in culture medium without antibiotics were transfected with recombinant constructs using Superfect (Qiagen), following manufacturer instructions. Briefly, 5 µg recombinant DNA was added in 30 µl Superfect reagent and 150µl of serum-free medium. The solution was incubated for 10 min at room temperature for complex formation. The mixture of recombinant DNA and Superfect was added to each dish in 1ml of complete cell growth medium, and cells were incubated for 2.5 h under normal conditions. After this time, cells were washed and fresh cell growth medium (containing serum and antibiotics) was added.

The transfected cells were incubated for 2 days. The cells were subsequently incubated with 500 μg/ml of geneticin (G418) for 3 days for further selection of cells transfected with the plasmids that harbor the neomycin resistance gene. The cells were then diluted 1:10 and plated in 1mg/ml of G418 for several days until the formation of clones was visible. Several clones were further amplified and analyzed. From each of these clones, total cells lysates were generated and the expression of 3XFLAG-Idol was analyzed by western blotting using a specific antibody against the FLAG tag (anti-FLAG M2 monoclonal antibody, Eastman Kodak). A clone of Raw264.7 cells that overexpressed 3XFLAG-Idol in an intermediate level was selected and used for subsequent studios (Figure 13). Overexpression of Idol in cells selected by culture with G418 was verified by q-RT-PCR.

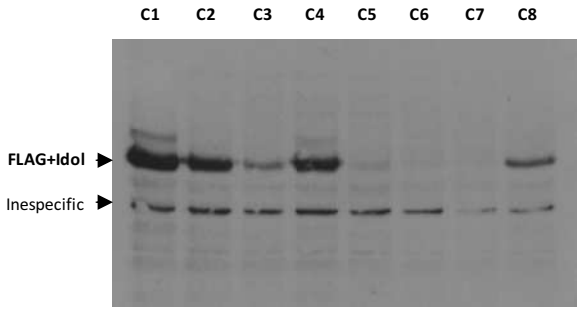

Figure 13. Selection by western blotting of the clone of Raw264.7 macrophages overexpressing 3XFLAG-Idol at intermediate level. Total cells lysates from 8 different clones were generated and the expression of 3XFLAG-Idol was analyzed by western blotting using a specific antibody against the FLAG tag. The clon from lane 2 (C2) showed an intermediate level of expression in comparison with the other clones, and it was chose to performed subsequent experiments.

Chromatin immunoprecipitation assays

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. Cells were rinsed with ice-cold PBS twice and collected into 100μM Tris.HCl (pH 9.4), 10mM DTT, incubated for 15 min at 30°C and centrifuged 5 min at 2000 *g*. Pellets were washed sequentially with ice-cold PBS, buffer I (0.25% Triton X-100, 10mM EDTA, 0.5mM EGTA, 10mM HEPES, pH 6.5) and buffer II (200mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM HEPES, pH 6.5) and centrifuged 5 min at 2000 *g*.

Cross-linked adducts were resuspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris.HCl, pH 8.1, 1X protease inhibitor cocktail) and sonicated, resulting in DNA fragments of 200 to 1200 bp. After sonication, samples were centrifuged for 10 min full speed, supernatant was collected and 1:10 diluted in dilution buffer (1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris.HCl, pH 8.1, 1X protease inhibitor cocktail). This soluble chromatin was immunocleared by incubating with 2µg sheared salmon sperm DNA (Amersham Bioscience, Munich, Germany), 5µg pre-immune serum and protein A-sepharose (GE Healthcare Europe) (50% slurry in 10mM Tris.HCl, pH 8.1, 1mM EDTA) overnight at 4°C. After this incubation, samples were quick centrifuged and supernatants were collected.

Immunoprecipitation was performed incubating samples with 1.5µg rabbit anti-LXR antibody (generated as described in Jakobsson et al., 2009, and kindly donated by Knut Steffensen, Karolinska Institutet, Stockholm, Sweden) or 1.5 µg rabbit anti-RXRα antibody (Santa Cruz Biotechnology) overnight at 4°C. Rabbit IgG (Sigma-Aldrich) was used as a control for nonspecific binding. Samples were then added 100µl protein A-sepharose (50%), 2µg of salmon sperm (Amersham Bioscience) and incubated for another 2h. Sepharose beads were harvested by centrifugation and washed sequentially for 10min each in TSE I (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris.HCl, pH 8.1, 150mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris.HCl, pH 8.1, 500mM NaCl), and buffer III (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris.HCl, pH 8.1). Sepharose beads were then washed twice with TE buffer and eluted three times with 1% SDS, 0.1 M NaHCO3. 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 60µl DNA extraction volume was used for q-RT-PCR analysis. q-RT-PCRs were performed using SYBERgreen (AppliedBiosystems). The primers shown in Table 6 were used to amplify the genomic regions analyzed in this study. *Aim* has been reported to be regulated by LXRα through a putative LXRE (LXR response element) at -5404 pb upstream in the *Aim* promoter (Joseph et al., 2004), however no LXR-RXR binding is observed in this region by ChIP-sequencing (ChIP-seq) data (deposited at http://genome.ucsc.edu by C. K. Glass, University of California San Diego (Heinz et al., 2010)). Thus, we designed primers

located in a region at -19750 pb upstream of the *Aim* transcriptional start site, were LXR and RXR binding has been described by ChiP-seq. For *Srebp1c*, we used primers surrounding the two LXREs located at positions -249 and -200 in the SREBP1c promoter (Yoshikawa et al., 2001). Binding of at least LXRβ to this genomic region has been also demonstrated by ChIP-sequencing. Moreover, these primers were previously used to analyze RXRα binding to the *Srebp1c* promoter. We also used beta-actin amplification as a control to normalize the specific detection of fragments of interest.

Table 6. Primers used for ChIP analysis.

Gen	Forward primer	Reverse primer
Aim	5'-TAGCGGAGCGGGTATCTCTA-3'	5'-TTTGTGGAAGTGCAAACTGG-3'
Srebp1c	5'-GAACCAGCGGTGGGAACACAGAGC-3'	5'-GACGGCGGCAGCTCGGGTTTCTC-3'
beta-actin	5'-ACTATTGGCAACGAGCGGTTC-3'	5'-AAGGAAGGCTGGAAAAGAGCC-3'

Phagocytosis assay

In vitro phagocytosis assays with Raw264.7 macrophages were carried out as described (A-Gonzalez et al., 2009). Briefly, 2 x 10^5 Raw264.7 cells were plated on sterile glass coverslips in DMEM supplemented with 10% FCS. To generate apoptotic thymocytes (AT), thymi from 4- to 5- week-old C57BL/6 mice were harvested and mechanically dissociated with the help of a 70µm Nylon Cell Strainer (BD Falcon, New Jersey). Gentle pressure was applied with the aid of a 1 ml syringe plunger. Stroma retained on the gauze was discarded. Thymocytes were pelleted and resuspended in DMEM supplemented with 10% FCS. Apoptosis was induced by treatment with 2 µM dexamethasone (Sigma-Aldrich) for 4h. This method resulted in 60%–80% thymocyte apoptosis, as measured with Propidium Iodade staining using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Fullerton, CA). AT were washed twice with PBS and labeled with CellTracker green during 30min at 37°C (Invitrogen) according to the manufacturer's instructions. Fluorescent AT were added to Raw macrophages at a 5:1 ratio (AT:macrophages) and cultured at 37°C for 90min in DMEM supplemented with 10% FBS. After incubation with AT, macrophages were gently washed several times with cold PBS and Cell Dissociation Buffer, Enzyme Free PBS-based (Gibco, Life

Technologies) to remove free AT. Alexa Fluor 594 WGA (Abcam; Cambridge, UK) was used to label plasma membranes (red). Cells were then fixed with 2% paraformaldehyde, DNA was stained with Hoechst solution (Sigma-Aldrich), and coverslips were mounted on a microscope slide with VECTASHIELD Mounting Media (Vector Laboratories; Burlingame, CA). Phagocytosis was scored with confocal fluorescent microscopy. Phagocytosis was expressed as % of phagocytosis : number of cells with ingested vesicles per total number of macrophages x 100. In some experiments, macrophages were treated with 1µM synthetic LXR agonist (GW3965) in DMEM supplemented with 0.5% FCS for 18h prior to addition of AT and phagocytosis assays were performed as described above in DMEM containing 10% FBS.

ELISA

CCL2 (MCP1) expression from Raw264.7 supernatants was analyzed by ELISA kit (Invitrogen). In brief, standard curve was prepared by serial dilutions of a recombinant mouse CCL2 (5000pg/ml) to obtain a standard range from 78.1pg/ml to 1250pg/ml. Standards, control and samples (without diluting) were led to bind to the immobilized (capture) antibody for 2h. After washing extensively, a biotinylated monoclonal antibody specific for CCL2 was added and incubated for 45min. Wells were washed extensively to remove the excess second antibody, and incubation with Streptavidin-Peroxidase during 45 min was carried out. This enzyme binds to the biotinylated antibody to complete the four member sandwich. After washing, a chromogen substrate was added and incubated for another 20 min. Stop solution was directly added to each well, and absorbance at 450nm was read.

CCL22 expression from IL-4 stimulated BMDM supernatants was measured with ELISA kit from R&D Systems (Minneapolis, MN). This kit use the same principle as explained above for CCL2. Standard curves for CCL22 were prepared to cover a range from 31.2pg/ml to 500pg/ml, and samples from wild type and from LXRαβ deficient cells were diluted 1:10 and 1:40, respectively, in calibrator diluent.

Experimental model of allergic asthma.

Sensitization to HDM aeroallergens.

Mice were exposed to a purified house dust mite (HDM) extract (provided by Alk-Abelló, Spain) with a low lipopolysaccharide content (<0.5 EU/dose, as determined by the Endosafe LAL Assay, Charles River Laboratories, Wilmington, MA, USA). The HDM extract was administered daily intranasally (i.n.) at a dose of 25 µg/mouse in a volume of 35 µl for 10 consecutive days under isoflurane-induced anaesthesia. Non-sensitized (control) animals were manipulated identically except that they received i.n. saline instead of the HDM extract. Airway reactions to aeroallergens were evaluated after the 10-day exposure protocol. LXR activation was induced by i.p. injection of GW3965 (20 mg/kg) 24h prior to the first HDM administration and during the next 9 days of sensitization. Controls were injected i.p. with vehicle (DMSO in PBS) (Figure 14).

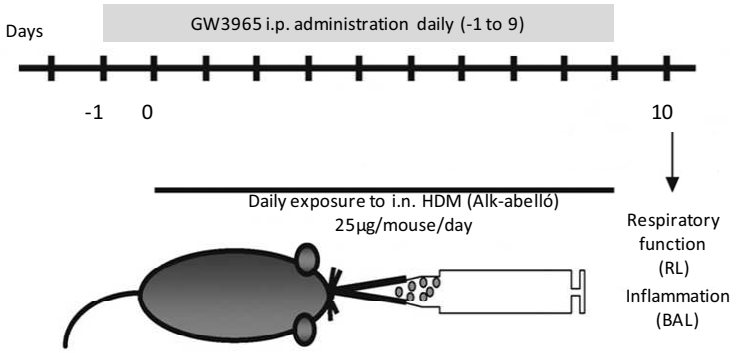

Figure 14. Murine model of allergic asthma and GW3965 treatment schedule. A schematic representation of the allergen challenge protocol. One group of mice also received daily i.p. injections of GW3965 just before HDM i.n. administration. RL: airway resistance; BAL: bronchoalveolar lavage.

Assessment of airway responsiveness

Airway reactivity was analyzed in all animals 24 h after the last exposure to HDM using an invasive technique to measure airway resistance with two Finepointe Series RC sites (Buxco Europe) as described in Torres et al., 2013. In brief, mice were anaesthetized with an i.p. injection of ketamine/xylazine. The trachea was exposed and cannulated.

The mice were mechanically ventilated at 120 breaths/min and tidal volumes of 12.5 ml/Kg. Baseline readings for resistance were recorded, and increasing doses of aerosolised methacholine were administered. The average of the maximum response for each dose was then calculated.

Quantification of airway inflammation

Animals were sacrificed after the assessment of airway responsiveness. Bronchoalveolar lavage (BAL) was performed slowly by infusing 0.3 mL PBS (2% foetal bovine serum) twice and recovering it by gentle aspiration after 30 s. An aliquot of the BAL cells was stained with Turk solution (0.01% crystal violet in 1% acetic acid) and analysed in a Neubauer chamber. Total airway cellularity was determined by counting cells in BAL samples from each mouse. Differential cell count was performed on BAL cytospins from each experimental group. The pelleted BAL cells were cytospun onto a slide using a cytocentrifuge (600 rpm, 6 min). The cells were then stained with Diff-Quick, and 300 leukocytes were analysed. The relative numbers of eosinophils, lymphocytes, macrophages, and neutrophils were determined.

Analysis of immunoregulatory cytokines and chemokines

Analysis of cytokine and chemokine gene expression was measured in lung tissue homogenate from mice sacrificed 10 days after initiating HDM exposure. RNA extraction, cDNA synthesis and quantitative real-time PCR analysis were performed as explained above.

Statistical Analysis

The data obtained from the airway responsiveness were compared between groups by a two-way repeated-measure ANOVA with a *post hoc* Bonferroni test. Other statistical analysis of data from gene expression analysis and ELISAS were also performed using a one way ANOVA with a *post hoc* Bonferroni test, Mann–Whitney U test, or two-tailed Student's *t*-test as specified.

Results

I. Study of LXR mediated transrepression of pro-inflammatory genes in macrophages. Evaluation of the effects of LXR agonists in foam cells versus primary macrophages.

Previous studies to the development of this thesis have demonstrated that activation of LXR exerts inhibitory effects on the induction of inflammatory gene expression by LPS (Joseph et al., 2003; Ogawa et al., 2005; Ghisletti et al., 2007). Additionally, recent work from our group demonstrated broad repressive effects of the LXR agonist GW3965 on the macrophage transcriptional response to IFN-γ (Pascual-García et al., 2013). From these studies we have determined sets of IFN-γ- and LPS-induced genes that are transrepressed specifically by LXR agonists in bone marrow derived macrophages.

To confirm the inhibitory effects of GW3965 on IFN-γ- and LPS- signaling we carried out transrepression assays in which primary macrophages were pretreated during 3 h with 1μM of the LXR agonist GW3965 and then stimulated with 5ng/ml IFN-γ or 5ng/ml LPS for 6h. We selected several genes involved in the immune response that have been previously seen to be repressed by GW3965 in microarray studies (Pascual-García et al., 2013; and Alameda D. and Ricote M., data not published). Examples of these genes are those that codify for prostaglandin synthase 2 (*Ptgs2*), several members of the chemokine (C-X-C motif) ligand (CXCL) family and of the chemokine (C-C motif) ligand (CCL) family, integrin alpha L (*Itgal*) and others. We also included the *inducible nitric oxide synthase* (*Nos2*) gene, as it is a thoroughly studied target for LXR-mediated transrepression (Ghisletti et al., 2007) (Figure 15).

The results indicate that the LXR transrepression capacity differs depending on the pro-inflammatory stimuli. In general, the IFNγ signal is partially inhibited by the treatment with GW3965 whereas the LPS signal is more robustly affected by the same agonist. For example, GW3965 resulted in 53% repression of the induction of Nos2 by IFN-γ and 93% inhibition of the induction of the same gene by LPS. The same tendency was observed for all the genes analyzed.

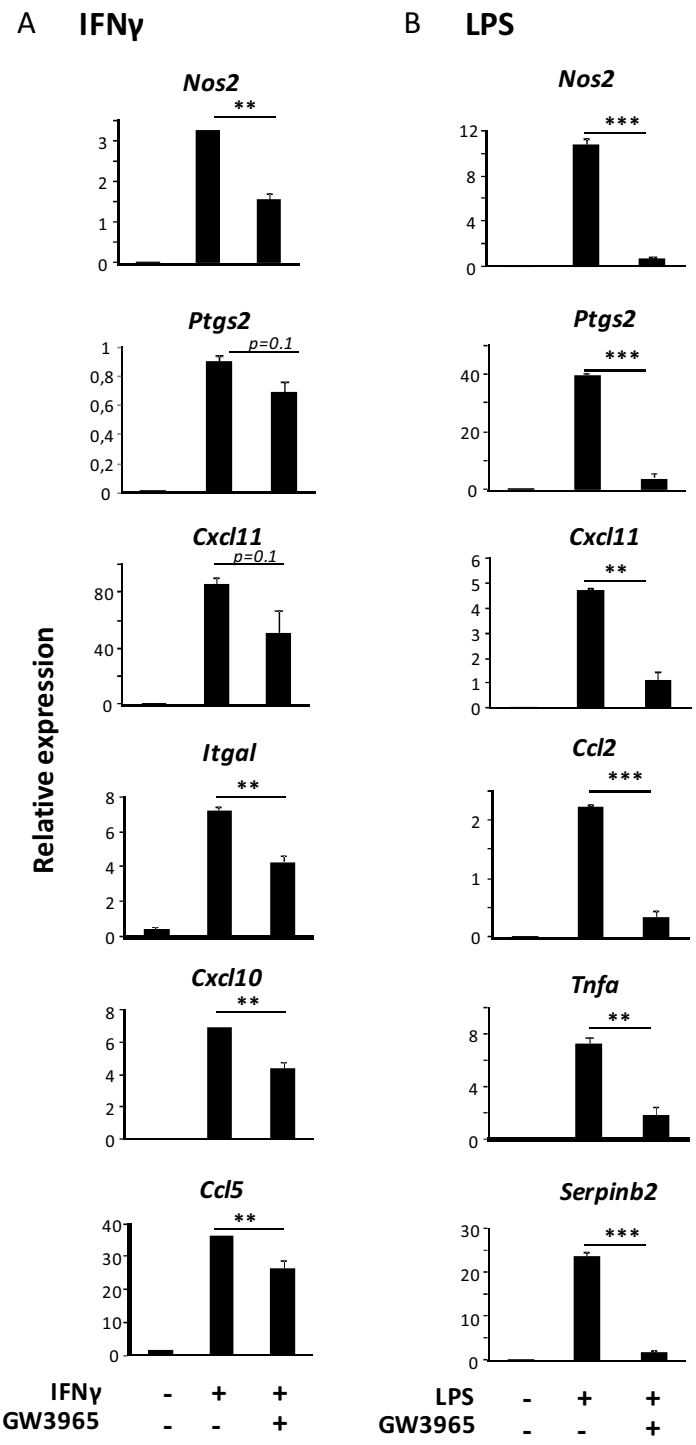

Figure 15. LXR mediated effects on IFNγ- and LPS- induced pro-inflammatory genes. BMDM were treated with 1μM GW3965 or vehicle for 3h and then stimulated with IFNγ (5ng/ml) (A) or LPS (5ng/ml) (B) in DMEM-1% FCS for 6h. Gene expression was measured by q-RT-PCR. Mean values of normalized relative expression ± SEM from three independent experiments are shown. A Student's *t* test was used for statistical comparisons. **p<0.01, ***p<0.0001.

Since chronic inflammation is a hallmark of atherosclerosis, we next evaluated the capability of LXR agonists to repress inflammatory responses induced by the cytokine IFN-γ or the bacterial component LPS in foam cells. For these experiments, foam cells were generated by loading BMDM with acetylated LDLs (acLDL, 10µg/ml) during 33h.

To confirm foam cell formation, macrophages incubated or not with acLDL were subjected to Oil-Red-O staining and visualized by light microscopy (Figure 16). The accumulation of red lipid droplets in acLDL treated cells is clearly observed, which confirms the acquisition of a lipid-loaded phenotype after this treatment.

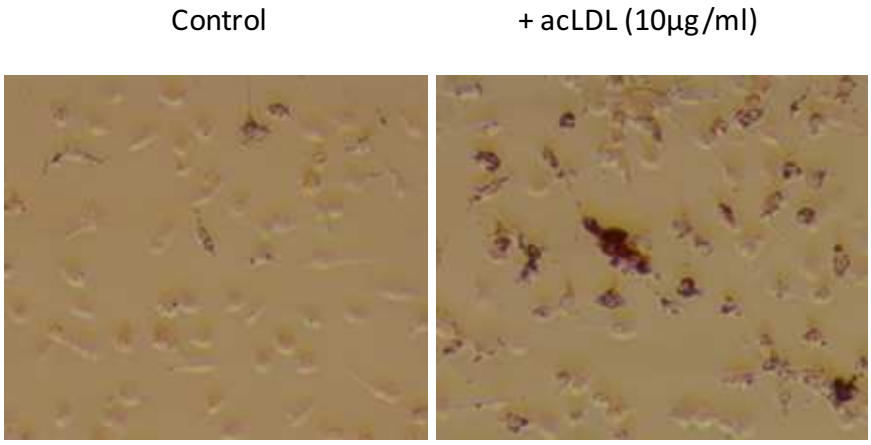

Figure 16. Accumulation of lipids in acLDL-treated macrophages. Macrophages were treated with acLDL (10µg/ml) (right) or PBS (control, left) in DMEM-1% FCS for 33h. Cells were then stained with Oil Red-O and observed in a light microscopy, 20X. Three independent experiments were analyzed; one representative panel of photos is shown.

To evaluate if the treatment of macrophages with acLDL was regulating the expression of genes involved in cholesterol and fatty acid metabolism, we analyzed the expression of the cholesterol transporters *Abca1*, *Abcg1* and of the transcription factor *Srebp1c* which is a master regulator of lipogenesis. Interestingly, the expression of *Abca1* and *Abcg1*, but not of *Srebp1c*, was induced by the treatment with acLDL. Increased expression of Abca1 and Abcg1 in these conditions is compatible with the existence of natural agonists for LXRs in acLDL (Figure 17).

We also analyzed the expression of LXR target genes after treatment of control macrophages and foam cells with the cytokine IFN-γ (Figure 17). Activation of both

types of cells by IFN-γ resulted in decreased expression of *Abca1* and *Srebp1c*, which indicates that inflammatory signals present at the atherosclerotic site alter the expression of genes involved in lipid homeostasis. Similar results have been obtained in our group by using the synthetic LXR agonists T1317 and GW3965 in BMDM (Pascual-García et al., 2013).

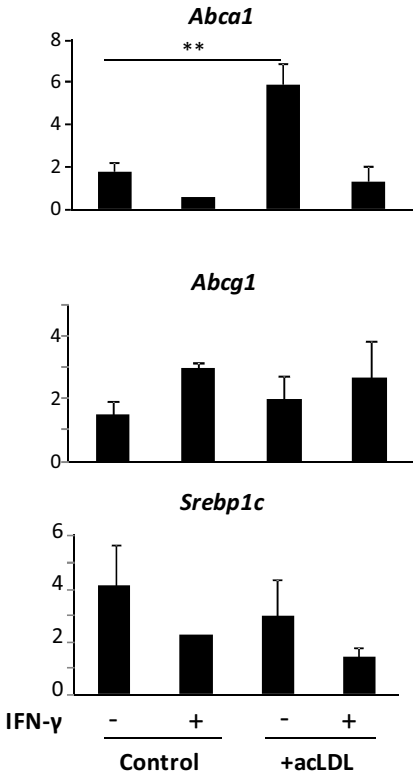

Figure 17. Effect of acLDL loading and proinflammatory stimulation of macrophages on the expression of cholesterol metabolism related genes. Macrophages were treated with acLDL (10μg/ml) or PBS in DMEM-1% FCS for 24h. The cells were then stimulated with IFN-γ (5ng/ml) for another 6h. *Abca1*, *Abcg1* and *Srebp1c* gene expression was measured by q-RT-PCR. Mean values of normalized relative expression ± SEM from three independent experiments are shown. A Student's *t*-test was used for statistical comparisons. *p<0.05; **p<0.01.

We next developed transrepression assays in these cells. Macrophages were treated with acLDL and then stimulated with IFN-γ (5ng/ml) or LPS (5ng/ml) in the presence or absence of the LXR agonist GW3965 (1μM). Our results show that foam cell formation did not alter the capability to mount a proinflammatory response to IFN-γ. The expression of most of the IFN-γ-induced proinflammatory genes evaluated in foam

cells was similar to that in control cells, with the exception of discrete downregulatory events such as diminished induction of *ItgaL* and *Cxcl11* expression (Figure 18A).

However, these observations contrast with the drastic impairment of the proinflammatory response to TLR signaling in acLDL-treated cells. All the inflammatory genes analyzed after the LPS-treatment in foam cells were remarkably suppressed (Figure 18B).

Interestingly, conversion to foam cells with acLDL did not prevent GW3965 from repressing proinflammatory gene expression in response to either IFN-γ or LPS, which indicates that despite acLDL may provide endogenous agonists for LXR, the high affinity agonist GW3965 is still able to potentiate the anti-inflammatory role of LXRs in foam cells (Figure 18). This observation strengthens the potential therapeutic use of LXR agonists in the context of atherosclerosis.

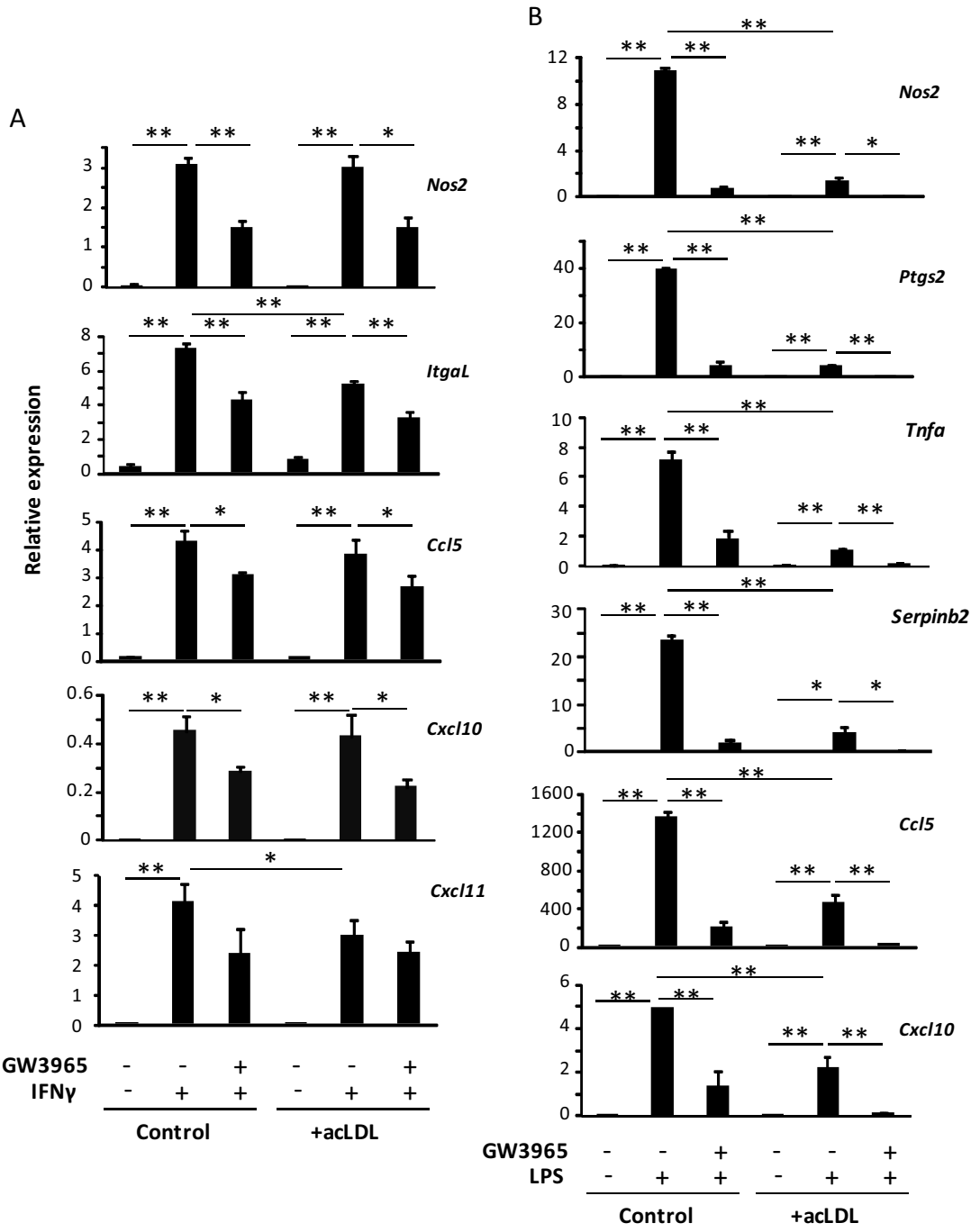

Figure 18. Lipid loaded macrophages show a suppression of the induction of LPS-responsive genes. Effect of LXR agonist Gw3965 on IFNγ-induced (A) and LPS-induced (B) gene expression in acLDL-loaded macrophages. Macrophages were treated with acLDL (10µg/ml) or PBS in DMEM-1% FCS for 24h. The cells were then treated with 1µM GW3965 or vehicle for 3h and stimulated with IFN-γ (5ng/ml) (A) or LPS (5ng/ml) (B) for 6h. Pro-inflammatory gene expression was measured by q-RT-PCR. Mean values of normalized relative expression ± SEM from three independent experiments are shown. A Student's *t*-test was used for statistical comparisons. *p<0.05; **p<0.01. *Serpinb2*: Serine (or cysteine) peptidase inhibitor.

In order to get more insight into how foam cell formation is affecting TLR signaling, we next studied if the activation of some of the molecules that participate in the LPS signaling pathway was impaired in foam cells. Macrophages loaded with acLDL during 24 h were stimulated with LPS during 15, 30 and 60 min. Phosphorylation levels of several members of the MAPK family, namely extracellular signal-regulated kinases 1 and 2 (ERK1/2), and the p38 mitogen activated protein kinase ($p38^{MAPK}$) were analyzed by western blotting. Also the phosphorylation of the inhibitor of NF-κB (IκBα) and its total expression levels were analyzed (Figure 19).

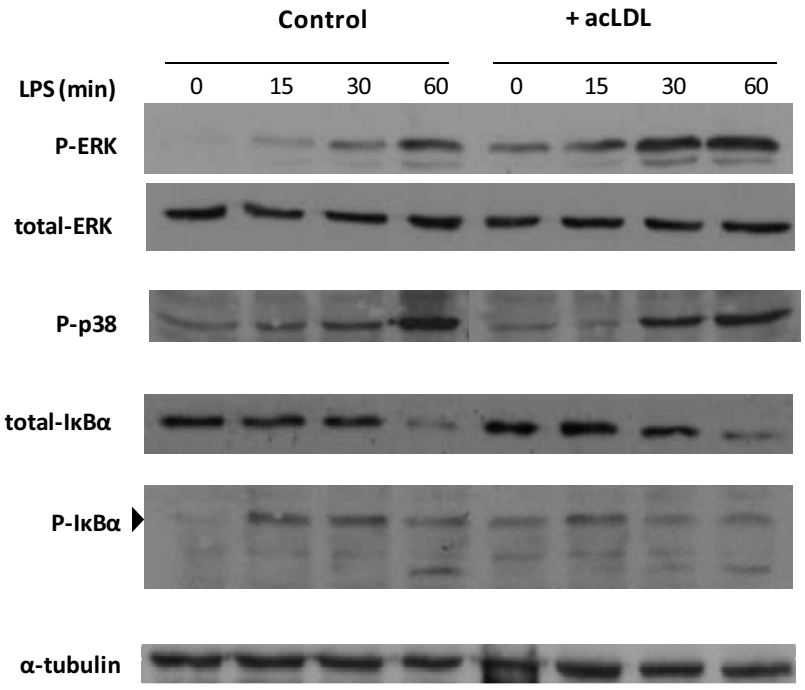

Figure 19. Loading of macrophages with acLDL does not affect the activation of the LPS signaling molecules ERK and p38 MAPK. Bone marrow derived macrophages were treated with acLDL (10μg/ml) or PBS in DMEM-1% FCS for 24h. The cells were then stimulated with LPS (5ng/ml) for 15, 30 and 60 minutes. Phosphorylation levels of ERK, p-38 MAPKs and IkB, as well as total ERK and total IkB levels were analyzed with specific antibodies by western blotting.

The response of BMDM to saturating concentrations of LPS is known to induce relatively early peaks of activation of ERK-1/2 and $p38^{MAPK}$ (Valledor et al., 2000a; Comalada et al., 2003). For example, ERK-1/2 activation by LPS at 100ng/ml has been shown to peak at 15-30min post-stimulation and decrease thereafter (Valledor et al., 2000a). In our conditions, however, treatment of BMDM with 5ng/ml LPS in 1%FBS-

containing medium resulted in slower activation of the ERK pathway. ERK phosphorylation was initially detected 15min after the start of stimulation and continued to increase up to at least one hour post-stimulation. Interestingly, higher basal ERK phosphorylation was observed in acLDL-treated cells. However, treatment with LPS was able to further induce ERK phosphorilation to the levels observed in control macrophages, which suggests that ERK activation by LPS is not impaired in acLDL-loaded macrophages.

Similarly, the phosphorylation of p38 was also relatively delayed in BMDM treated with subsaturant concentrations of LPS and loading with acLDL did not interfere negatively with such activation.

Moreover, the pattern of phosphorylation of IκBα, and its degradation (total IκBα), an indirect measurement of NFκB activation, was similar in foam cells and in control macrophages indicating that this branch of the LPS signaling machinery is not affected either.

Another possible manner by which TLR signaling can be suppressed in foam cells is by the fact that accumulation of high levels of lipids that are metabolized and can lead to the formation of natural ligands for other nuclear receptors that have also anti-inflammatory effects, such as lipid metabolites that activate PPAR-γ (Nagy et al., 1998). Having this in mind, we decided to investigate in more detail the crosstalk between LXR and PPAR-γ in the transrepression of LPS-inducible genes.

With the aim to identify the specific contribution of LXR and PPAR-γ on the negative regulation of proinflammatory gene expression, thioglycollate-elicited peritoneal macrophages were obtained from wild type and LXRα- and β- deficient (LXRαβ DKO) mice. The reason why we used peritoneal macrophages for these assays is because they express higher basal levels of PPAR-γ (Ricote et al., 1998). Cells were preincubated with ligands for LXR and PPAR-γ, GW3965 and rosiglitazone respectively, during 18h previous to the stimulation with LPS for 6h.

In collaboration with Daniel Alameda and Mercedes Ricote at CNIC, Madrid, we used the information from expression profiling assays developed by their group to select

several LPS-inducible target genes that were found to be transrepressed by both nuclear receptors or only by one of them in primary macrophages. Our results indicate that there is a large number of genes that are down regulated similarly by both LXR and PPAR-γ, including *Nos2*, *Ptgs2* and *Ccl2* (Figure 20 and data not shown). There is another group of genes that are selectively transrepressed by the PPAR-γ agonist, but not significantly affected by LXR, that include *Ccl17*, *Il12b* and *Il6.* And lastly, there is a reduced number of genes that are only sensitive to the LXR ligand, and not to the PPAR-γ ligand, including serine peptidase inhibitor (*Serpinb2/Pai2)*, podoplanin (*Pdpn*) and *Il1b*.

The use of LXR-deficient macrophages revealed that the repressive actions of the agonist GW3965 are LXR-specific under the conditions used on these experiments. Moreover, the inhibitory effects of the PPAR-γ agonist rosiglitazone are still observed in the absence of functional LXRs. This is true for genes that are only affected by the PPAR-γ agonist, but also for genes that can be repressed by both PPAR-γ and LXR agonists, which suggests that both nuclear receptors can transrepress the same genes independently of each other. These observations may help explain why macrophage lipid loading after treatment with modified LDLs leads to strong inhibition of many LPS-inflammatory targets and yet, the synthetic LXR agonist GW3965 can further repress those transcriptional responses (Figure 18B).

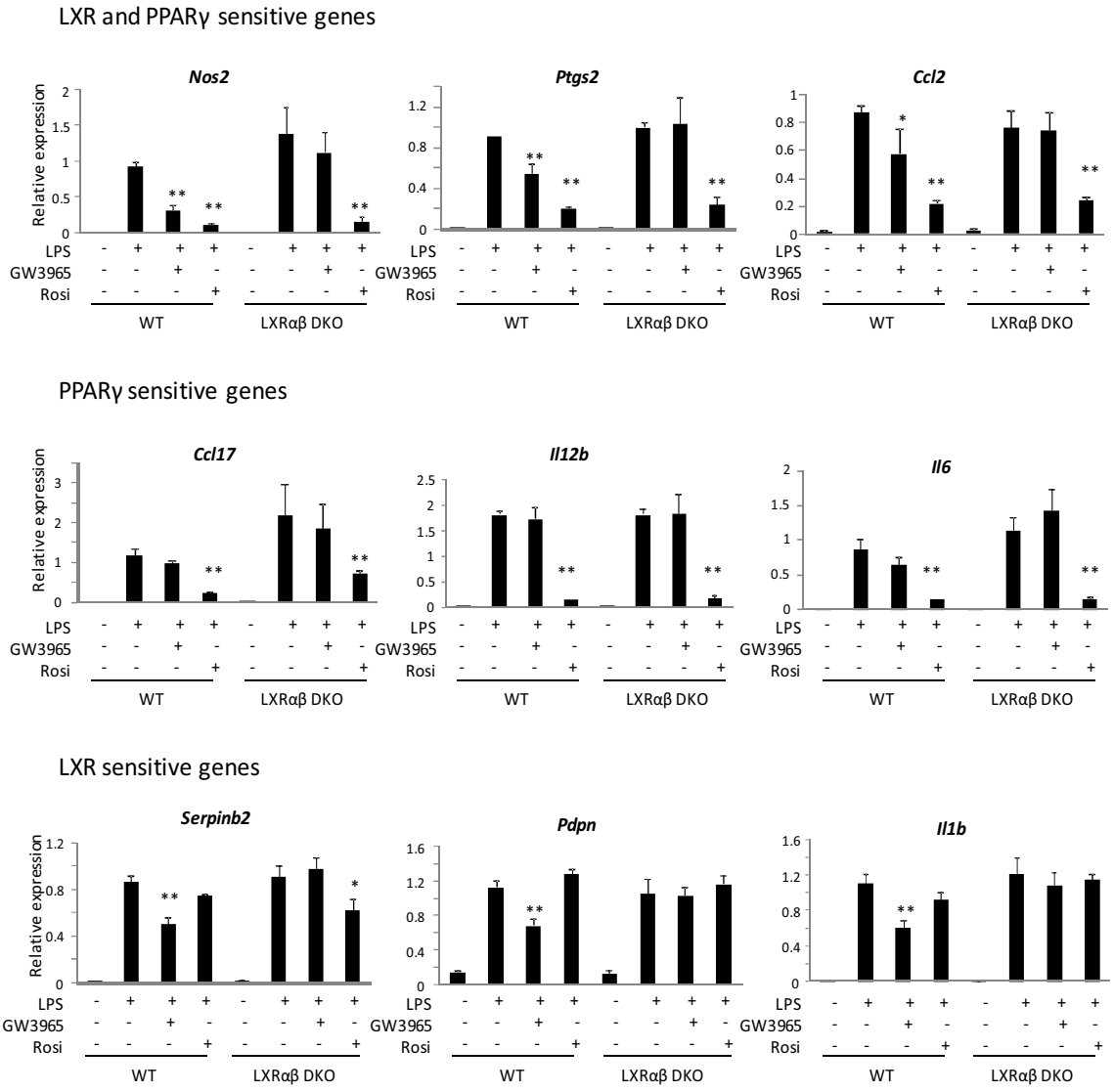

Figure 20. Receptor-specific transrepression mediated by LXR and PPARγ. Selected representation of genes susceptible to transrepression by LXR or PPARγ agonists in macrophages stimulated with 5ng/ml LPS (except for Il1b, 100ng/ml LPS). Peritoneal macrophages were obtained from wild type (WT) or LXRα-and β-deficient (LXRαβ DKO) mice. Cells were preincubated for 18 h with each ligand or vehicle before stimulation with LPS during 6 h. The genes chosen for these studies were determined in collaboration with Daniel Alameda and Mercedes Ricote (CNIC, Madrid) based on expression profiling assays and subsequent validation experiments. Genes that undergo at least 30% repression by a nuclear receptor agonist are considered sensitive to the action of that nuclear receptor. Mean values of normalized relative expression ± SEM from at least two independent experiments with triplicate samples in each experiment are represented. Statistical analysis was performed using a Student *t* test ; *p <0.05, **p < 0.01 versus LPS.

II. Analysis of a potential role for Idol in the transrepression of proinflammatory genes

Throughout development of the studies with peritoneal macrophages we observed that prolonged treatments with GW3965 had more robust effects on transrepression than short treatments. An illustrative example of these observations is shown in Figure 21A. In these experiments, the cells were preincubated for 2h and 18h with GW3965 and then stimulated with LPS. Although transrepression can be already detected with a 2h preincubation time with GW3965, prolonged activation of the LXR pathway resulted in enhanced repression of most of the genes studied.

We next tested, whether cooperation with the LXR heterodimeric partner RXR contributed to the transrepression potential of prolonged LXR activation. In collaboration with the group directed by Dr. M. Ricote, CNIC, Madrid, we analyzed the effect of GW3965 in wild type (WT) cells and in macrophages with a combined deficiency in the isoforms RXRα and β (RXRαβ DKO) (Figure 21B). Interestingly, expression of functional RXRs was required for LXR-mediated transrepression of several genes. A more extended overview of the list of genes that require heterodimerization with RXR for LXR-mediated transrepression has been generated by Daniel Alameda in Dr. Ricote's group and will be included in his thesis defense work.

Taking these observations together, we raised the hypothesis that transcriptional positive regulation of an LXR/RXR target gene could contribute as a mechanism for LXR-mediated repression of inflammatory responses. A potential candidate for this search was the LXR target gene Idol, an E3 ubiquitin ligase whose function has been recently associated with the post-transcriptional regulation of members of the LDL-receptor family (Zelcer et al., 2009; Hong et al., 2010).

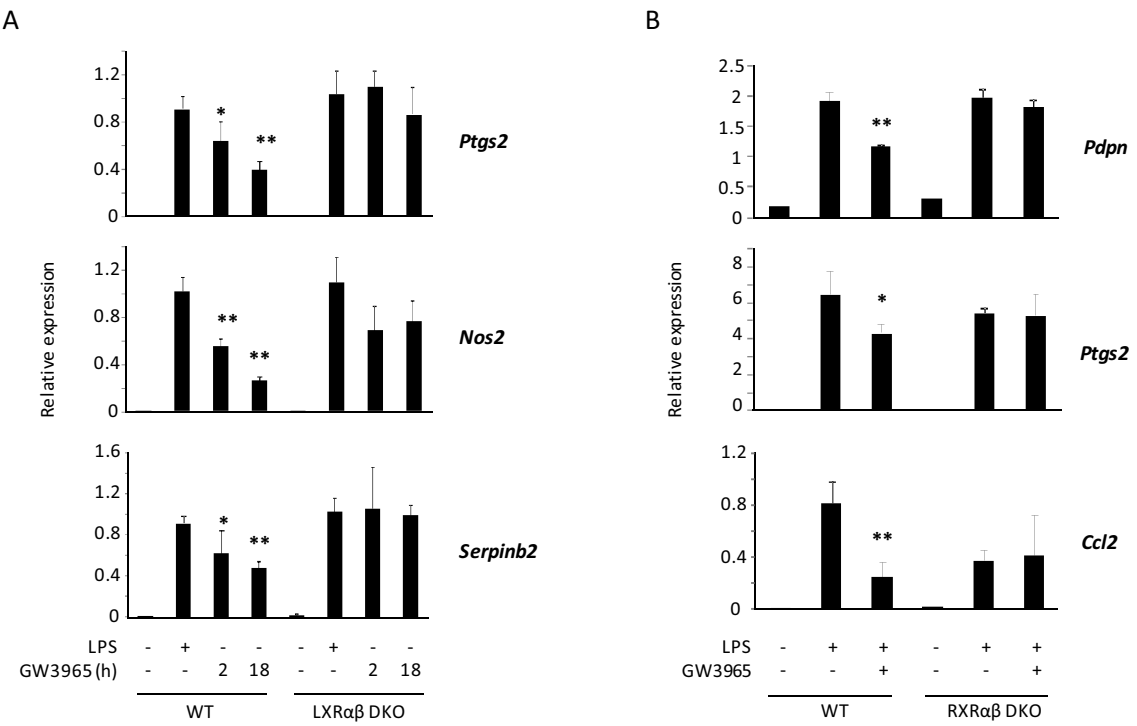

Figure 21. LXR-mediated transrepression is enhanced by prolonged pretreatment with the agonist and cooperation between LXR and RXR contributes to LXR-mediated transrepression. Peritoneal macrophages were obtained from wildtype (WT), LXRαβ-deficient (LXRαβ DKO) (A) or RXRαβ-deficient (RXRαβ DKO) (B) mice. In A, the efficiency of repression by GW3965 (1 μM) was analyzed at different time points of pretreatment with the agonist (2 and 18h). In B, the cells were pretreated with GW3965 for 18h. After pretreatment, the cells were stimulated with LPS (5ng/ml) for 6h. Analysis of gene expression was assessed by qRT-PCR. Results are expressed as the average of at least two independent experiments each of them performed in triplicates (A) or the average of triplicates (B). The genes included in these studies had been previously selected based on the specificity of GW3965 in LXRαβ deficient macrophages and at least 30% repression by the agonist. Statistical analysis was performed using a Mann–Whitney U test, *p < 0.05 and **p < 0.01 versus LPS. These data were obtained in collaboration with Daniel Alameda and Mercedes Ricote (CNIC, Madrid).

To confirm the induction of Idol by the LXR pathway, BMDMs were stimulated with LXR and /or RXR agonists for different periods of time and Idol mRNA expression was assessed by q-RT-PCR. In our hands, expression of *Idol* was upregulated 2 fold by a 24h treatment with an LXR agonist (T1317) or an RXR agonist (LG268), with synergistic effects when a combination of both ligands was used (Figure 22A)

Based on the capability of Idol to post-transcriptionally affect specific gene expression, we decided to investigate if this protein could be also facilitating repression of proinflammatory genes through an indirect mechanism involving regulatory protein

degradation. For this purpose, we cloned the Idol coding sequence in the expression vector pcDNA3.1 (Invitrogen) and generated a stably transfected Raw 264.7 macrophage cell line that overexpresses Idol (Raw264.7-Idol). As a control, we also generated a Raw 264.7 line that is stably transfected with the empty vector (Raw264.7-empty). The overexpression of Idol in the Raw264.7-Idol transfectants was confirmed by western blotting using antibodies anti-FLAG tag (Figure 13; Materials and Methods) and by q-RT-PCR using specific primers against *Idol* (Figure 22B). Activation of LXR further potentiated Idol overexpression in the Raw264.7-Idol transfectant.

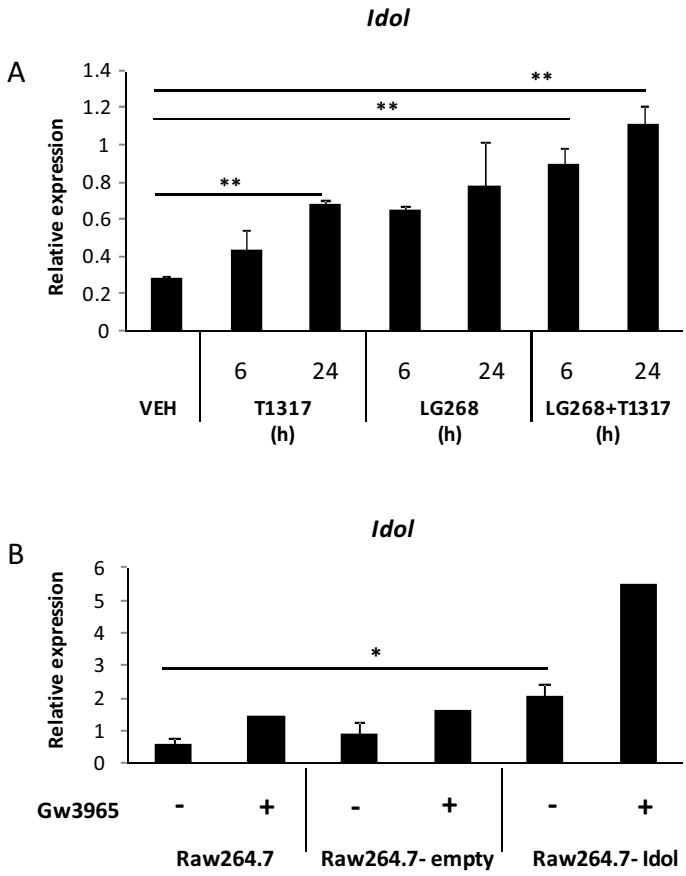

Figure 22. Idol is induced upon LXR activation in BMDM and in Raw 264.7 macrophages. A) LXR/RXR-mediated induction of Idol in BMDM. BMDM were incubated with the LXR ligand T1317 (1μM), the RXR ligand LG268 (1μM), or a combination of both LG268+T1317 during 6 or 24 h. Control cells were treated with vehicle for 24h. **B) Expression of Idol in Raw 264.7 macrophages stably transfected with pcDNA3-3XFLAG-Idol vector.** Cells were preincubated for 18 h with the LXR ligand GW3965 (1μM) or vehicle. Data of vehicle-treated cells is represented as mean values of normalized relative expression ± SEM from three independent experiments. Statistical analysis was performed using a Student *t* test ; *p <0.05, **p < 0.01.

To evaluate if the Raw264.7-Idol macrophages display any difference in either the capability to mount a proinflammatory response or in the capability of LXR to mediate transrepression, normal non-transfected Raw264.7 cells, Raw264.7-empty and Raw264.7-Idol transfectant clones were incubated with LPS for 6h in the presence or absence of the LXR agonist GW3965.

In general, overexpression of Idol did not interfere negatively with the induction of inflammatory genes by LPS (Figure 23A). In fact, increased expression of several proinflammatory mediators was observed upon overexpression of Idol in comparison with cells transfected with the empty vector.

Moreover, in the Idol-overexpressing macrophages we observed that LXR mediated transrepression of the different proinflammatory genes analyzed, without leading to evident differences in comparison with the Raw 264.7 control cells (Figure 23A). These results indicate that the LXR target gene Idol is not apparently involved in the transrepression mechanism mediated by LXR on LPS-inducible genes.

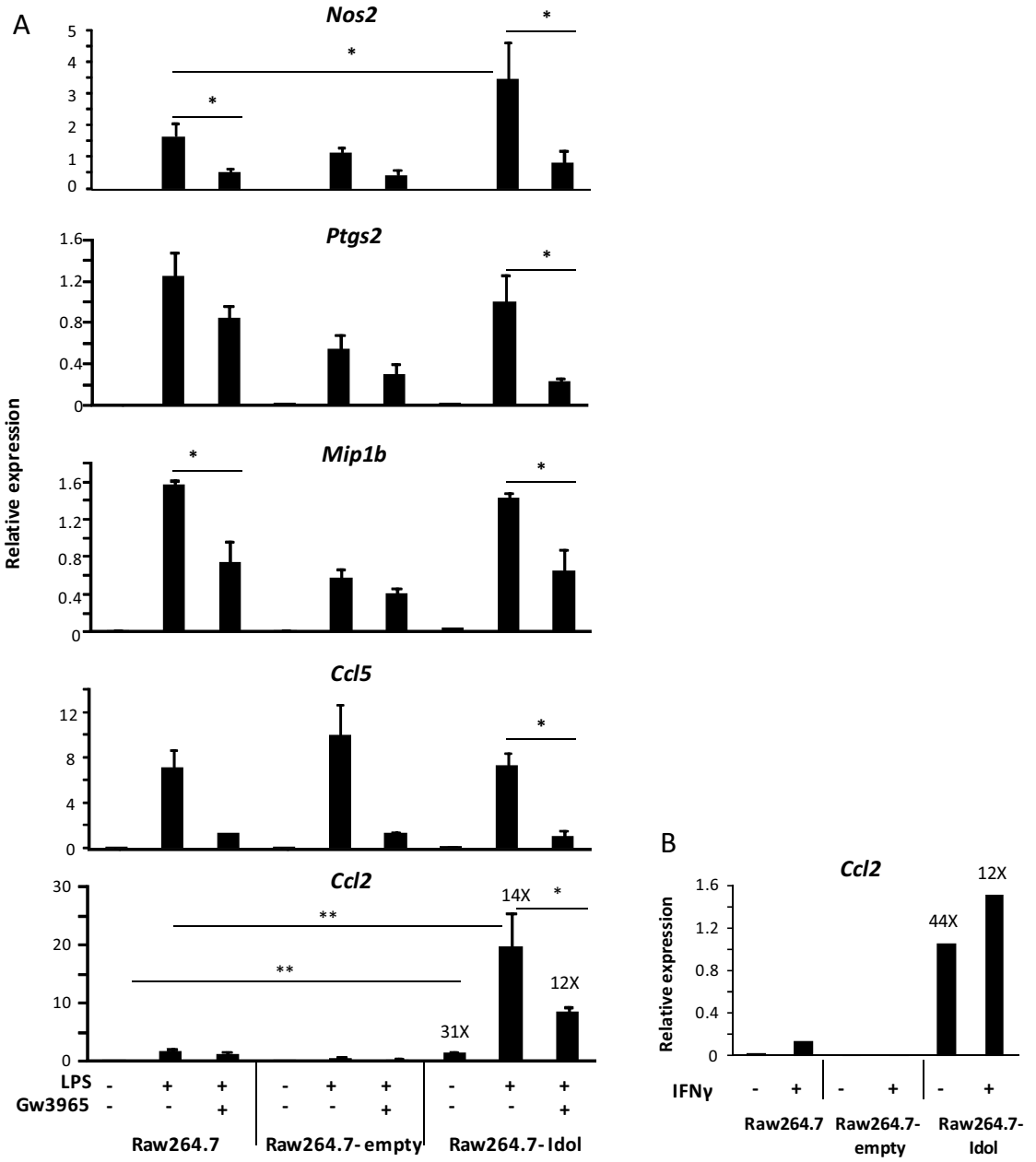

Figure 23. Idol overexpression does not affect the LXR-mediated transrepression of pro-inflammatory genes. Cells were preincubated or not for 18 h with 1μM of the LXR ligand GW3965 or vehicle (DMSO) before stimulation with 5ng/ml LPS (**A**) or 5ng/ml IFN-γ (**B**) during 6 h. **A)** Mean values of normalized relative expression ± SEM from three independent experiments are represented. Statistical analysis was performed using a Student *t* test ; *p <0.05, **p < 0.01. **B)** *Ccl2* is also induced by IFN-γ in Raw264.7 macrophages. Cells were stimulated with 5ng/ml IFN-γ during 6 h. Normalized relative expression is represented. X means the fold in comparison to the correspondent treatment in Raw264.7 cells.

However, an unexpected finding was observed while developing these assays. Expression of the LPS-inducible gen *Ccl2* (*mcp-1*) was remarkably over-induced in the Idol-overexpressing macrophages. This effect was observed in non-stimulated cells

(31-fold in comparison to control Raw264.7 cells), and after the treatment with LPS (14-fold) (Figure 23A) or IFNγ induction (Figure 23B). To test whether upregulation of the mRNA levels of *Ccl2* is translated into higher secretion of this chemokine to the medium, the concentration of CCL2 in supernatants of the Raw264.7-Idol, Raw264.7-empty and normal Raw264.7 macrophages was analyzed by ELISA. The results show a significant increase in the concentration of CCL2 in supernatants from Raw264.7-Idol in comparison with the Raw264.7 control cells (Figure 24A). Interestingly, LXR activation was able to repress the LPS-induced secretion of CCL2 in all the cells tested, in correlation with the inhibitory effects exerted by this nuclear receptor on the *Ccl2* mRNA levels (Figure 23A). These observations suggest that LXR and Idol exert opposite actions on the production of the chemokine CCL2 in macrophages.

Treatment with CCL2 has been recently shown to induce the expression of monocyte chemotactic protein (MCP-1)-induced protein (MCPIP) in monocytes, a novel zinc-finger protein with multiple functions including the post-transcriptional regulation of proinflammatory gene expression, the regulation of autophagy and others (Reviewed in Kolattukudy and Niu 2012). In order to test whether the upregulation of *Ccl2* in Idol-overexpressing macrophages is detected autocrinally by Raw264.7 cells, the expression of *Mcpip1* was analyzed in Raw264.7-Idol, Raw264.7-empty and normal Raw264.7 cells. We observed a significant increase in the *Mcpip1* levels in the Raw264.7-Idol cells in comparison with the controls, suggesting that the endogenous production of CCL2 by the Raw264.7-Idol macrophages is indeed enhancing, in an autocrine manner, *Mcpip1* expression (Figure 24B).

Taken together, our results suggest that Idol activity positively regulates *Ccl2* expression, which in turn is able to stimulate specific gene expression in macrophages in an autocrine fashion.

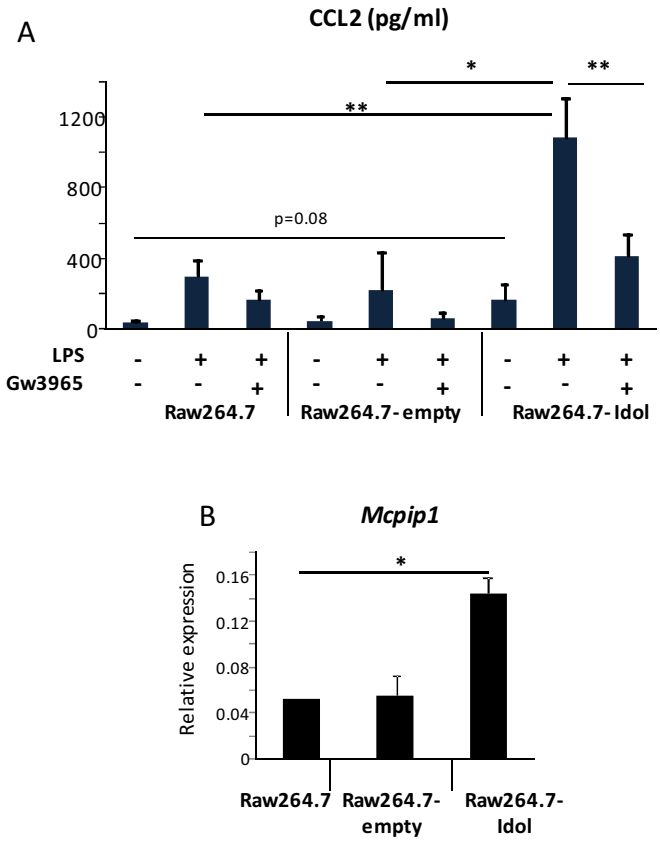

Figure 24. A) Idol overexpression in Raw264.7 macrophages results in increased secretion of CCL2. Cells were stimulated with LPS (5ng/ml) during 6 h in the presence or absence of GW3965 (1μM). CCL2 (MCP-1) concentration was analyzed by ELISA. The total protein concentration in each sample was used to normalize the data. Mean normalized values ± SEM from three independent experiments are represented. **B) Raw-Idol macrophages express higher levels of *Mcpip1*.** The mRNA levels of *Mcpip* were analyzed in control Raw264.7 cells and in cells transfected with empty vector or Idol-overexpressing vector. Mean values of normalized relative expression ± SEM from two independent experiments are represented. Statistical analysis was performed using a Student *t* test ; *p <0.05, **p < 0.01.

Given the fact that Idol regulates the half life of the LDL-receptor, we questioned if the increase in *Ccl2* expression observed upon everexpression of Idol would be indirectly mediated by diminished expression of the LDL receptor. To evaluate this possibility, we analyzed *Ccl2* expression in LDLR deficient (LDLR-/-) macrophages in comparison to wild type cells. If the LDLR was to be involved in *Ccl2* regulation, we would expect upregulation of the levels of *Ccl2* in LDLR-/- cells, similarly to the effects obtained after overexpression of *Idol*. However, LDLR-/- cells express the same levels of *Ccl2* than the wild type macrophages in response to LPS (Figure 25). Therefore, we can conclude that

the increase of *Ccl2* expression mediated by Idol is independent on the effects of Idol on the LDLR.

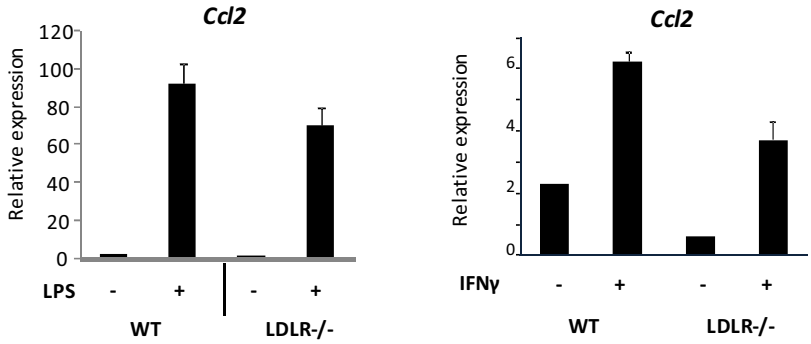

Figure 25. LDLR-/- macrophages do not overexpress *Ccl2*. BMDM obtained from wild type (WT) and LDLR deficient (LDLR-/-) mice were stimulated with LPS (5ng/ml) (left panel) or IFN-γ (5ng/ml) (right panel) in DMEM-1% FCS for 6h. *Ccl2* expression was measured by q-RT-PCR. Mean values ± SD of normalized relative expression of triplicates are shown.

III. Evaluation of the role of Idol in promoting phagocytosis of apoptotic cells

Apoptotic cell death is an important pathophysiological mechanism in many diseases including atherosclerosis. Also the clearance of apoptotic bodies is associated with normal resolution of inflammation.

Based on initial observations that the LXR target gene Idol could be interacting with the myosin regulatory light chain protein (MRLC) (Bornhauser et al., 2003b), and the implication of the MRLC in cytoskeleton restructuring during phagosome formation, we questioned whether Idol could be playing a role in macrophage mediated phagocytosis.

We used the stably transfected Raw 264.7-Idol cells to evaluate if Idol has a role in the clearance of dying thymocytes after dexamethasone (dex)-induced apoptosis *in vitro*. Apoptotic thymocytes (ATs) were fluorescently labeled before incubation with

Raw264.7 macrophages for 90min. In order to distinguish the macrophage cell membrane, we immunostained the cells with WGA (red). Cellular nuclei was visualized by Hoechst staining (blue). Interestingly, we observed that macrophages overexpressing Idol show a higher capacity for attaching ATs, which is further enhanced by LXR activation with GW3965 (Figure 26). This result suggests that Idol activity could be increasing the ability of macrophages to attach to apoptotic cells for subsequent phagocytosis.

Previous reports have shown that LXR activation can enhance phagocytosis in macrophages, due to induction of three different mediators of phagocytosis: ABCA-1, transglutaminase 2, and c-Mer tyrosine kinase (Hamon et al., 2000; Rébé et al., 2009; A-Gonzalez et al., 2009). We next wanted to test whether overexpression of Idol in Raw 264.7 macrophages potentiates phagocytosis along with LXR activation. Preincubation of cells during 18h with GW3965 before the challenge with ATs increased their capacity for both attaching and phagocyting ATs in the different cell clones evaluated in these studies. The highest % of phagocytosis was observed in Raw264.7-Idol cells stimulated with an LXR agonist (Figure 26). These results suggest a promising role for Idol in clearance of apoptotic cells although a higher number of experiments are required for statistical purposes.

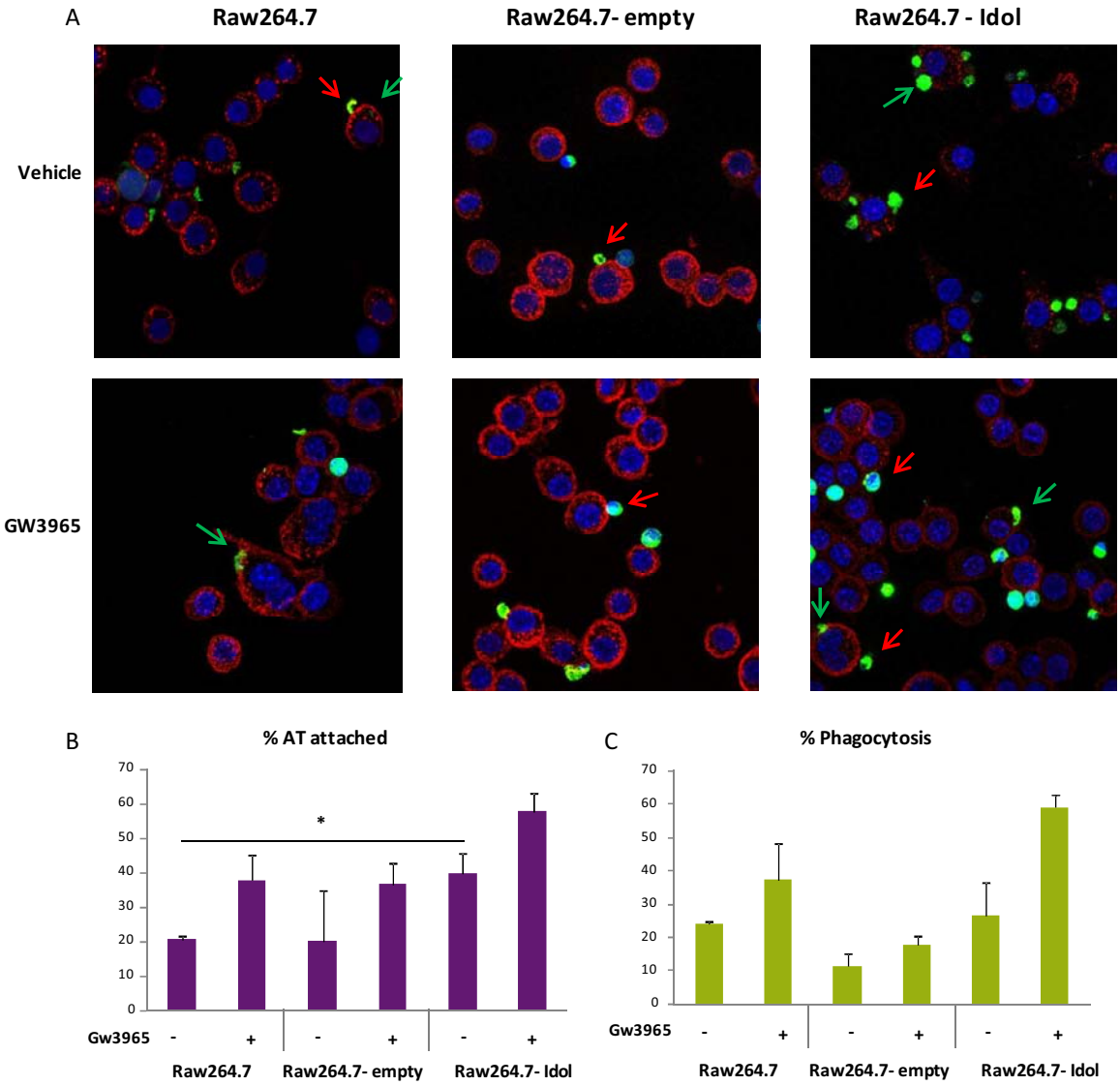

Figure 26. Phagocytosis of apoptotic thymocytes (ATs) by stably transfected Raw264.7–Idol macrophages. A) Phagocytosis of CellTracker Green CMFDA-labeled ATs was evaluated in Raw264.7, Raw264.7-empty or Raw264.7-Idol macrophages. ATs were cultured with macrophages for 90min, a ratio of 5 ATs:1 macrophage was used. Attached and engulfed cells were distinguished by extensive washing with cold PBS and Enzyme Free Cell Dissociation Buffer to remove free ATs. Macrophage phagocytosis was evaluated by fluorescence confocal microscopy. Red arrows indicate attached ATs, green arrows indicate engulfed ATs. Macrophages are observed in red, ATs in green in cell nuclei in blue. Quantification of attachment (B) and phagocytosis (C) is expressed as % of AT attached/Phagocytosis: number of cells with ATs attached/ingested vesicles per total number of macrophages x 100. Results are the average ± SEM of three independent experiments for vehicle treated cells, and duplicate experiments for GW3965 treated cells.

IV. LXR mediated effects in alternative activation of macrophages

The role of LXR in the regulation of inflammatory responses mediated by classically activated macrophages has been the focus of numerous studies; however, the relationship between LXR and the alternative activation of macrophages has not been described. In the next part of this work we evaluated whether LXR activation affects the expression of alternative activation marker genes, with a special focus on the study of the effects of LXR activation in the IL-4-mediated response of macrophages.

First we performed an IL-4-stimulation time-course, preincubating BMDM for 6 h with the LXR ligand GW3965 before the incubation with IL-4 during 24, 36 and 48 h. We observed that the expression of the main genes associated with alternative macrophage activation, namely *Ym1*, *Arg1*, *Mgl1* and *Mrc1*, were not affected by the treatment with the LXR ligand. However, incubation of macrophages with GW3965 significantly inhibited the expression of the genes *Ccl22* and *Ccl17,* that codify for the chemokines CCL22 (MDC) and CCL17 (TARC), at 12 h of stimulation with IL-4, and it continued to reduce their expression after 24 and 36 h of stimuli (Figure 27).

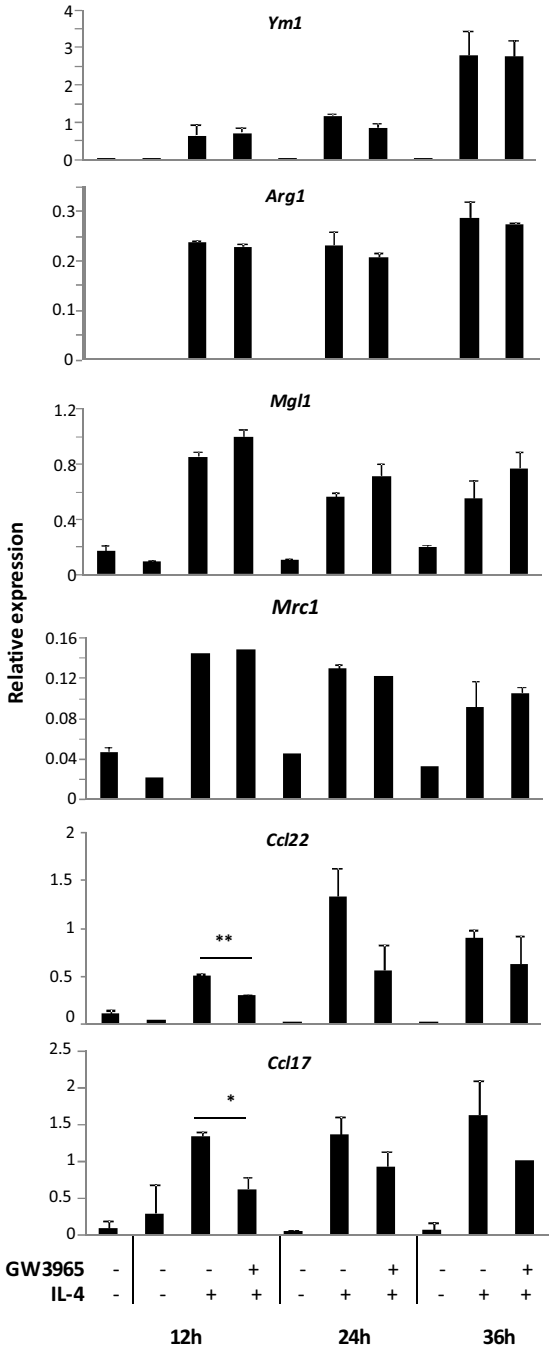

Figure 27. LXR activation inhibits IL-4-mediated expression of *Ccl22* and *Ccl17* without broadly affecting the macrophage alternative activation-program. BMDM obtained from WT mice were preincubated with GW3965 for 6 h before treatment with IL-4 (10 ng/ml) during 12, 24 and 36 h. mRNA levels were determined using qRT-PCR. Mean values of normalized relative expression ± SEM from three independent experiments are represented. Statistical analysis was performed using a Student *t* test; *p <0.05, **p < 0.01.

The next question we wanted to assess was whether the presence of functional LXR is necessary or affects the induction of alternative activation marker genes. Figure 28

shows the relative expression of IL-4-induced genes in BMDM from wild-type (WT) and LXRαβ deficient (LXRαβ DKO) mice stimulated during 12, 24 and 36 h with IL-4.

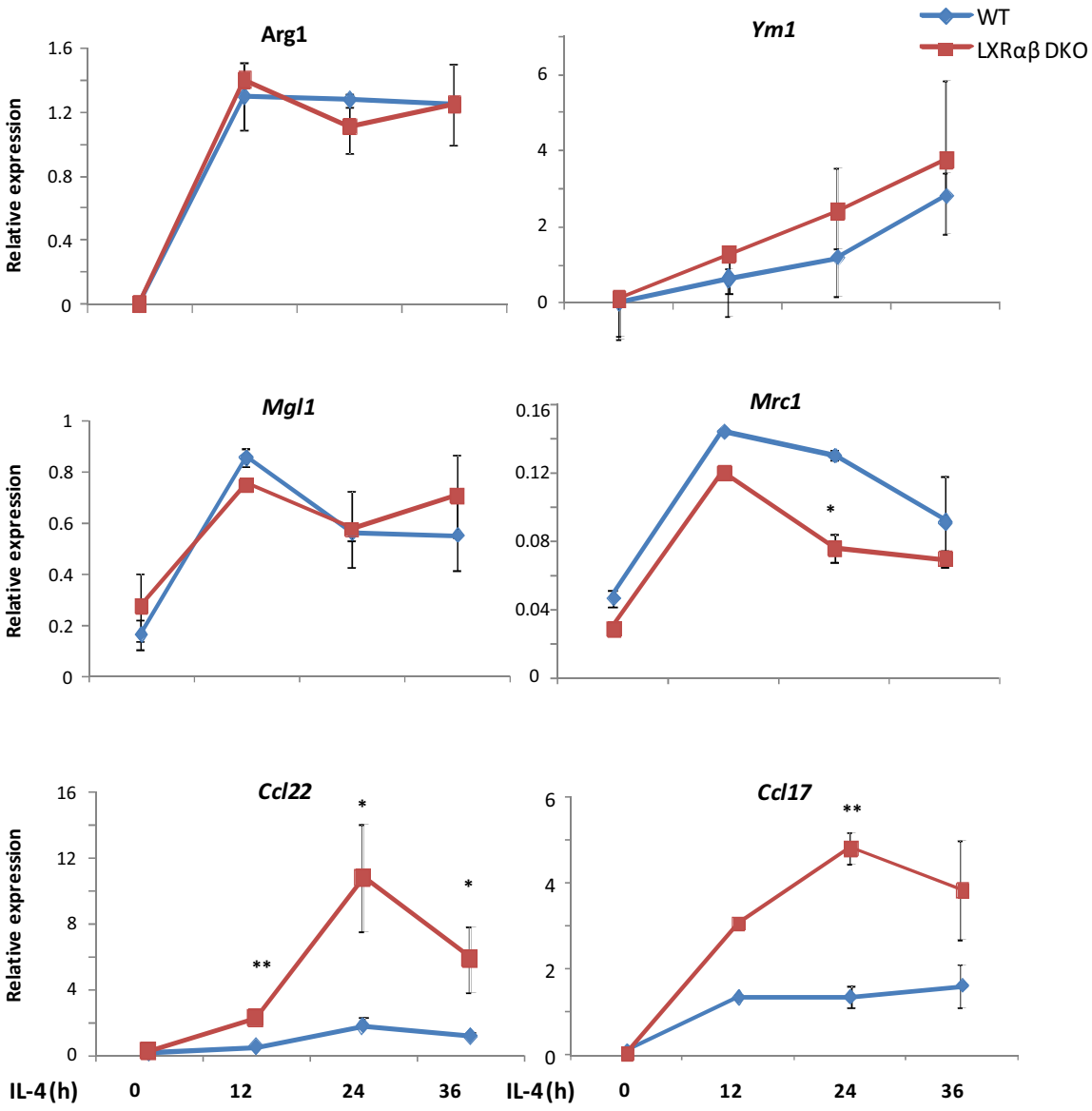

Figure 28. Ccl22 and Ccl17 are overexpressed in LXRαβ deficient macrophages. BMDM were obtained from WT or LXRα and LXRβ deficient (LXRαβ DKO) mice. Cells were treated with IL-4 (10 ng/ml) or PBS during 12, 24 and 36 h. mRNA levels were determined using qRT-PCR. Mean values of normalized relative expression ± SEM from three independent experiments are represented. Statistical analysis of WT versus LXRαβ DKO was performed using a Student *t* test; *p <0.05, **p < 0.01.

In general, the lack of LXR does not affect the pattern of expression of macrophage alternative activation-marker genes. However, the IL-4 induced-genes that were affected by preincubation with LXR ligand, namely *Ccl22* and *Ccl17*, showed robust

significant overexpression in IL-4-treated LXRαβ deficient macrophages (Figure 28). Together, these results indicate that LXR exert selective negative regulatory actions on *Ccl22* and *Ccl17* expression; both genes are repressed by LXR activation, and they are overinduced by IL-4 in the absence of functional LXRs.

To evaluate if *Ccl22* overexpression in IL-4-stimulated LXRαβ deficient macrophages results in increased secretion of this chemokine to the medium, supernatants of IL-4 stimulated macrophages were analysed by ELISA. As expected, the concentration of CCL22 was higher in the supernatants from LXRαβ DKO cells than in those from WT cells (Figure 29), in correlation with the observations at the mRNA level (Figure 28).

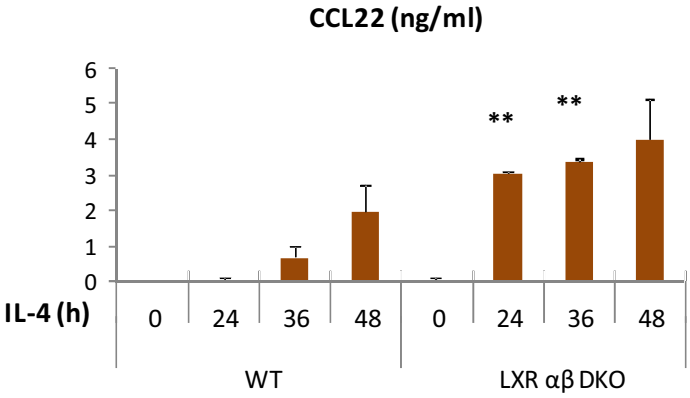

Figure 29. CCL22 production from IL-4 stimulated-BMDM *in vitro*. BMDM from WT and LXRαβ deficient mice were stimulated with IL-4 (20ng/ml) for 24, 36 and 48 h. CCL22 secretion in supernatants was measured by ELISA. Mean values ± SEM from three independent experiments is represented. Statistical analysis was performed using a Student *t* test on normalized relative expression values of LXRαβ DKO versus WT; **p < 0.01.

With the aim of further evaluating the specific contribution of each LXR isoform in the inhibition of the expression of *Ccl22* and *Ccl17*, we used macrophages obtained from either LXRα deficient (LXRα KO), LXRβ deficient (LXRβ KO) or both LXRαβ deficient mice (LXRαβ DKO). BMDM were pretreated with 1μM of the LXR ligands T1317 and GW3965 during 6 h before incubation with IL-4 for 12 h.

Our results indicate that both isoforms are required to mediate the ligand-dependent repression of *Ccl22,* whereas repression of *Ccl17* by LXR synthetic agonists is mainly mediated by LXRα. However, both LXRα and LXRβ isoforms, are required in the

absence of synthetic agonists, to keep under control the expression of *Ccl22* and *Ccl17* in response to IL-4 (Figure 30).

Moreover, the inhibitory effects obtained with the ligands disappeared completely in the macrophages deficient for both isoforms, demonstrating that the T1317 and GW3965 effects are specifically mediated by the LXR pathway.

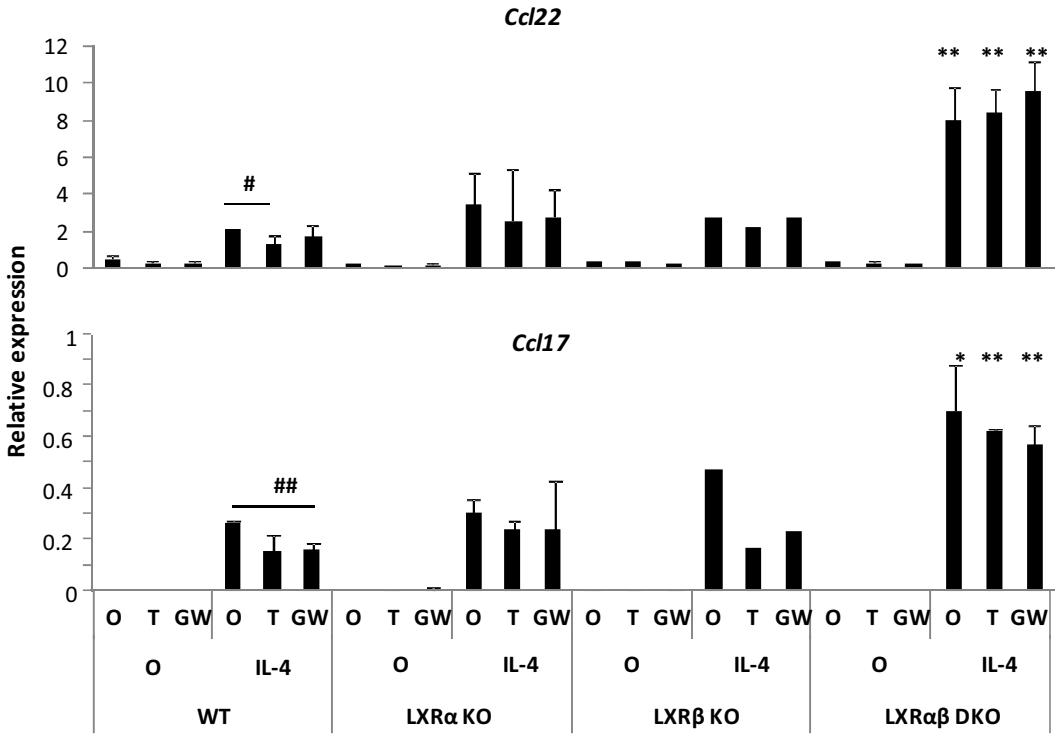

Figure 30. Both LXR isoforms mediate inhibition of Ccl22 and Ccl17 expression. BMDM were obtained from WT mice or mice deficient for either LXRα (LXRα KO), LXRβ (LXRβ KO), or both LXRα and LXRβ (LXRαβ DKO). Cells were prestimulated with vehicle (O), T01317 (T) or GW3965 (GW) for 6h and then treated with IL-4 (20 ng/ml) during 12 h and control cells were left untreated. mRNA levels were determined using qRT-PCR. Mean values of normalized relative expression ± SEM from at least two independent experiments are represented. Statistical analysis was performed using a Student *t* test on normalized relative expression values of LXRαβ DKO versus WT, **p < 0.01. # p <0.05, ## p < 0.01 versus IL-4 treated cells.

IL-4 signaling is mediated mainly by the activation of the transcription factor STAT-6, which becomes phosphorylated and translocates to the nucleus to activate the transcription of selective genes. In order to test whether LXR activation has an impact on the IL-4 signaling pathway, and more specifically on STAT6 activation, we analysed

STAT-6 phosphorylation by western-blotting. A time course treatment with IL-4, in the presence or absence of GW3965, was carried out in BMDM obtained from wild type and LXRαβ DKO animals and phosphor-STAT-6 was analyzed from protein extracts using specific antibodies (Figure 31).

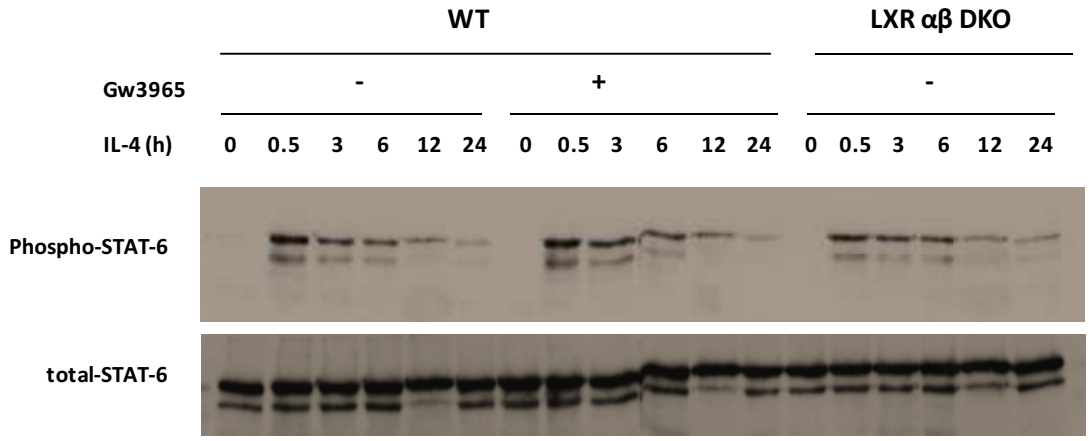

Figure 31. The LXR pathway does not alter the STAT-6 phosphorylation pattern. Immunoblot analysis of phosphorylated and total STAT-6 from protein extracts of BMDM obtained from WT or LXRα and LXRβ deficient (LXRαβ DKO) mice. Cells were preincubated with GW3965 or vehicle for 18 h and then stimulated with IL-4 (20 ng/ml) or PBS during 0.5, 3, 6, 12, and 24 h before protein extraction.

In wild type macrophages we observed that STAT-6 is phosphorylated at 30 min and the signal diminishes gradually with time, as seen at 3, 6, 12 and 24 h after IL-4 stimulation. Preincubation with the LXR agonist GW3965 does not modify the phosphorylation pattern of STAT-6. There are no observable differences between wild type and LXRαβ deficient cells either. This result correlates with the previous observations that LXR does not affect the general transcriptional activities of IL-4 in macrophages, but rather exerts gene-specific effects.

Another way in which LXR could be affecting the expression of the genes *Ccl17* and *Ccl22* is through interfering the binding of STAT-6 to its specific response elements (GAS elements) in the regulatory regions of these genes. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) assays using macrophages from wild type and LXR deficient cells, stimulated with IL-4 during 2 h, and evaluated the binding of STAT-6 to different regions located in the promoters of *Ccl17* and *Ccl22*. We did not obtain any enrichment of immunoprecipitated chromatin with anti-STAT-6 from those

regions (data not shown), therefore it is necessary to explore other possible STAT-6-interacting sequences in the regulatory regions of these genes that allow us to determine whether LXR exerts any type of interfering activity on the binding of STAT-6.

Role of LXR in a murine model of allergic asthma

The chemokines CCL17 and CCL22 have been demonstrated to be involved in allergic inflammation diseases such as asthma (Bisset and Schmid-Grendelmeier, 2005). Based on the effects of LXRs on the regulation of these chemokines, we decided to evaluate the role of LXR in a murine model of allergic asthma. In collaboration with Dr. De Mora's group (Universitat Autònoma de Barcelona) we performed an experiment in which *wild type* and LXRαβ double *knockout* mice were exposed to house dust mite (HDM) extract by intranasal (i.n.) administration during 10 days. 24 h after the last HDM administration, the respiratory function was evaluated and the animals were sacrificed.

We observed that the HDM-sensitized LXRαβ deficient animals showed a significantly impaired respiratory function, detected by direct invasive measurements of airway resistance (R_L) and compliance (Cdyn) to increasing dosis of methacholine. LXRαβ deficient mice showed an enhanced airway resistance curve that correlates with a significantly diminished compliance response in comparison with HDM-sensitized *wild type* mice (Figure 32).

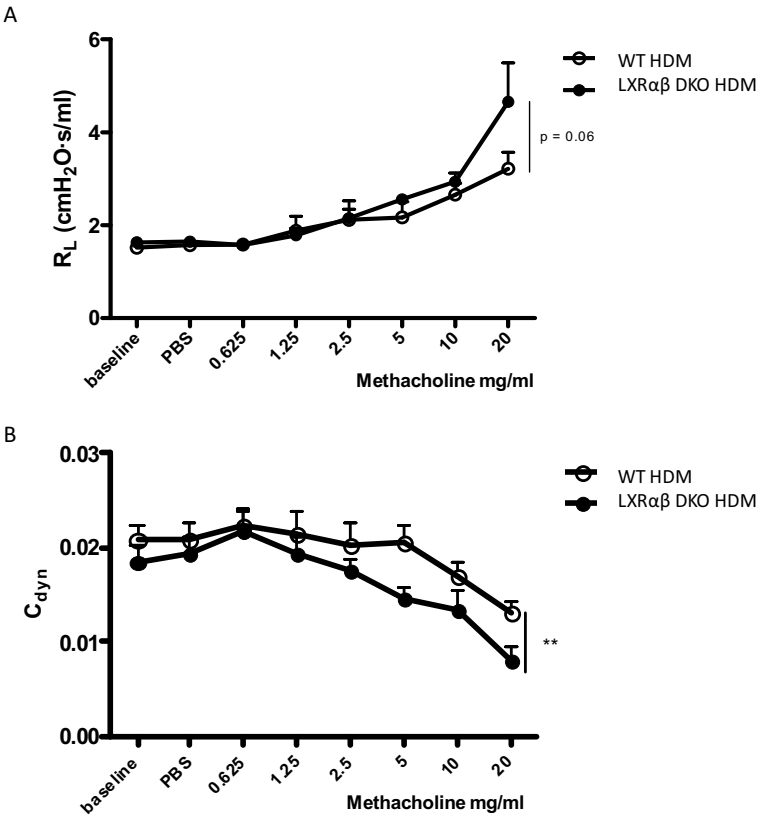

Figure 32. HDM-induced airway resistance in WT and LXRαβ deficient mice. Airway resistance to increasing doses of methacholine was assessed through an invasive procedure (Buxco) 24h after the last HDM exposure (10 days). A) Lung Resistance (R_L) and B) Compliance (Cdyn) data were compared between groups by a two-way repeated-measure ANOVA with a *post hoc* Bonferroni test. ** p<0.01. (n=5 WT animals; 4 LXRαβ DKO animals). This experiment was developed in collaboration with Dr. Fernando de Mora's group, Department of Pharmacology, Universitat Autònoma de Barcelona.

We next assessed whether LXR activation by intraperitoneal (i.p.) injection of GW3965 could have a beneficial effect in the same model of allergic asthma. To evaluate this, a separate experimental group of *wild type* mice were HDM-sensitized and treated with i.p. injections of the LXR ligand GW3965, 24 h before and once daily during the 10 day course of allergen exposure. Another group of HDM-sensitized was treated i.p. with vehicle (DMSO) and a control group of mice was left non-sensitized. In all cases, 24 h after the last HDM administration we evaluated the respiratory function. The animals were then sacrificed and samples of BAL, blood and lungs were isolated.

As expected, exposure to HDM induced significant airway resistance (R_L) after the last challenge in the vehicle-treated mice (HDM+VEH, Figure 33) as compared with non-sensitized mice (NS+VEH). Importantly, we observed that HDM-sensitized mice, that were treated with GW3965 (HDM+GW3965) showed an airway resistance to methacholine that remained close to baseline levels, similar to the lung response of non-sensitized mice. This result suggests that GW3965 treatment provides beneficial effects in preventing the pulmonary deficiency in this model of allergic asthma disease.

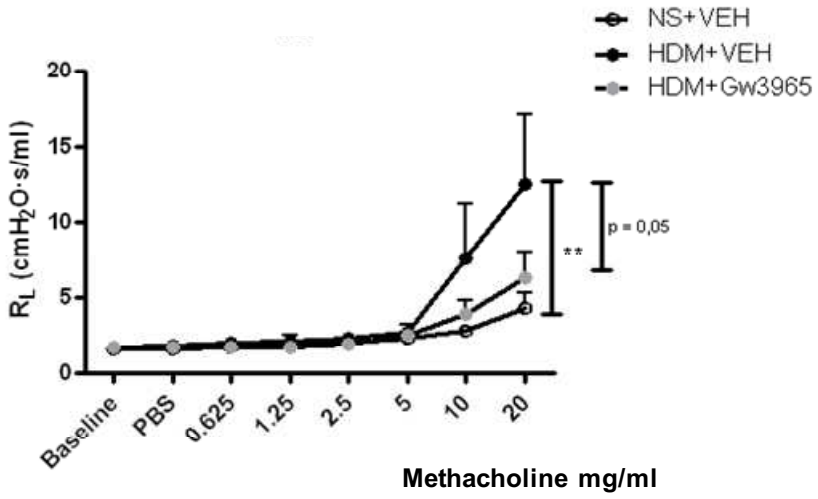

Figure 33. Effect of the LXR ligand GW3965 on the airway response of HDM-sensitized mice. Three groups of animals were evaluated: NS (non-sensitized) and i.p. injected with vehicle (NS+VEH), HDM-sensitized and i.p. injected with vehicle (HDM+VEH) and HDM-sensitized and i.p. injected with GW3965 (20mg/kg) (HDM+GW3965). Airway resistance to increasing doses of methacholine was assessed through an invasive procedure (Buxco) 24h after the last HDM exposure. Lung Resistance (R_L) data were compared between groups by a two-way repeated-measure ANOVA with a *post hoc* Bonferroni test, ** $p<0.01$, (n=6 animals per group). This experiment was developed in collaboration with Dr. Fernando de Mora's group, Department of Pharmacology Universitat Autònoma de Barcelona.

In order to identify some of the airway inflammatory players affected by GW3965, the mRNA expression levels of several chemokines, chemokine receptors and cytokines in the lung tissues were evaluated by q-RT-PCR.

We observed significantly increased expression of the mRNA levels of the chemokines *Ccl17*, *Ccl22*, and *Ccl2* as well as of *Il4* and *Ccr8* (the receptor for CCL1) in the lungs of

HDM-sensitized mice. Interestingly, the GW3965-treated mice displayed a diminished cytokine expression pattern in comparison with the vehicle-treated HDM-sensitized mice. A solid reduction of the levels of *Ccl22* and *Ccl17* was observed in the group of animals treated with the LXR ligand, and in a lower extent, the expression of *Ccl2*, *Ccr8*, and *Il4* was also diminished in this group (Figure 34).

In addition to the gene expression analysis in the lung tissues, serum and BAL fluid samples were analysed for CCL22 and CCL17 chemokine levels by ELISA. As expected, these two chemokines were augmented in the HDM-sensitized mice in comparison with non-sensitized mice in both BAL fluid and serum. However, no differences were observed between the GW3965 treated and non-treated groups (data not shown), suggesting that GW3965 administration has a local effect in lungs rather than affecting chemokine production at systemic levels.

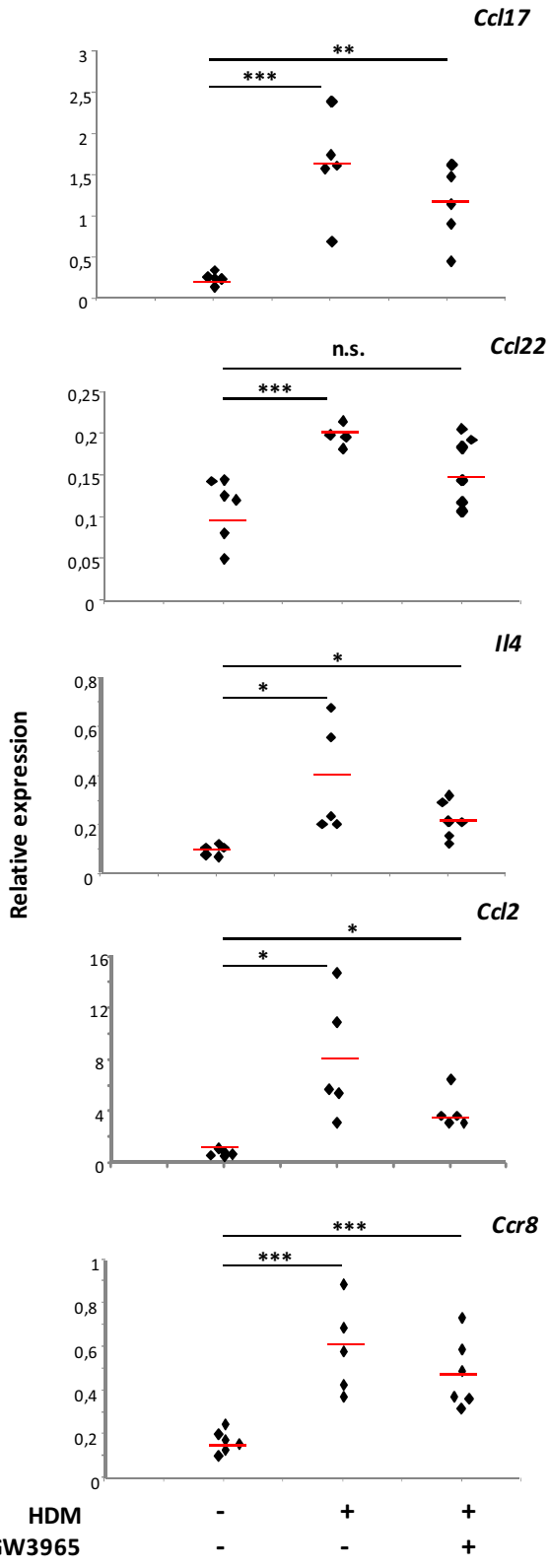

Figure 34. GW3965 local inhibitory effects in lungs of 10-day HDM-sensitized mice. LXR activation was induced by i.p. injection of GW3965 (20mg/kg) from -1 to 9 days parallel to the HDM i.n. administration. Lung homogenates were analysed by q-RT-PCR (n=6 animals per group). Comparisons between groups were made by one-way ANOVA test. *p<0.05, ** p<0.01, ***p<0.001.

In order to evaluate the inflammatory changes induced by GW3965 treatment in the HDM-sensitized lungs, the degree of bronchovascular cell infiltration was assessed. Of note, the total inflammatory cell count in BAL fluid of HDM-sensitized mice was significantly augmented, and it was partially reduced in the GW3965-treated group. Additionally, the different inflammatory cells in BAL fluid were counted. The result shows a significantly higher number of eosinophils in the mice exposed to HDM aeroallergens in comparison with non-sensitized animals. Similarly, in GW3965 treated mice we observed a trend towards a lower number of eosinophils and monocytes when compared to animals that were HDM-sensitized but injected with vehicle alone (Figure 35).

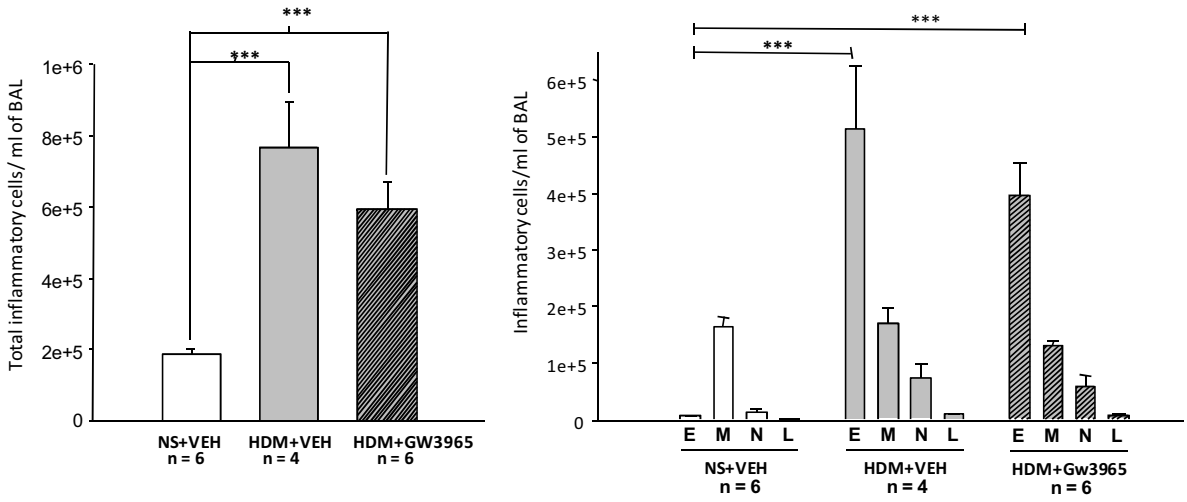

Figure 35. Total and differential inflammatory cell counts in BAL fluid. Samples were taken 24 h after 10 days of exposure to HDM. Data are expressed as mean ± SEM. Comparisons between groups were made by Student's *t* test. This data was obtained in collaboration with Dr. Fernando de Mora's group, Department of Pharmacology Universitat Autònoma de Barcelona. E, eosinophils; M, monocytes; N, neutrophils; L, lymphocytes; NS, non-sensitized (white bars); HDM+VEH, HDM-sensitized (grey bars); HDM+ GW3965, GW3965-treated HDM-sensitized (dark grey bars) (***$p < 0.001$).

In general, our results suggest that LXR activation is able to exert a local anti-inflammatory role in the lungs of HDM-sensitized mice, without robust effects on systemic production of inflammatory mediators. Such local effects are sufficient to inhibit airway resistance induced by HDM exposure.

Reciprocal effects of macrophage alternative activation on the expression of LXR target genes

As a complementation to these studies, we also explored if stimulation with IL-4 has a reciprocal impact on the expression of LXR target genes. For these experiments, we pretreated BMDM with IL-4 for 18h and then activated the LXR pathway with 1μM of the agonists T1317 and GW3965 during 24 h. In this set of experiments we also compared the effects of IL-4 with those of deactivating cytokines, such as IL-10 and TGF-β.

In macrophages previously stimulated with IL-10 and TGF-β, we analysed the mRNA expression level of the LXR target genes *Abca1*, *Abcg1*, *Srebp1c*, *Idol*, *Apoc2*, and *Apoe* and we did not observed any significant change in their expression compared to the untreated cells (Figure 36). Only *Aim* (*Spα*) seems to be slightly increased in the IL-10 treated cells, and lightly inhibited by the TGF-β pretreatment.

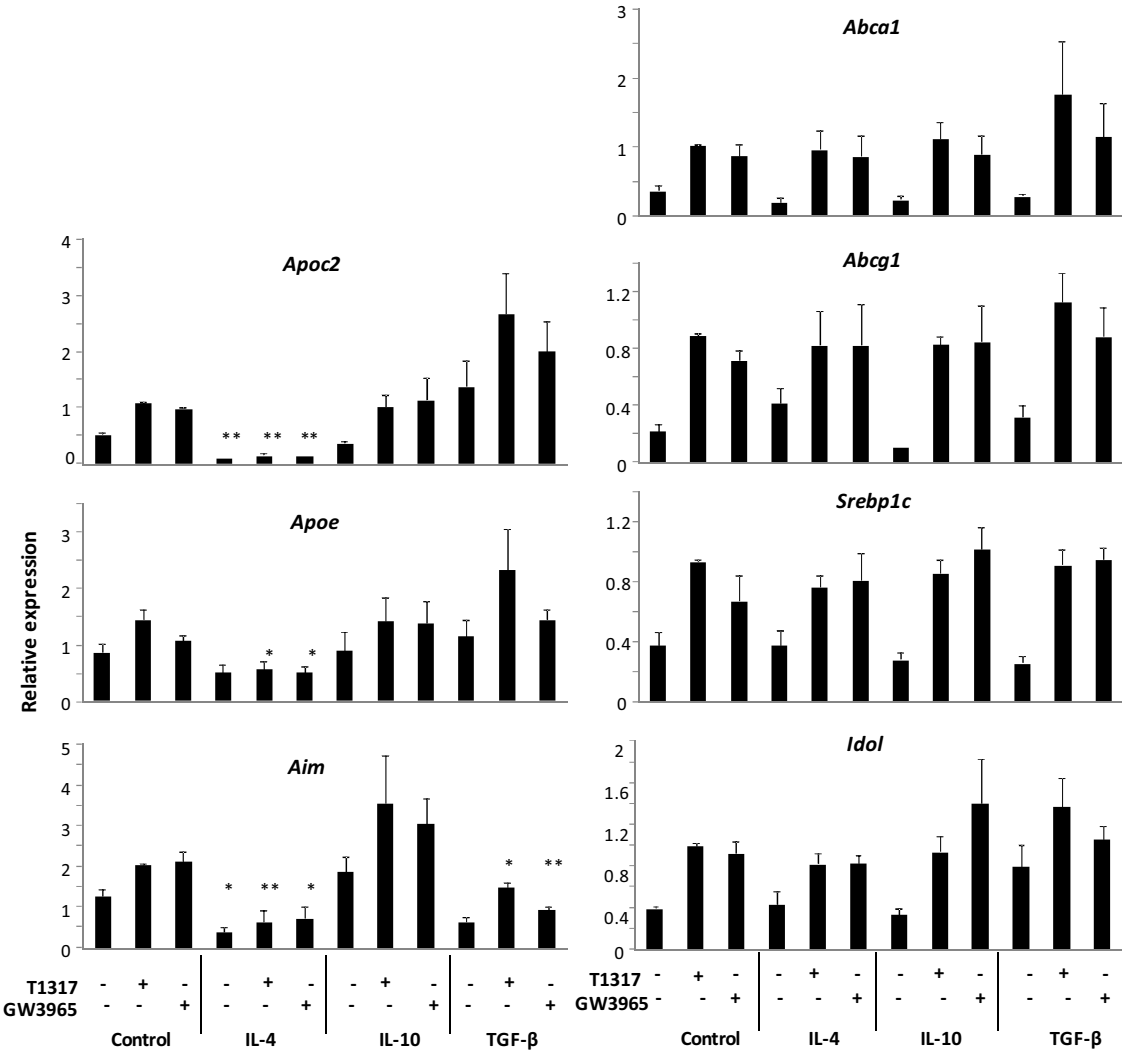

Figure 36. Reciprocal IL-4-mediated inhibition of specific LXR target genes. BMDM obtained from WT mice were preincubated with either IL-4 (20 ng/ml), IL-10 (10 ng/ ml), or TGF-β (2 ng/ml) for 18 h and then treated with T1317 or GW3965 for 24 h. mRNA levels were determined using qRT-PCR. Mean values of normalized relative expression ± SEM from three independent experiments are represented. Statistical analysis was performed using a Student *t* test; *p<0.05, **p< 0.01 versus IL-4 control.

Interestingly, the treatment with IL-4 significantly inhibited the expression of the genes *Apoc2*, *Apoe* and *Aim* at the basal level and in response to the LXR ligands T1317 and GW3965. However, the induction of other LXR targets, such as *Abca1*, *Abcg1*, *Srebp1c* and *Idol* was not diminished with the IL-4 stimulation. The fact that IL-4 does not inhibit all the genes induced by LXR suggests that the interference of IL-4 is gene specific and it does not imply an effect over all the general activity of LXR.

To evaluate the implication of STAT-6 in the inhibitory effects of IL-4 over LXR induced genes, we studied the expression of the LXR target genes in BMDM obtained from wild

type or STAT-6 deficient mice (Stat6 KO). We observed that the inhibition of *Apoc2*, *Apoe* and *Aim* by IL-4 is lost in the Stat6 KO macrophages (Figure 37), indicating that IL-4 represses the LXR-mediated induction of *Apoc2*, *Apoe* and *Aim* in a STAT-6 dependent manner.

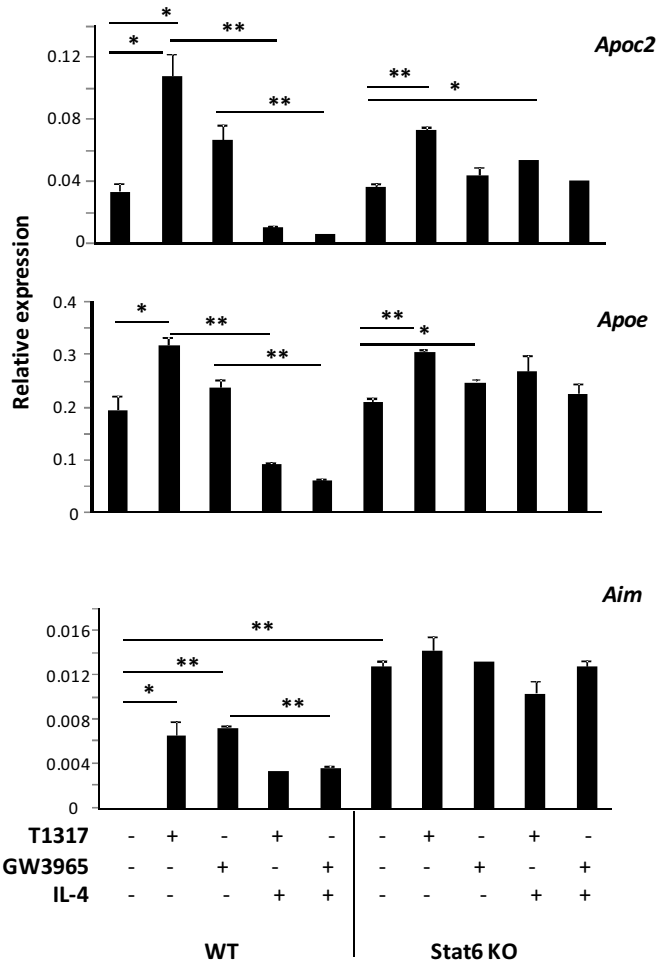

Figure 37. IL-4-mediated inhibition of *Apoc2, Apoe* and *Aim* LXR target genes is STAT-6 dependent. BMDM obtained from WT and STAT-6 deficient mice were preincubated with IL-4 (20 ng/ml) for 18 h and then treated with T1317 or GW3965 for 24 h in triplicates. mRNA levels were determined using qRT-PCR. Statistical analysis was performed using a Student *t* test; *p<0.05, **p< 0.01.

In order to get more insight into the mechanism by which IL-4 inhibits the expression of specific LXR target genes, the binding of LXR to a regulatory site in the promoter of *Aim* was evaluated. The putative binding site of LXR/RXR heterodimers on the regulatory region of *Aim* was chosen by analysis of chromatin immunoprecipitation (ChIP)-Sequencing data (see materials and methods). We performed ChIP assays in macrophages pretreated with IL-4 during 12 h before the stimulation with a

combination of LXR and RXR ligands for 90min, and the relative enrichment of specific binding of LXR and RXR to the region of interest was assessed by q-RT-PCR (Figure 38). The binding of LXR and RXR to the promoter of *Srebp1c* (whose expression is not affected by IL-4) was analysed as a control.

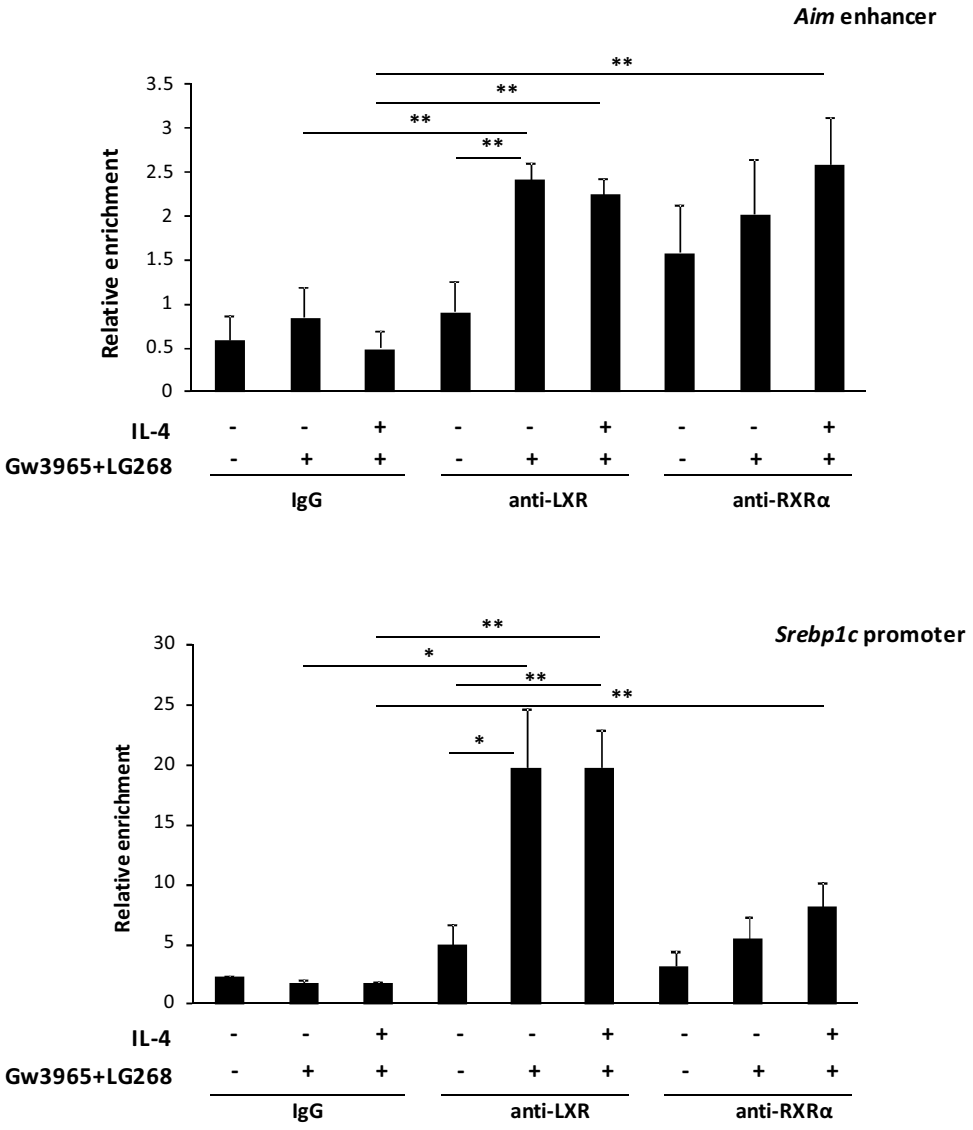

Figure 38. LXR and RXR binding to the *Aim* regulatory region are not affected by IL-4 stimulation. BMDM were pretreated with IL-4 for 12 h and then stimulated with GW3965 + LG268 or vehicle for 90 min. LXR and RXR binding to the promoter region of *Aim* and *Srebp1c* was analysed by ChIP assay. Total LXR and RXR were immunoprecipitated using specific antibodies. Rabbit IgG was used as a control. The presence of specific DNA fragments was determined by qRT-PCR. Mean results of enrichment of LXR- or RXR-bound DNA ± SEM are represented normalized by the levels of β-actin and each respective input DNA; n = 4 independent experiments. Statistical analysis was performed using a Student *t* test; *p<0.05, **p< 0.01.

The results in Figure 38 show that the binding of LXR to the *Aim* regulatory region is enhanced by the combined treatment with the LXR/RXR ligands. However, such binding is not inhibited in the presence of IL-4. Similar results were also observed for the binding of LXR to the promoter region of *Srebp1c*. These results indicate that IL-4 is able to inhibit the expression of *Aim*, without affecting negatively the LXR ability to bind to the *Aim* enhancer region.

Discussion

Strong evidence has been gathered in the past decade that supports the notion that LXRs exert key inhibitory roles in the control of inflammation (reviewed in A-González and Castrillo, 2011). More specifically, several studies have focused on the capability of LXR agonists to repress inflammatory signaling in response to bacterial LPS (Joseph et al., 2003; Ghisletti et al., 2007, 2009), with important contributions to the understanding of the molecular mechanisms governing this process. Furthermore, previous work in our laboratory has served to demonstrate that LXR ligands are able to repress a large extent of the pro-inflammatory genes that are induced by IFN-γ in macrophages and microglia, thus ameliorating the acquisition of a macrophage M1 phenotype in response to an endogenous inflammatory cytokine (Pascual-García et al., 2013).

In the first part of the current study, we have continued to explore some of the aspects underlying LXR-mediated repression of classically activated macrophages. We have observed that the efficiency of transcriptional repression by LXRs agonists differs depending on the inflammatory stimulus that is used. In bone marrow-derived macrophages, activation of LXR resulted in partial repression on IFN-γ-inducible inflammatory genes whereas more robust inhibitory effects were observed on LPS-inducible genes. In fact, these results are in agreement with previous reports that suggest that the mechanism/s used for transrepression differ depending on the pro-inflammatory trigger (Ghisletti et al., 2007, 2009).

It is probable that what we understand collectively as transrepression is indeed a combination of mechanisms that are signal-specific and gene-specific. A mechanism based in the SUMOylation of LXRs in the presence of LXR agonists has been proposed to be required for repression of a subset of LPS-inducible genes. SUMOylated LXRs interact with a corepressor complex harbouring NCoR to prevent the recruitment of the ubiquitin conjugating enzyme UbcH5 and the 19S proteasome subunit, which are required to release the corepressor complex from the promoter of LPS-induced genes (reviewed in Glass and Saijo 2010). However, whether or not LXR affects IFN-γ-mediated release of corepressor complexes from target gene promoters is not clear. Our previous work indicated that LXR, in a SUMOylation-dependent and NCoR-

independent manner, interferes with the capability of STAT1 to directly bind to specific genomic regulatory regions.

Notably, in our studies we have observed that conversion of macrophages to foam cells resulted in dramatic inhibition of the inflammatory response to LPS, but not to IFN-γ. While this work was in progress, a study demonstrated that peritoneal macrophages obtained from LDLR knockout animals fed with a high-cholesterol, high-fat diet displayed a "deactivated" phenotype, with marked suppression of TLR4 target genes. Induction of several pro-inflammatory markers by Kdo2 lipid A, a structurally defined saccharolipidic component of LPS, was also down-regulated by in vitro cholesterol loading of macrophages from murine and human origin (Spann et al., 2012). In the same line of evidence, treatment of Raw264.7 cells with oxLDL markedly inhibited production of LPS-induced inflammatory mediators (Min et al., 2012).

Moreover, it has been proposed that oxidized phospholipid-treated murine macrophages develop into a phenotype (Mox) that is different from conventional M1 and M2 macrophage phenotypes. Compared to M1 and M2, Mox macrophages show a different gene expression pattern, as well as decreased phagocytotic and chemotactic capacity. Apparently, Mox macrophages comprise 30% of all macrophages in advanced atherosclerotic lesions of LDLR-deficient mice (Kadl et al., 2010).

Our results with LPS-treated foam cells support the hypothesis that cholesterol accumulation suppresses, rather than stimulates, TLR-dependent activation of pro-inflammatory gene expression in macrophages. However, the fact that the inflammatory response to IFN-γ remains almost unchanged after cholesterol loading is intriguing and may help explain why the most abundant phenotype within established atherosclerotic lesions, approximately 40% of foam cells, is still the classically activated or M1 macrophage (Kadl et al., 2010).

The mechanisms that mediate the drastic suppression of TLR4-induced genes after acLDL-induced foam cell formation are still not clear. We have explored whether well-established LPS signaling pathways are affected in these cells, based on the fact that a previous report had demonstrated that conversion of Raw264.7 macrophages to foam cells by oxidized LDL treatment resulted in reduced degradation of IκB and subsequent

blockage of the activation and nuclear translocation of NF-κB (Min et al., 2012). Moreover, it has been proposed that structural components within modified LDLs can be recognized by TLR4 (Mogilenko et al., 2012; Miller et al., 2005). Based on these observations, competition for TLR4 binding has been considered as a potential mechanism by which modified LDLs negatively affect LPS-induced signaling. However, in our system, we have not observed that acLDL treatment leads to significant changes in the capability of LPS to induce either degradation of IκB or stimulation of other signaling pathways such as p38 and ERK. Therefore, our data does not support the theory that acLDL may use a competition strategy to partially block TLR4-dependent responses.

Another hypothesis that we have considered is that macrophage lipid loading may result in the generation of natural agonists for nuclear receptors, including LXR itself and perhaps PPAR-γ, that can interfere with transcriptional events occurring downstream of NFκB activation. Activation of the LXR pathway upon cholesterol loading by treatment with modified LDLs has been extensively documented (Reviewed in Pascual-García and Valledor 2012) and, in our hands, acLDL caused upregulation of the LXR target *Abca1*. Moreover, specific lipid components of oxLDL, namely 9-HODE and 13-HODE, have been also shown to represent natural agonists for PPAR-γ (Nagy et al., 1998). We believe that this type of components may also be generated after incorporation of acLDL particles, especially under highly oxidative conditions such as the macrophage response to LPS.

Previous expression profiling studies have demonstrated that LXR and PPARγ can both transrepress a large number of genes induced by LPS in macrophages (Ogawa et al., 2005). Through extensive gene expression profiling analysis performed in collaboration with Dr. Ricote's group at CNIC, Madrid, we have characterized LXR- and PPAR-γ-agonist specific effects in the transrepression of LPS-inducible genes. Our results indicate that LXR and PPAR-γ share the capability to transrepress a large number of genes, including, for example, *Nos2*, *Ptgs2* (C*ox2*) and *Ccl2* (*Mcp1*). There is a subset of genes that are only sensitive to the PPAR-γ agonist and another group, of reduced size, that can only be repressed by the LXR agonist. Of note, a previous report described *Il12b* and *Il6* as genes potentially inhibited by the LXR agonist GW3965 (Ghisletti et al.,

2009; Joseph et al., 2003). However, in our hands, these effects were only observed with high doses of LPS, never reached 30% of transrepression and were not LXR-specific, as they were also present in LXRαβ-deficient cells (data not shown). In fact, the use of LXR-deficient mice is an important strength of our study, with which the GW3965-mediated effects were tested for LXR specificity, an aspect that has been missing in many previous reports.

Although LXR and PPARγ share many transrepression targets, our data indicate that PPARγ is able to repress those genes independently of the presence of functional LXRs, which suggests that both nuclear receptors act in parallel. The possible involvement of PPARγ in the anti-inflammatory actions of acLDL is, at this point, only speculation, as we have not been able to use PPARγ-deficient cells for these assays. Remarkably, beyond the anti-inflammatory action of acLDL loading *per se*, the high-affinity synthetic LXR agonist GW3965 was able to further repress IFN-γ– and LPS-dependent responses in acLDL-induced foam cells, which is in agreement with previous reports demonstrating anti-inflammatory actions of synthetic LXR agonists within the artery wall *in vivo* (Joseph et al., 2003; Terasaka et al., 2005) and helps support the therapeutic value of synthetic LXR ligands in the context of atherosclerosis.

Interestingly, and again in collaboration with Dr. Ricote's group, we have observed that LXR-mediated transrepression of several genes, e.g. *Pdpn*, *Ptgs2* and *Ccl2*, is significantly impaired in RXRαβ deficient macrophages. This observation suggests that LXR and RXR cooperate for some of the transrepressive effects mediated by LXRs. Whether nor not heterodimerization between LXR and RXR contributes to transrepression mediated by both nuclear receptors is currently being investigated by Dr. Ricote's group.

Part of this doctoral thesis work has focused on the analysis of the role of the LXR/RXR target gene Idol in regulation of inflammatory gene expression. In our hands, overexpression of Idol did not inhibit the inflammatory response to LPS and did not lead to general changes in the capability of LXR to transrepress pro-inflammatory genes. However, striking enhancement of the basal and signal-induced expression of *Ccl2* (Mcp1) was observed in Idol-overexpressing cells.

One of the features of Idol is its role as an E3 ubiquitin ligase, specifically implicated so far in the post-translational control of members of the LDLR family (Zelcer et al., 2009; Hong et al., 2010). In our hands, overexpression of *Ccl2* is independent of the inhibitory role that Idol exerts on the LDLR, as macrophages deficient in LDLR did not overexpress *Ccl2*. Altogether our results suggest a selective role of Idol in the mechanism of positive transcriptional regulation of *Ccl2*, independent of the transrepression effects that LXR exerts on the same gene. Inflammatory signal-dependent clearance of NCoR-containing complexes from target gene promoters involves the action of proteins that display E3 ubiquitin ligase activity, e.g. transducin (beta)-like 1 (TBL1) and TBL related 1 (TBLR1), that are core components of the corepressor complex itself. Their E3 ubiquitin ligase activity leads to recruitment of the UbcH5/19S proteasome machinery and the subsequent ubiquitylation and dismissal of the corepressor complexes (Ogawa et al., 2004). Therefore, future studies from our group will be address to examine the potential involvement of Idol E3 ubiquitin ligase activity in the specific regulation of the *Ccl2* promoter.

Previous reports endorse another regulatory role of Idol in the processes of cell morphology and motility, apparently through the regulation of the degradation of the myosin regulatory light chain protein (MRLC) (Olsson et al., 1999). MRLC is part of a protein complex that controls the prime mover of myosin molecules and regulates the activity of the actomyosin complex both in skeletal and non-skeletal muscle cells (Howard, 1997). In general, the actions exerted by MRLC serve to restructure the actomyosin cytoskeleton which, depending on the cell type and the inducing signal that is implicated, result in both positive and negative effects on the formation of structures that facilitate cell motility (Bornhauser et al., 2003b; a; Nagano et al., 2006).

Local restructuring of the cytoskeleton plays an important role in engulfment and in the generation of phagosomes during phagocytosis. Moreover, LXRs have been shown to promote phagocytosis of apoptotic cells (A-Gonzalez et al., 2009). These premises prompted us to explore the phagocytic capacity of Idol-overexpressing cells. Macrophages that overexpress Idol displayed higher capacity to attach apoptotic thymocytes in their surface and higher levels of phagocytosis upon LXR activation in comparison to control cells. Although a larger number of similar experiments are

required to reach statistical significance, our data suggest a promising role of Idol in mediating apoptotic cell attachment and uptake. This role would add on the already described contributing roles of other molecules involved in phagocytosis that are upregulated by LXR agonists, namely the receptor c-Mer tyrosine kinase, Abca1 and transglutaminase-2 (A-Gonzalez et al., 2009; Hamon et al., 2000; Rébé et al., 2009). Interestingly, the chemokine CCL2 has been shown to enhance apoptotic cell uptake by macrophages in vitro and in vivo through a Rac1/phosphatidylinositol 3-kinase-dependent mechanism (Tanaka et al., 2010). Therefore, it will also be of interest to determine whether the E3 ubiquitin ligase activity and the transcriptional control of *Ccl2* are part of the role of Idol in regulation of phagocytosis.

In the second part of this study we evaluated the role of LXRs in the transcriptional control of M2 or alternative activation-marker genes induced by IL-4. Unlike the strong and broad repressive actions carried out by LXRs on M1-associated genes, the stimulation of macrophages with LXR ligands only affected a discrete subset of genes induced by IL-4. Most of the genes tested that exert important functions in macrophage-mediated tissue repair were not downregulated upon LXR activation. When comparing the effects of LXR on members of the STAT family of transcription factors, LXR is able to lead to broader interfering actions on the IFN-γ-STAT1 axis (Pascual-García et al., 2013) than on the IL-4-STAT6 pathway, which is in agreement with the general idea that LXRs exert a major role in negative control of inflammation and in resolution of inflammation (Reviewed in Pascual-García and Valledor, 2012).

Another group, however, has reported that the expression of Arginase 1, considered a tissue remodelling enzyme and a marker gene of alternatively activated macrophages, is increased by LXR agonists in regressive atherosclerotic lesions and in cultured macrophages (Pourcet et al., 2011). The authors propose a mechanism of regulation of *Arginase1* mediated through the binding of the transcription factors PU.1 and IRF8 to a novel site in the *Arginase1* promoter in an LXR ligand–dependent manner, and that LXR enhances the interaction between IRF8 and PU.1 in part through the induction of IRF8 gene expression. In our hands, the *Arginase1* mRNA expression levels were not upregulated by activation of LXR in IL-4-treated BMDM. We do not have a clear explanation for this discrepancy. Perhaps, the differences in our cellular model and

experimental conditions do not support the induction of the IRF8-Arginase 1 axis by LXR agonists.

Nonetheless, we found that LXR exerts a rather specific inhibitory effect on the IL-4 mediated expression of the chemokines CCL22 and CCL17. On one side, we have observed that the LXR ligand GW3965 down regulates *Ccl22* and *Ccl17* expression in wild type macrophages stimulated with IL-4. On the other side, we observed over-expression of these genes in IL-4-stimulated LXRαβ deficient macrophages. The negative regulation of *Ccl22* and *Ccl17* is mostly mediated by both isoforms of LXR since it is in the absence of both LXRα and LXRβ when their over-induction by IL-4 is observed.

Both chemokines, CCL22 and CCL17, have been implicated in establishment of numerous diseases including allergen-induced pulmonary inflammation, atopic dermatitis and lymphoma (Jacobsen et al., 2008; Hirota et al., 2011; Chang et al., 2012). Leukocyte recruitment to the lung during allergic inflammation is partially dependent on chemokines, and a considerable amount of work has shown that CCL22 and CCL17 play a role in recruiting Th2-type T cells to sites of allergic inflammation (reviewed in Bisset and Schmid-Grendelmeier 2005). Based on the LXR-dependent negative control of the expression of these chemokines, we decided to study the potential relevance of the LXR pathway on the development of allergic asthma using a model of responsiveness to HDM allergens.

Our results suggest that LXR could have a protective role in the allergen-induced murine model of experimental asthma given the fact that wild type mice that were intraperitoneally treated with GW3965 showed a higher respiratory capacity after induction of the asthmatic response. Correspondently, LXRαβ deficient mice developed significantly worsened airway responses to methacholine in comparison with the wild type animals. Our results contrast with a previous report in which oral administration with GW3965 increased the airway reactivity in a model of ovalbumin-induced asthma via induction of airway smooth muscle growth (Birrell et al., 2008). It is possible though that the route of administration of the agonist or the type of allergen used may be responsible for the differences observed between these two studies.

Murine models of allergic airway inflammation have demonstrated that increased expression of Th2-associated cytokines, specifically IL-4 and IL-13, is sufficient for the induction of a number of chemokines in the lung and the development of pulmonary eosinophilia (Li et al., 1998; Wills-Karp et al., 1998). However, other studies have shown that allergic airway inflammation is a more complex scenario, with production of IFN-γ being required for the expression of the allergen-induced chemokines CXCL9 and CXCL10. Infiltration of leukocytes, in particular eosinophils, is indeed regulated by the coordinated interplay of STAT6 and the IFN-γ-STAT1 axis (Fulkerson et al., 2004) and the interplay of a large number of chemokines, with distinct kinetics and transcriptional regulation, is what ultimately orchestrates allergic airway inflammation.

By analysing local responses in lung tissues from our animal model of allergic disease we found that the levels of *Il4* and of the IL-4-induced chemokines *Ccl17* and *Ccl22* were upregulated in the HDM-sensitized mice. We also observed increased expression of the chemokine *Ccl2*. Interestingly, the levels of these mediators of airway inflammation were reduced in the GW3965-treated animals, although a higher number of animals per group is necessary to determine whether the differences are statistically significant. It remains to be determined whether the expression of a larger set of inflammatory mediators and other asthma-related changes are also affected in the GW3965-treated lungs.

Altogether, our results suggest that modulation of LXR activity offers prospects for new therapeutic approaches in the treatment of asthma and perhaps other inflammatory respiratory diseases, which is supported by the fact that LXR is also able to suppress inflammatory responses in human airway smooth muscle cells in vitro (Birrell et al. 2007) and in an experimental model of LPS-driven airway inflammation (Delvecchio et al. 2007).

Finally, our findings indicate that IL-4, in a STAT6-dependent manner, exerts negative reciprocal actions on a specific subset of LXR inducible genes, disclosing a negative relationship between both transcription factors. More specifically, the genes that codify for *Apoe*, *Apoc2* and *Aim* are sensitive to STAT6-mediated repression, while *Abca1*, *Abcg1*, *Srebp1c* and *Idol* do not undergo such down regulation.

The analysis of the binding of LXRs and RXRα to a genomic regulatory region upstream of the *Aim* gene revealed, on one side, that combined treatment with LXR and RXR agonists results in increased recruitment of both nuclear receptors to this region and, on the other side, that down regulation of *Aim* by IL-4 is not mediated by interference on such recruitment. A similar scenario has been recently described by our group in the context of the macrophage response to IFN-γ. LXR and STAT1 establish a negative reciprocal crosstalk, and STAT1 activation leads to the selective inhibition of some of the genes induced by LXR, in this case *Abca1*, *Srebp1c* and *Aim*, without affecting the binding capacity of LXR and RXR to their response elements (Pascual-García et al., 2013).

Curiously, the activation of STAT1 and STAT6 negatively affects different subsets of LXR-dependent genes, suggesting that interference of these two pathways with the LXR transcriptional response involves distinct molecular mechanisms that may operate in a gene-specific manner. In this sense, overexpression of the coactivator CBP, but not of PGC-1α or SRC-1, counteracted partially the inhibitory effect of IFN-γ signaling on the *Abca1* promoter (Pascual-García et al., 2013). This suggests that competition for a specific coactivator may be a mechanism by which IFN-γ signaling molecules interfere with LXR-mediated gene expression. Future studies in the group will be addressed to evaluate if LXRs use different coactivators in a gene-specific manner and the molecular mechanisms underlying the differential actions of STAT1 and STAT6 on LXR target gene expression.

Conclusions

1. AcLDL-loading of macrophages leads to robust repression of the induction of LPS-responsive genes, without negatively interfering with the IFN-γ induced response.

2. Loading of macrophages with acLDL does not affect the activation of the LPS signaling molecules ERK-1/2 and p38 MAPK, nor the degradation of IκBα.

3. PPAR-γ shares with LXR the capability to repress common sets of inflammatory genes, although functional LXR is not required for PPAR-γ mediated transrepression.

4. LXR and RXR cooperate in GW3965-induced transrepression.

5. Overexpression of the LXR target gene Idol does not inhibit the transrepression of proinflammatory genes mediated by LXR, results in increased expression and secretion of CCL2, independently of its effects on the LDLR, and leads to increased attachment of apoptotic thymocytes.

6. LXR activation selectively inhibits IL-4-mediated expression of the chemokines CCL22 and CCL17 without altering the STAT-6 phosphorylation pattern.

7. GW3965 treatment has inhibitory effects on airway reactivity and lung inflammatory gene expression in a murine model of allergen-induced asthma.

8. IL-4, via STAT-6 activation, reciprocally interferes with the expression of a selective subset of LXR-dependent genes (*Apoc2, Apoe* and *Aim*). At least on the *Aim* regulatory region, this effect is not mediated by decreased recruitment of LXR/RXR.

References

A-Gonzalez, N., S.J. Bensinger, C. Hong, S. Beceiro, M.N. Bradley, N. Zelcer, J. Deniz, C. Ramirez, M. Díaz, G. Gallardo, C.R. de Galarreta, J. Salazar, F. Lopez, P. Edwards, J. Parks, M. Andujar, P. Tontonoz, and A. Castrillo. 2009. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity*. 31:245–58.

A-González, N., and A. Castrillo. 2011. Liver X receptors as regulators of macrophage inflammatory and metabolic pathways. *Biochimica et biophysica acta*. 1812:982–94.

Alexopoulou, L., A.C. Holt, R. Medzhitov, and R.A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*. 413:732–8.

Anthonisen, E.H., L. Berven, S. Holm, M. Nygård, H.I. Nebb, and L.M. Grønning-Wang. 2010. Nuclear receptor liver X receptor is O-GlcNAc-modified in response to glucose. *The Journal of biological chemistry*. 285:1607–15.

Arai, S., J.M. Shelton, M. Chen, M.N. Bradley, A. Castrillo, A.L. Bookout, P. a Mak, P. a Edwards, D.J. Mangelsdorf, P. Tontonoz, and T. Miyazaki. 2005. A role for the apoptosis inhibitory factor AIM/Spalpha/Api6 in atherosclerosis development. *Cell metabolism*. 1:201–13.

Aranda, A., and A. Pascual. 2001. Nuclear hormone receptors and gene expression. *Physiological reviews*. 81:1269–304.

Austenaa, L.M., H. Carlsen, K. Hollung, H.K. Blomhoff, and R. Blomhoff. 2009. Retinoic acid dampens LPS-induced NF-kappaB activity: results from human monoblasts and in vivo imaging of NF-kappaB reporter mice. *The Journal of nutritional biochemistry*. 20:726–34.

Baldán, A., D.D. Bojanic, and P.A. Edwards. 2009. The ABCs of sterol transport. *Journal of lipid research*. 50 Suppl:S80–5.

Barton, G.M., and J.C. Kagan. 2009. A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nature reviews. Immunology*. 9:535–42.

Bauer, M., K. Heeg, H. Wagner, and G.B. Lipford. 1999. DNA activates human immune cells through a CpG sequence-dependent manner. *Immunology*. 97:699–705.

Benoit, M., B. Desnues, and J.-L. Mege. 2008. Macrophage polarization in bacterial infections. *Journal of immunology (Baltimore, Md. : 1950)*. 181:3733–9.

Bhatia, S., M. Fei, M. Yarlagadda, Z. Qi, S. Akira, S. Saijo, Y. Iwakura, N. van Rooijen, G.A. Gibson, C.M. St Croix, A. Ray, and P. Ray. 2011. Rapid host defense against Aspergillus fumigatus involves alveolar macrophages with a predominance of alternatively activated phenotype. *PloS one*. 6:e15943.

Birrell, M. a, J. De Alba, M.C. Catley, E. Hardaker, S. Wong, M. Collins, D.L. Clarke, S.N. Farrow, T.M. Willson, J.L. Collins, and M.G. Belvisi. 2008. Liver X receptor agonists increase airway reactivity in a model of asthma via increasing airway smooth muscle growth. *Journal of immunology (Baltimore, Md. : 1950)*. 181:4265–71.

Birrell, M. a, M.C. Catley, E. Hardaker, S. Wong, T.M. Willson, K. McCluskie, T. Leonard, S.N. Farrow, J.L. Collins, S. Haj-Yahia, and M.G. Belvisi. 2007. Novel role for the liver X nuclear

receptor in the suppression of lung inflammatory responses. *The Journal of biological chemistry*. 282:31882–90.

Bisset, L.R., and P. Schmid-Grendelmeier. 2005. Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective. *Current opinion in pulmonary medicine*. 11:35–42.

Blaschke, F., Y. Takata, E. Caglayan, A. Collins, P. Tontonoz, W.A. Hsueh, and R.K. Tangirala. 2006. A nuclear receptor corepressor-dependent pathway mediates suppression of cytokine-induced C-reactive protein gene expression by liver X receptor. *Circulation research*. 99:e88–99.

Bode, J.G., C. Ehlting, and D. Häussinger. 2012. The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. *Cellular signalling*. 24:1185–94.

Boehm, U., T. Klamp, M. Groot, and J.C. Howard. 1997. Cellular responses to interferon-gamma. *Annual review of immunology*. 15:749–95.

Bornhauser, B.C., C. Johansson, and D. Lindholm. 2003a. Functional activities and cellular localization of the ezrin, radixin, moesin (ERM) and RING zinc finger domains in MIR. *FEBS letters*. 553:195–9.

Bornhauser, B.C., P.-A. Olsson, and D. Lindholm. 2003b. MSAP is a novel MIR-interacting protein that enhances neurite outgrowth and increases myosin regulatory light chain. *The Journal of biological chemistry*. 278:35412–20.

Brandt, E., G. Woerly, A.B. Younes, S. Loiseau, and M. Capron. 2000. IL-4 production by human polymorphonuclear neutrophils. *Journal of leukocyte biology*. 68:125–30.

Brizzard, B., and R. Chubet. 2001. Epitope tagging of recombinant proteins. *Current protocols in neuroscience / editorial board, Jacqueline N. Crawley ... [et al.]*. Chapter 5:Unit 5.8.

Brown, M.S., and J.L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science (New York, N.Y.)*. 232:34–47.

Bruhn, K.W., C. Marathe, A.C. Maretti-Mira, H. Nguyen, J. Haskell, T.A. Tran, V. Vanchinathan, U. Gaur, M.E. Wilson, P. Tontonoz, and N. Craft. 2010. LXR deficiency confers increased protection against visceral Leishmania infection in mice. *PLoS neglected tropical diseases*. 4:e886.

Cauwels, A., and P. Brouckaert. 2007. Survival of TNF toxicity: dependence on caspases and NO. *Archives of biochemistry and biophysics*. 462:132–9.

Coleman, J.W. 2001. Nitric oxide in immunity and inflammation. *International immunopharmacology*. 1:1397–406.

Collins, J.L., A.M. Fivush, M.A. Watson, C.M. Galardi, M.C. Lewis, L.B. Moore, D.J. Parks, J.G. Wilson, T.K. Tippin, J.G. Binz, K.D. Plunket, D.G. Morgan, E.J. Beaudet, K.D. Whitney, S.A. Kliewer, and T.M. Willson. 2002. Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. *Journal of medicinal chemistry*. 45:1963–6.

Comalada, M., J. Xaus, A.F. Valledor, C. López-López, D.J. Pennington, and A. Celada. 2003. PKC epsilon is involved in JNK activation that mediates LPS-induced TNF-alpha, which induces apoptosis in macrophages. *American journal of physiology. Cell physiology*. 285:C1235–45.

Conrad, D.J., H. Kuhn, M. Mulkins, E. Highland, and E. Sigal. 1992. Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proceedings of the National Academy of Sciences of the United States of America*. 89:217–21.

Croker, B.A., H. Kiu, and S.E. Nicholson. 2008. SOCS regulation of the JAK/STAT signalling pathway. *Seminars in cell & developmental biology*. 19:414–22.

Cuadrado, A., and A.R. Nebreda. 2010. Mechanisms and functions of p38 MAPK signalling. *The Biochemical journal*. 429:403–17.

Cuchel, M., and D.J. Rader. 2006. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? *Circulation*. 113:2548–55.

Cha, J.-Y., and J.J. Repa. 2007. The liver X receptor (LXR) and hepatic lipogenesis. The carbohydrate-response element-binding protein is a target gene of LXR. *The Journal of biological chemistry*. 282:743–51.

Chang, D.-K., J. Sui, S. Geng, A. Muvaffak, M. Bai, R.C. Fuhlbrigge, A.S. Lo, A. Yammanuru, L. Hubbard, J. Sheehan, J.J. Campbell, Q. Zhu, T.S. Kupper, and W. a Marasco. 2012. Humanization of an anti-CCR4 antibody that kills Cutaneous T Cell Lymphoma cells and abrogates suppression by T regulatory cells. *Molecular cancer therapeutics*.

Chawla-Sarkar, M., D.J. Lindner, Y.-F. Liu, B.R. Williams, G.C. Sen, R.H. Silverman, and E.C. Borden. 2003. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis : an international journal on programmed cell death*. 8:237–49.

Chen, J.D., and R.M. Evans. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature*. 377:454–7.

Chen, M., M.N. Bradley, S.W. Beaven, and P. Tontonoz. 2006. Phosphorylation of the liver X receptors. *FEBS letters*. 580:4835–41.

Chizzolini, C., R. Rezzonico, C. De Luca, D. Burger, and J.M. Dayer. 2000. Th2 cell membrane factors in association with IL-4 enhance matrix metalloproteinase-1 (MMP-1) while decreasing MMP-9 production by granulocyte-macrophage colony-stimulating factor-differentiated human monocytes. *Journal of immunology (Baltimore, Md. : 1950)*. 164:5952–60.

Chow, J.C., D.W. Young, D.T. Golenbock, W.J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *The Journal of biological chemistry*. 274:10689–92.

Darnell, J.E. 1997. STATs and gene regulation. *Science (New York, N.Y.)*. 277:1630–5.

Darnell, J.E., I.M. Kerr, and G.R. Stark. 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science (New York, N.Y.)*. 264:1415–21.

DeBose-Boyd, R.A., J. Ou, J.L. Goldstein, and M.S. Brown. 2001. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proceedings of the National Academy of Sciences of the United States of America*. 98:1477–82.

Delphin, S., and J. Stavnezer. 1995. Regulation of antibody class switching to IgE: characterization of an IL-4-responsive region in the immunoglobulin heavy-chain germline epsilon promoter. *Annals of the New York Academy of Sciences*. 764:123–35.

Delvecchio, C.J., P. Bilan, K. Radford, J. Stephen, B.L. Trigatti, G. Cox, K. Parameswaran, and J.P. Capone. 2007. Liver X receptor stimulates cholesterol efflux and inhibits expression of proinflammatory mediators in human airway smooth muscle cells. *Molecular endocrinology (Baltimore, Md.)*. 21:1324–34.

Dickensheets, H., N. Vazquez, F. Sheikh, S. Gingras, P.J. Murray, J.J. Ryan, and R.P. Donnelly. 2007. Suppressor of cytokine signaling-1 is an IL-4-inducible gene in macrophages and feedback inhibits IL-4 signaling. *Genes and immunity*. 8:21–7.

Dinarello, C.A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology*. 27:519–50.

Doyle, A.G., G. Herbein, L.J. Montaner, A.J. Minty, D. Caput, P. Ferrara, and S. Gordon. 1994. Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *European journal of immunology*. 24:1441–5.

Durbin, J.E., R. Hackenmiller, M.C. Simon, and D.E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell*. 84:443–50.

Von Eckardstein, A., J.R. Nofer, and G. Assmann. 2001. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arteriosclerosis, thrombosis, and vascular biology*. 21:13–27.

Eder, C. 2009. Mechanisms of interleukin-1beta release. *Immunobiology*. 214:543–53.

Edwards, J.P., X. Zhang, K.A. Frauwirth, and D.M. Mosser. 2006. Biochemical and functional characterization of three activated macrophage populations. *Journal of leukocyte biology*. 80:1298–307.

Edwards, P.A., H.R. Kast, and A.M. Anisfeld. 2002. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. *Journal of lipid research*. 43:2–12.

Eilers, A., and T. Decker. 1995. Activity of Stat family transcription factors is developmentally controlled in cells of the macrophage lineage. *Immunobiology*. 193:328–33.

Feng, G.S., K. Chong, A. Kumar, and B.R. Williams. 1992. Identification of double-stranded RNA-binding domains in the interferon-induced double-stranded RNA-activated p68 kinase. *Proceedings of the National Academy of Sciences of the United States of America*. 89:5447–51.

Finkelman, F.D., I.M. Katona, T.R. Mosmann, and R.L. Coffman. 1988. IFN-gamma regulates the isotypes of Ig secreted during in vivo humoral immune responses. *Journal of immunology (Baltimore, Md. : 1950)*. 140:1022–7.

Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *Journal of immunology (Baltimore, Md. : 1950)*. 147:3815–22.

Foell, D., H. Wittkowski, and J. Roth. 2007. Mechanisms of disease: a "DAMP" view of inflammatory arthritis. *Nature clinical practice. Rheumatology*. 3:382–90.

Fulkerson, P.C., N. Zimmermann, L.M. Hassman, F.D. Finkelman, and M.E. Rothenberg. 2004. Pulmonary chemokine expression is coordinately regulated by STAT1, STAT6, and IFN-gamma. *Journal of immunology (Baltimore, Md. : 1950)*. 173:7565–74.

Geissmann, F., M.G. Manz, S. Jung, M.H. Sieweke, M. Merad, and K. Ley. 2010. Development of monocytes, macrophages, and dendritic cells. *Science*. 327:656–661.

Ghisletti, S., W. Huang, K. Jepsen, C. Benner, G. Hardiman, M.G. Rosenfeld, and C.K. Glass. 2009. Cooperative NCoR/SMRT interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways. *Genes & development*. 23:681–93.

Ghisletti, S., W. Huang, S. Ogawa, G. Pascual, M.-E. Lin, T.M. Willson, M.G. Rosenfeld, and C.K. Glass. 2007. Parallel SUMOylation-dependent pathways mediate gene- and signal-specific transrepression by LXRs and PPARgamma. *Molecular cell*. 25:57–70.

Glass, C.K., and S. Ogawa. 2006. Combinatorial roles of nuclear receptors in inflammation and immunity. *Nature reviews. Immunology*. 6:44–55.

Glass, C.K., and M.G. Rosenfeld. 2000. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes & development*. 14:121–41.

Glass, C.K., and K. Saijo. 2010. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nature reviews. Immunology*. 10:365–76.

Gobin, S.J., M. van Zutphen, A.M. Woltman, and P.J. van den Elsen. 1999. Transactivation of classical and nonclassical HLA class I genes through the IFN-stimulated response element. *Journal of immunology (Baltimore, Md. : 1950)*. 163:1428–34.

Gobin, S.J.P., P. Biesta, and P.J. Van den Elsen. 2003. Regulation of human beta 2-microglobulin transactivation in hematopoietic cells. *Blood*. 101:3058–64.

Goerdt, S., and C.E. Orfanos. 1999. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity*. 10:137–42.

Gordon, S. 2003. Alternative activation of macrophages. *Nature reviews. Immunology*. 3:23–35.

Gratchev, A., P. Guillot, N. Hakiy, O. Politz, C.E. Orfanos, K. Schledzewski, and S. Goerdt. 2001. Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. *Scandinavian journal of immunology*. 53:386–92.

Haller, O., S. Stertz, and G. Kochs. 2007. The Mx GTPase family of interferon-induced antiviral proteins. *Microbes and infection / Institut Pasteur*. 9:1636–43.

Hamon, Y., C. Broccardo, O. Chambenoit, M.F. Luciani, F. Toti, S. Chaslin, J.M. Freyssinet, P.F. Devaux, J. McNeish, D. Marguet, and G. Chimini. 2000. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nature cell biology*. 2:399–406.

Hazen, S.L. 2008. Oxidized phospholipids as endogenous pattern recognition ligands in innate immunity. *The Journal of biological chemistry*. 283:15527–31.

Heim, M.H., I.M. Kerr, G.R. Stark, and J.E. Darnell. 1995. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. *Science (New York, N.Y.)*. 267:1347–9.

Heinz, S., C. Benner, N. Spann, E. Bertolino, Y.C. Lin, P. Laslo, J.X. Cheng, C. Murre, H. Singh, and C.K. Glass. 2010. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Molecular cell*. 38:576–89.

Hirota, T., H. Saeki, K. Tomita, S. Tanaka, K. Ebe, M. Sakashita, T. Yamada, S. Fujieda, A. Miyatake, S. Doi, T. Enomoto, N. Hizawa, T. Sakamoto, H. Masuko, T. Sasaki, T. Ebihara, M. Amagai, H. Esaki, S. Takeuchi, M. Furue, E. Noguchi, N. Kamatani, Y. Nakamura, M. Kubo, and M. Tamari. 2011. Variants of C-C motif chemokine 22 (CCL22) are associated with susceptibility to atopic dermatitis: case-control studies. *PloS one*. 6:e26987.

Hoberg, J.E., F. Yeung, and M.W. Mayo. 2004. SMRT derepression by the IkappaB kinase alpha: a prerequisite to NF-kappaB transcription and survival. *Molecular cell*. 16:245–55.

Hölscher, C., B. Arendse, A. Schwegmann, E. Myburgh, and F. Brombacher. 2006. Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing BALB/c mice. *Journal of immunology (Baltimore, Md. : 1950)*. 176:1115–21.

Hong, C., S. Duit, P. Jalonen, R. Out, L. Scheer, V. Sorrentino, R. Boyadjian, K.W. Rodenburg, E. Foley, L. Korhonen, D. Lindholm, J. Nimpf, T.J.C. van Berkel, P. Tontonoz, and N. Zelcer. 2010. The E3 ubiquitin ligase IDOL induces the degradation of the low density lipoprotein receptor family members VLDLR and ApoER2. *The Journal of biological chemistry*. 285:19720–6.

Hörlein, A.J., A.M. Näär, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Söderström, and C.K. Glass. 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature*. 377:397–404.

Howard, J. 1997. Molecular motors: structural adaptations to cellular functions. *Nature*. 389:561–7.

Hsia, E.Y., M.L. Goodson, J.X. Zou, M.L. Privalsky, and H.-W. Chen. 2010. Nuclear receptor coregulators as a new paradigm for therapeutic targeting. *Advanced drug delivery reviews*. 62:1227–37.

Hu, X., S.D. Chakravarty, and L.B. Ivashkiv. 2008. Regulation of interferon and Toll-like receptor signaling during macrophage activation by opposing feedforward and feedback inhibition mechanisms. *Immunological reviews*. 226:41–56.

Hu, X., and L.B. Ivashkiv. 2009. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity*. 31:539–50.

Hu, X., P.K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T.T. Lu, J.R. Woodgett, and L.B. Ivashkiv. 2006. IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. *Immunity*. 24:563–74.

Huang, W., S. Ghisletti, K. Saijo, M. Gandhi, M. Aouadi, G.J. Tesz, D.X. Zhang, J. Yao, M.P. Czech, B.L. Goode, M.G. Rosenfeld, and C.K. Glass. 2011. Coronin 2A mediates actin-dependent de-repression of inflammatory response genes. *Nature*. 470:414–8.

Igarashi, K., G. Garotta, L. Ozmen, A. Ziemiecki, A.F. Wilks, A.G. Harpur, A.C. Larner, and D.S. Finbloom. 1994. Interferon-gamma induces tyrosine phosphorylation of interferon-gamma receptor and regulated association of protein tyrosine kinases, Jak1 and Jak2, with its receptor. *The Journal of biological chemistry*. 269:14333–6.

Ikeda, H., L.J. Old, and R.D. Schreiber. 2002. The roles of IFN gamma in protection against tumor development and cancer immunoediting. *Cytokine & growth factor reviews*. 13:95–109.

Ikonen, E. 2008. Cellular cholesterol trafficking and compartmentalization. *Nature reviews. Molecular cell biology*. 9:125–38.

Isaacs, A., and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character. Royal Society (Great Britain)*. 147:258–67.

Jacobsen, E.A., S.I. Ochkur, R.S. Pero, A.G. Taranova, C.A. Protheroe, D.C. Colbert, N.A. Lee, and J.J. Lee. 2008. Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *The Journal of experimental medicine*. 205:699–710.

Jakobsson, T., E. Treuter, J.-Å. Gustafsson, and K.R. Steffensen. 2012. Liver X receptor biology and pharmacology: new pathways, challenges and opportunities. *Trends in pharmacological sciences*. 33:394–404.

Jakobsson, T., N. Venteclef, G. Toresson, A.E. Damdimopoulos, A. Ehrlund, X. Lou, S. Sanyal, K.R. Steffensen, J.-A. Gustafsson, and E. Treuter. 2009. GPS2 is required for cholesterol efflux by triggering histone demethylation, LXR recruitment, and coregulator assembly at the ABCG1 locus. *Molecular cell*. 34:510–8.

Janeway, C.A., P. Travers, M. Walport, and M.J. Shlomchik. 2001. Immunobiology: The Immune System in Health and Disease. 6. C.A.J. Janeway, P. Travers, M. Walport, and M.J. Shlomchik, editors. Garland Science.

Janowski, B.A., P.J. Willy, T.R. Devi, J.R. Falck, and D.J. Mangelsdorf. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*. 383:728–31.

Joseph, S.B., M.N. Bradley, A. Castrillo, K.W. Bruhn, P.A. Mak, L. Pei, J. Hogenesch, R.M. O'connell, G. Cheng, E. Saez, J.F. Miller, and P. Tontonoz. 2004. LXR-dependent gene expression is important for macrophage survival and the innate immune response. *Cell*. 119:299–309.

Joseph, S.B., A. Castrillo, B. a Laffitte, D.J. Mangelsdorf, and P. Tontonoz. 2003. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nature medicine*. 9:213–9.

Joseph, S.B., B.A. Laffitte, P.H. Patel, M.A. Watson, K.E. Matsukuma, R. Walczak, J.L. Collins, T.F. Osborne, and P. Tontonoz. 2002a. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *The Journal of biological chemistry*. 277:11019–25.

Joseph, S.B., E. McKilligin, L. Pei, M.A. Watson, A.R. Collins, B.A. Laffitte, M. Chen, G. Noh, J. Goodman, G.N. Hagger, J. Tran, T.K. Tippin, X. Wang, A.J. Lusis, W.A. Hsueh, R.E. Law, J.L. Collins, T.M. Willson, and P. Tontonoz. 2002b. Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proceedings of the National Academy of Sciences of the United States of America*. 99:7604–9.

Justesen, J., R. Hartmann, and N.O. Kjeldgaard. 2000. Gene structure and function of the 2'-5'-oligoadenylate synthetase family. *Cellular and molecular life sciences : CMLS*. 57:1593–612.

Kadl, A., A.K. Meher, P.R. Sharma, M.Y. Lee, A.C. Doran, S.R. Johnstone, M.R. Elliott, F. Gruber, J. Han, W. Chen, T. Kensler, K.S. Ravichandran, B.E. Isakson, B.R. Wamhoff, and N. Leitinger. 2010. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. *Circulation research*. 107:737–46.

Kalinski, P. 2012. Regulation of immune responses by prostaglandin E2. *Journal of immunology (Baltimore, Md. : 1950)*. 188:21–8.

Kaplan, M.H., U. Schindler, S.T. Smiley, and M.J. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity*. 4:313–9.

Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature immunology*. 11:373–84.

Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 34:637–50.

Kelchtermans, H., A. Billiau, and P. Matthys. 2008. How interferon-gamma keeps autoimmune diseases in check. *Trends in immunology*. 29:479–86.

Kim, E.Y., J.T. Battaile, A.C. Patel, Y. You, E. Agapov, M.H. Grayson, L.A. Benoit, D.E. Byers, Y. Alevy, J. Tucker, S. Swanson, R. Tidwell, J.W. Tyner, J.D. Morton, M. Castro, D. Polineni, G.A. Patterson, R.A. Schwendener, J.D. Allard, G. Peltz, and M.J. Holtzman. 2008. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nature medicine*. 14:633–40.

Kim, K.H., J.M. Yoon, A.H. Choi, W.S. Kim, G.Y. Lee, and J.B. Kim. 2009. Liver X receptor ligands suppress ubiquitination and degradation of LXRalpha by displacing BARD1/BRCA1. *Molecular endocrinology (Baltimore, Md.)*. 23:466–74.

Kindt, T.J., R.A. Goldsby, and B.A. Osborne. 2007. Kuby Immunology. 6. R.A. Goldsby, T.J. Kindt, and B.A. Osborne, editors. W.H. Freeman.

Kliewer, S.A., J.M. Lehmann, and T.M. Willson. 1999. Orphan nuclear receptors: shifting endocrinology into reverse. *Science (New York, N.Y.)*. 284:757–60.

Kodelja, V., C. Müller, O. Politz, N. Hakij, C.E. Orfanos, and S. Goerdt. 1998. Alternative macrophage activation-associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-1 alpha with a Th2-associated expression pattern. *Journal of immunology (Baltimore, Md. : 1950)*. 160:1411–8.

Kolattukudy, P.E., and J. Niu. 2012. Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. *Circulation research*. 110:174–89.

Korf, H., S. Vander Beken, M. Romano, K.R. Steffensen, B. Stijlemans, J.-A. Gustafsson, J. Grooten, and K. Huygen. 2009. Liver X receptors contribute to the protective immune response against Mycobacterium tuberculosis in mice. *The Journal of clinical investigation*. 119:1626–37.

Krebs, D.L., and D.J. Hilton. 2001. SOCS proteins: negative regulators of cytokine signaling. *Stem cells (Dayton, Ohio)*. 19:378–87.

Kurokawa, J., S. Arai, K. Nakashima, H. Nagano, A. Nishijima, K. Miyata, R. Ose, M. Mori, N. Kubota, T. Kadowaki, Y. Oike, H. Koga, M. Febbraio, T. Iwanaga, and T. Miyazaki. 2010. Macrophage-derived AIM is endocytosed into adipocytes and decreases lipid droplets via inhibition of fatty acid synthase activity. *Cell metabolism*. 11:479–92.

Kurowska-Stolarska, M., B. Stolarski, P. Kewin, G. Murphy, C.J. Corrigan, S. Ying, N. Pitman, A. Mirchandani, B. Rana, N. van Rooijen, M. Shepherd, C. McSharry, I.B. McInnes, D. Xu, and F.Y. Liew. 2009. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *Journal of immunology (Baltimore, Md. : 1950)*. 183:6469–77.

Laffitte, B.A., L.C. Chao, J. Li, R. Walczak, S. Hummasti, S.B. Joseph, A. Castrillo, D.C. Wilpitz, D.J. Mangelsdorf, J.L. Collins, E. Saez, and P. Tontonoz. 2003. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. *Proceedings of the National Academy of Sciences of the United States of America*. 100:5419–24.

Laffitte, B.A., S.B. Joseph, R. Walczak, L. Pei, D.C. Wilpitz, J.L. Collins, and P. Tontonoz. 2001. Autoregulation of the human liver X receptor alpha promoter. *Molecular and cellular biology*. 21:7558–68.

Lamont, A.G., and L. Adorini. 1996. IL-12: a key cytokine in immune regulation. *Immunology today*. 17:214–7.

Lee, J.H., S.M. Park, O.S. Kim, C.S. Lee, J.H. Woo, S.J. Park, E. Joe, and I. Jou. 2009. Differential SUMOylation of LXRalpha and LXRbeta mediates transrepression of STAT1 inflammatory signaling in IFN-gamma-stimulated brain astrocytes. *Molecular cell*. 35:806–17.

Lee, M.H., K. Lu, S. Hazard, H. Yu, S. Shulenin, H. Hidaka, H. Kojima, R. Allikmets, N. Sakuma, R. Pegoraro, A.K. Srivastava, G. Salen, M. Dean, and S.B. Patel. 2001. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nature genetics*. 27:79–83.

Lehmann, J.M., S.A. Kliewer, L.B. Moore, T.A. Smith-Oliver, B.B. Oliver, J.L. Su, S.S. Sundseth, D.A. Winegar, D.E. Blanchard, T.A. Spencer, and T.M. Willson. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *The Journal of biological chemistry*. 272:3137–40.

LeibundGut-Landmann, S., J.-M. Waldburger, M. Krawczyk, L.A. Otten, T. Suter, A. Fontana, H. Acha-Orbea, and W. Reith. 2004. Mini-review: Specificity and expression of CIITA, the master regulator of MHC class II genes. *European journal of immunology*. 34:1513–25.

Li, A.C., and C.K. Glass. 2002. The macrophage foam cell as a target for therapeutic intervention. *Nature medicine*. 8:1235–42.

Li, A.C., and W. Palinski. 2006. Peroxisome proliferator-activated receptors: how their effects on macrophages can lead to the development of a new drug therapy against atherosclerosis. *Annual review of pharmacology and toxicology*. 46:1–39.

Li, L., Y. Xia, A. Nguyen, L. Feng, and D. Lo. 1998. Th2-induced eotaxin expression and eosinophilia coexist with Th1 responses at the effector stage of lung inflammation. *Journal of immunology (Baltimore, Md. : 1950)*. 161:3128–35.

Li, M.O., Y.Y. Wan, S. Sanjabi, A.-K.L. Robertson, and R.A. Flavell. 2006. Transforming growth factor-beta regulation of immune responses. *Annual review of immunology*. 24:99–146.

Li, N., R.C. Salter, and D.P. Ramji. 2011. Molecular mechanisms underlying the inhibition of IFN-γ-induced, STAT1-mediated gene transcription in human macrophages by simvastatin and agonists of PPARs and LXRs. *Journal of cellular biochemistry*. 112:675–83.

Li, X., S. Zhang, G. Blander, J.G. Tse, M. Krieger, and L. Guarente. 2007. SIRT1 deacetylates and positively regulates the nuclear receptor LXR. *Molecular cell*. 28:91–106.

Li, Y., C. Bolten, B.G. Bhat, J. Woodring-Dietz, S. Li, S.K. Prayaga, C. Xia, and D.S. Lala. 2002. Induction of human liver X receptor alpha gene expression via an autoregulatory loop mechanism. *Molecular endocrinology (Baltimore, Md.)*. 16:506–14.

Loke, P., I. Gallagher, M.G. Nair, X. Zang, F. Brombacher, M. Mohrs, J.P. Allison, and J.E. Allen. 2007. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *Journal of immunology (Baltimore, Md. : 1950)*. 179:3926–36.

Lu, K., M.H. Lee, S. Hazard, A. Brooks-Wilson, H. Hidaka, H. Kojima, L. Ose, A.F. Stalenhoef, T. Mietinnen, I. Bjorkhem, E. Bruckert, A. Pandya, H.B. Brewer, G. Salen, M. Dean, A. Srivastava, and S.B. Patel. 2001. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin-2, encoded by ABCG5 and ABCG8, respectively. *American journal of human genetics*. 69:278–90.

Lu, Y.-C., W.-C. Yeh, and P.S. Ohashi. 2008. LPS/TLR4 signal transduction pathway. *Cytokine*. 42:145–51.

MacMicking, J., Q.W. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annual review of immunology*. 15:323–50.

MacMicking, J.D. 2004. IFN-inducible GTPases and immunity to intracellular pathogens. *Trends in immunology*. 25:601–9.

Mandal, M., D. Bandyopadhyay, T.M. Goepfert, and R. Kumar. 1998. Interferon-induces expression of cyclin-dependent kinase-inhibitors p21WAF1 and p27Kip1 that prevent activation of cyclin-dependent kinase by CDK-activating kinase (CAK). *Oncogene*. 16:217–25.

Mangelsdorf, D.J., C. Thummel, M. Beato, P. Herrlich, G. Schütz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R.M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell*. 83:835–9.

Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends in immunology*. 25:677–86.

Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in immunology*. 23:549–55.

Marathe, C., M.N. Bradley, C. Hong, F. Lopez, C.M. Ruiz de Galarreta, P. Tontonoz, and A. Castrillo. 2006. The arginase II gene is an anti-inflammatory target of liver X receptor in macrophages. *The Journal of biological chemistry*. 281:32197–206.

Martinez, F.O., S. Gordon, M. Locati, and A. Mantovani. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *Journal of immunology (Baltimore, Md. : 1950)*. 177:7303–11.

Martinez, F.O., L. Helming, and S. Gordon. 2009. Alternative activation of macrophages: an immunologic functional perspective. *Annual review of immunology*. 27:451–83.

Martinez, F.O., L. Helming, R. Milde, A. Varin, B.N. Melgert, C. Draijer, B. Thomas, M. Fabbri, A. Crawshaw, L.P. Ho, N.H. Ten Hacken, V. Cobos Jiménez, N. a Kootstra, J. Hamann, D.R. Greaves, M. Locati, A. Mantovani, and S. Gordon. 2013. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. *Blood*. 121:e57–69. doi:10.1182/blood-2012-06-436212.

Martínez-Pomares, L., and S. Gordon. 1999. Potential role of the mannose receptor in antigen transport. *Immunology letters*. 65:9–13.

Matsumoto, M., N. Tanaka, H. Harada, T. Kimura, T. Yokochi, M. Kitagawa, C. Schindler, and T. Taniguchi. 1999. Activation of the transcription factor ISGF3 by interferon-gamma. *Biological chemistry*. 380:699–703.

Maurer, M., and E. von Stebut. 2004. Macrophage inflammatory protein-1. *The international journal of biochemistry & cell biology*. 36:1882–6.

McKercher, S.R., B.E. Torbett, K.L. Anderson, G.W. Henkel, D.J. Vestal, H. Baribault, M. Klemsz, A.J. Feeney, G.E. Wu, C.J. Paige, and R.A. Maki. 1996. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *The EMBO journal*. 15:5647–58.

Merad, M., M.G. Manz, H. Karsunky, A. Wagers, W. Peters, I. Charo, I.L. Weissman, J.G. Cyster, and E.G. Engleman. 2002. Langerhans cells renew in the skin throughout life under steady-state conditions. *Nature immunology*. 3:1135–41.

Mildner, A., H. Schmidt, M. Nitsche, D. Merkler, U.-K. Hanisch, M. Mack, M. Heikenwalder, W. Brück, J. Priller, and M. Prinz. 2007. Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. *Nature neuroscience*. 10:1544–53.

Miloux, B., P. Laurent, O. Bonnin, J. Lupker, D. Caput, N. Vita, and P. Ferrara. 1997. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. *FEBS letters*. 401:163–6.

Miller, Y.I., S. Viriyakosol, D.S. Worrall, A. Boullier, S. Butler, and J.L. Witztum. 2005. Toll-like receptor 4-dependent and -independent cytokine secretion induced by minimally oxidized low-density lipoprotein in macrophages. *Arteriosclerosis, thrombosis, and vascular biology*. 25:1213–9.

Min, K., K.-H. Cho, and T.K. Kwon. 2012. The effect of oxidized low density lipoprotein (oxLDL)-induced heme oxygenase-1 on LPS-induced inflammation in RAW 264.7 macrophage cells. *Cellular signalling*. 24:1215–21.

Mogilenko, D.A., I. V Kudriavtsev, A.S. Trulioff, V.S. Shavva, E.B. Dizhe, B. V Missyul, A. V Zhakhov, A.M. Ischenko, A.P. Perevozchikov, and S. V Orlov. 2012. Modified low density lipoprotein stimulates complement C3 expression and secretion via liver X receptor and Toll-like receptor 4 activation in human macrophages. *The Journal of biological chemistry*. 287:5954–68.

Montaner, L.J., R.P. da Silva, J. Sun, S. Sutterwala, M. Hollinshead, D. Vaux, and S. Gordon. 1999. Type 1 and type 2 cytokine regulation of macrophage endocytosis: differential activation by IL-4/IL-13 as opposed to IFN-gamma or IL-10. *Journal of immunology (Baltimore, Md. : 1950)*. 162:4606–13.

Moresco, E.M.Y., D. LaVine, and B. Beutler. 2011. Toll-like receptors. *Current biology : CB*. 21:R488–93.

Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology*. 8:958–69.

Munder, M., K. Eichmann, J.M. Morán, F. Centeno, G. Soler, and M. Modolell. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *Journal of immunology (Baltimore, Md. : 1950)*. 163:3771–7.

Murata, T., J. Taguchi, and R.K. Puri. 1998. Interleukin-13 receptor alpha' but not alpha chain: a functional component of interleukin-4 receptors. *Blood*. 91:3884–91.

Murray, P.J., and T. a Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nature reviews. Immunology*. 11:723–37.

Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature immunology*. 3:667–72.

Nagano, K., B.C. Bornhauser, G. Warnasuriya, A. Entwistle, R. Cramer, D. Lindholm, and S. Naaby-Hansen. 2006. PDGF regulates the actin cytoskeleton through hnRNP-K-mediated activation of the ubiquitin E3-ligase MIR. *The EMBO journal*. 25:1871–82.

Nagarkar, D.R., E.R. Bowman, D. Schneider, Q. Wang, J. Shim, Y. Zhao, M.J. Linn, C.L. McHenry, B. Gosangi, J.K. Bentley, W.C. Tsai, U.S. Sajjan, N.W. Lukacs, and M.B. Hershenson. 2010. Rhinovirus infection of allergen-sensitized and -challenged mice induces eotaxin release from functionally polarized macrophages. *Journal of immunology (Baltimore, Md. : 1950)*. 185:2525–35.

Nagy, L., A. Szanto, I. Szatmari, and L. Széles. 2012. Nuclear hormone receptors enable macrophages and dendritic cells to sense their lipid environment and shape their immune response. *Physiological reviews*. 92:739–89.

Nagy, L., P. Tontonoz, J.G. Alvarez, H. Chen, and R.M. Evans. 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell*. 93:229–40.

Naka, T., N. Nishimoto, and T. Kishimoto. 2002. The paradigm of IL-6: from basic science to medicine. *Arthritis research*. 4 Suppl 3:S233–42.

Nelms, K., A.D. Keegan, J. Zamorano, J.J. Ryan, and W.E. Paul. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annual review of immunology*. 17:701–38.

Neurath, M.F., and S. Finotto. 2011. IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer. *Cytokine & growth factor reviews*. 22:83–9.

Ogawa, M. 1993. Differentiation and proliferation of hematopoietic stem cells. *Blood*. 81:2844–53.

Ogawa, S., J. Lozach, C. Benner, G. Pascual, R.K. Tangirala, S. Westin, A. Hoffmann, S. Subramaniam, M. David, M.G. Rosenfeld, and C.K. Glass. 2005. Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell*. 122:707–21.

Ogawa, S., J. Lozach, K. Jepsen, D. Sawka-Verhelle, V. Perissi, R. Sasik, D.W. Rose, R.S. Johnson, M.G. Rosenfeld, and C.K. Glass. 2004. A nuclear receptor corepressor transcriptional checkpoint controlling activator protein 1-dependent gene networks required for macrophage activation. *Proceedings of the National Academy of Sciences of the United States of America*. 101:14461–6.

Olsson, P.A., L. Korhonen, E.A. Mercer, and D. Lindholm. 1999. MIR is a novel ERM-like protein that interacts with myosin regulatory light chain and inhibits neurite outgrowth. *The Journal of biological chemistry*. 274:36288–92.

Paliard, X., R. de Waal Malefijt, H. Yssel, D. Blanchard, I. Chrétien, J. Abrams, J. de Vries, and H. Spits. 1988. Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones. *Journal of immunology (Baltimore, Md. : 1950)*. 141:849–55.

Pålsson-McDermott, E.M., and L. a J. O'Neill. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*. 113:153–62.

Palvimo, J.J. 2007. PIAS proteins as regulators of small ubiquitin-related modifier (SUMO) modifications and transcription. *Biochemical Society transactions*. 35:1405–8.

Panina-Bordignon, P., A. Papi, M. Mariani, P. Di Lucia, G. Casoni, C. Bellettato, C. Buonsanti, D. Miotto, C. Mapp, A. Villa, G. Arrigoni, L.M. Fabbri, and F. Sinigaglia. 2001. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *The Journal of clinical investigation*. 107:1357–64.

Pascual-García, M., J.M. Carbó, T. León, J. Matalonga, R. Out, T. Van Berkel, M.-R. Sarrias, F. Lozano, A. Celada, and A.F. Valledor. 2011. Liver X receptors inhibit macrophage proliferation through downregulation of cyclins D1 and B1 and cyclin-dependent kinases 2 and 4. *Journal of immunology (Baltimore, Md. : 1950)*. 186:4656–67.

Pascual-García, M., L. Rué, T. León, J. Julve, J.M. Carbó, J. Matalonga, H. Auer, A. Celada, J.C. Escolà-Gil, K.R. Steffensen, E. Pérez-Navarro, and A.F. Valledor. 2013. Reciprocal Negative Cross-Talk between Liver X Receptors (LXRs) and STAT1: Effects on IFN-γ-Induced Inflammatory Responses and LXR-Dependent Gene Expression. *Journal of immunology (Baltimore, Md. : 1950)*.

Pascual-García, M., and A.F. Valledor. 2012. Biological roles of liver x receptors in immune cells. *Archivum immunologiae et therapiae experimentalis*. 60:235–49.

Patterson, J.B., D.C. Thomis, S.L. Hans, and C.E. Samuel. 1995. Mechanism of interferon action: double-stranded RNA-specific adenosine deaminase from human cells is inducible by alpha and gamma interferons. *Virology*. 210:508–11.

Peet, D.J., S.D. Turley, W. Ma, B.A. Janowski, J.M. Lobaccaro, R.E. Hammer, and D.J. Mangelsdorf. 1998. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell*. 93:693–704.

Perussia, B. 1991. Lymphokine-activated killer cells, natural killer cells and cytokines. *Current opinion in immunology*. 3:49–55.

Pestka, S., C.D. Krause, and M.R. Walter. 2004. Interferons, interferon-like cytokines, and their receptors. *Immunological reviews*. 202:8–32.

Pourcet, B., J.E. Feig, Y. Vengrenyuk, A.J. Hobbs, D. Kepka-Lenhart, M.J. Garabedian, S.M. Morris, E. a Fisher, and I. Pineda-Torra. 2011. LXRα regulates macrophage arginase 1 through PU.1 and interferon regulatory factor 8. *Circulation research*. 109:492–501.

Raes, G., L. Brys, B.K. Dahal, J. Brandt, J. Grooten, F. Brombacher, G. Vanham, W. Noël, P. Bogaert, T. Boonefaes, A. Kindt, R. Van den Bergh, P.J.M. Leenen, P. De Baetselier, and G.H. Ghassabeh. 2005. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. *Journal of leukocyte biology*. 77:321–7.

Raes, G., W. Noël, A. Beschin, L. Brys, P. de Baetselier, and G.H.G. Hassanzadeh. 2002. FIZZ1 and Ym as tools to discriminate between differentially activated macrophages. *Developmental immunology*. 9:151–9.

Rakesh, K., and D.K. Agrawal. 2005. Controlling cytokine signaling by constitutive inhibitors. *Biochemical pharmacology*. 70:649–57.

Rébé, C., M. Raveneau, A. Chevriaux, D. Lakomy, A.-L. Sberna, A. Costa, G. Bessède, A. Athias, E. Steinmetz, J.M.A. Lobaccaro, G. Alves, A. Menicacci, S. Vachenc, E. Solary, P. Gambert, and D. Masson. 2009. Induction of transglutaminase 2 by a liver X receptor/retinoic acid receptor alpha pathway increases the clearance of apoptotic cells by human macrophages. *Circulation research*. 105:393–401.

Reese, T.A., H.-E. Liang, A.M. Tager, A.D. Luster, N. Van Rooijen, D. Voehringer, and R.M. Locksley. 2007. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature*. 447:92–6.

Reichel, M., B.H. Nelson, P.D. Greenberg, and P.B. Rothman. 1997. The IL-4 receptor alpha-chain cytoplasmic domain is sufficient for activation of JAK-1 and STAT6 and the induction of IL-4-specific gene expression. *Journal of immunology (Baltimore, Md. : 1950)*. 158:5860–7.

Reiner, S.L., and R.M. Locksley. 1995. The regulation of immunity to Leishmania major. *Annual review of immunology*. 13:151–77.

Remick, D.G. 2005. Interleukin-8. *Critical care medicine*. 33:S466–7.

Repa, J.J., K.E. Berge, C. Pomajzl, J.A. Richardson, H. Hobbs, and D.J. Mangelsdorf. 2002. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *The Journal of biological chemistry*. 277:18793–800.

Repa, J.J., G. Liang, J. Ou, Y. Bashmakov, J.M. Lobaccaro, I. Shimomura, B. Shan, M.S. Brown, J.L. Goldstein, and D.J. Mangelsdorf. 2000a. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes & development*. 14:2819–30.

Repa, J.J., and D.J. Mangelsdorf. 2000. The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annual review of cell and developmental biology*. 16:459–81.

Repa, J.J., S.D. Turley, J.A. Lobaccaro, J. Medina, L. Li, K. Lustig, B. Shan, R.A. Heyman, J.M. Dietschy, and D.J. Mangelsdorf. 2000b. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science (New York, N.Y.)*. 289:1524–9.

Ricote, M., A.C. Li, T.M. Willson, C.J. Kelly, and C.K. Glass. 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature*. 391:79–82.

Van Rijt, L.S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H.C. Hoogsteden, and B.N. Lambrecht. 2005. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *The Journal of experimental medicine*. 201:981–91.

Rosenzweig, S.D., and S.M. Holland. 2005. Defects in the interferon-gamma and interleukin-12 pathways. *Immunological reviews*. 203:38–47.

Rossi, A., A.M. Acquaviva, F. Iuliano, R. Di Paola, S. Cuzzocrea, and L. Sautebin. 2005. Up-regulation of prostaglandin biosynthesis by leukotriene C4 in elicited mice peritoneal macrophages activated with lipopolysaccharide/interferon-{gamma}. *Journal of leukocyte biology*. 78:985–91.

Rückerl, D., M. Hessmann, T. Yoshimoto, S. Ehlers, and C. Hölscher. 2006. Alternatively activated macrophages express the IL-27 receptor alpha chain WSX-1. *Immunobiology*. 211:427–36.

Ryan, J.J., L.J. McReynolds, A. Keegan, L.H. Wang, E. Garfein, P. Rothman, K. Nelms, and W.E. Paul. 1996. Growth and gene expression are predominantly controlled by distinct regions of the human IL-4 receptor. *Immunity*. 4:123–32.

Sabat, R. 2010. IL-10 family of cytokines. *Cytokine & growth factor reviews*. 21:315–24.

Sad, S., R. Marcotte, and T.R. Mosmann. 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity*. 2:271–9.

Saha, B., S. Jyothi Prasanna, B. Chandrasekar, and D. Nandi. 2010. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine*. 50:1–14.

Scott, R.S., E.J. McMahon, S.M. Pop, E.A. Reap, R. Caricchio, P.L. Cohen, H.S. Earp, and G.K. Matsushima. 2001. Phagocytosis and clearance of apoptotic cells is mediated by MER. *Nature*. 411:207–11.

Schroder, K., P.J. Hertzog, T. Ravasi, and D.A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*. 75:163–89.

Schroder, K., M.J. Sweet, and D.A. Hume. 2006. Signal integration between IFNgamma and TLR signalling pathways in macrophages. *Immunobiology*. 211:511–24.

Schultz, J.R., H. Tu, A. Luk, J.J. Repa, J.C. Medina, L. Li, S. Schwendner, S. Wang, M. Thoolen, D.J. Mangelsdorf, K.D. Lustig, and B. Shan. 2000. Role of LXRs in control of lipogenesis. *Genes & development*. 14:2831–8.

Seder, R.A., J.L. Boulay, F. Finkelman, S. Barbier, S.Z. Ben-Sasson, G. Le Gros, and W.E. Paul. 1992. CD8+ T cells can be primed in vitro to produce IL-4. *Journal of immunology (Baltimore, Md. : 1950)*. 148:1652–6.

Sevov, M., L. Elfineh, and L.B. Cavelier. 2006. Resveratrol regulates the expression of LXR-alpha in human macrophages. *Biochemical and biophysical research communications*. 348:1047–54.

Sher, A., and R.L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annual review of immunology*. 10:385–409.

Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *The Journal of experimental medicine*. 189:1777–82.

Shimoda, K., J. van Deursen, M.Y. Sangster, S.R. Sarawar, R.T. Carson, R.A. Tripp, C. Chu, F.W. Quelle, T. Nosaka, D.A. Vignali, P.C. Doherty, G. Grosveld, W.E. Paul, and J.N. Ihle. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature*. 380:630–3.

Shindou, H., S. Ishii, M. Yamamoto, K. Takeda, S. Akira, and T. Shimizu. 2005. Priming effect of lipopolysaccharide on acetyl-coenzyme A:lyso-platelet-activating factor acetyltransferase is MyD88 and TRIF independent. *Journal of immunology (Baltimore, Md. : 1950)*. 175:1177–83.

Sica, A., and A. Mantovani. 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*. 122:787–95.

Snapper, C.M., C. Peschel, and W.E. Paul. 1988. IFN-gamma stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *Journal of immunology (Baltimore, Md. : 1950)*. 140:2121–7.

Song, E., N. Ouyang, M. Hörbelt, B. Antus, M. Wang, and M.S. Exton. 2000. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. *Cellular immunology*. 204:19–28.

Spann, N.J., L.X. Garmire, J.G. McDonald, D.S. Myers, S.B. Milne, N. Shibata, D. Reichart, J.N. Fox, I. Shaked, D. Heudobler, C.R.H. Raetz, E.W. Wang, S.L. Kelly, M.C. Sullards, R.C. Murphy, A.H. Merrill, H.A. Brown, E. a Dennis, A.C. Li, K. Ley, S. Tsimikas, E. Fahy, S. Subramaniam, O. Quehenberger, D.W. Russell, and C.K. Glass. 2012. Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses. *Cell*. 151:138–52.

Staples, K.J., T.S.C. Hinks, J. a Ward, V. Gunn, C. Smith, and R. Djukanović. 2012. Phenotypic characterization of lung macrophages in asthmatic patients: Overexpression of CCL17. *The Journal of allergy and clinical immunology*. 1.

Stark, G.R., I.M. Kerr, B.R. Williams, R.H. Silverman, and R.D. Schreiber. 1998. How cells respond to interferons. *Annual review of biochemistry*. 67:227–64.

Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine*. 176:287–92.

Stout, R.D., and J. Suttles. 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *Journal of leukocyte biology*. 76:509–13.

Street, S.E.A., J.A. Trapani, D. MacGregor, and M.J. Smyth. 2002. Suppression of lymphoma and epithelial malignancies effected by interferon gamma. *The Journal of experimental medicine*. 196:129–34.

Svensson, P.-A., D.A. Hägg, M. Jernås, M.C.O. Englund, L.M. Hulten, B.G. Ohlsson, J. Hulthe, O. Wiklund, B. Carlsson, B. Fagerberg, and L.M.S. Carlsson. 2004. Identification of genes predominantly expressed in human macrophages. *Atherosclerosis*. 177:287–90.

Szabo, S.J., N.G. Jacobson, A.S. Dighe, U. Gubler, and K.M. Murphy. 1995. Developmental commitment to the Th2 lineage by extinction of IL-12 signaling. *Immunity*. 2:665–75.

Talukdar, S., and F.B. Hillgartner. 2006. The mechanism mediating the activation of acetyl-coenzyme A carboxylase-alpha gene transcription by the liver X receptor agonist T0-901317. *Journal of lipid research*. 47:2451–61.

Tanaka, T., M. Terada, K. Ariyoshi, and K. Morimoto. 2010. Monocyte chemoattractant protein-1/CC chemokine ligand 2 enhances apoptotic cell removal by macrophages through Rac1 activation. *Biochemical and biophysical research communications*. 399:677–82.

Terasaka, N., A. Hiroshima, A. Ariga, S. Honzumi, T. Koieyama, T. Inaba, and T. Fujiwara. 2005. Liver X receptor agonists inhibit tissue factor expression in macrophages. *The FEBS journal*. 272:1546–56.

Terasaka, N., A. Hiroshima, T. Koieyama, N. Ubukata, Y. Morikawa, D. Nakai, and T. Inaba. 2003. T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice. *FEBS letters*. 536:6–11.

Töröcsik, D., H. Bárdos, L. Nagy, and R. Adány. 2005. Identification of factor XIII-A as a marker of alternative macrophage activation. *Cellular and molecular life sciences : CMLS*. 62:2132–9.

Torra, I.P., N. Ismaili, J.E. Feig, C.-F. Xu, C. Cavasotto, R. Pancratov, I. Rogatsky, T.A. Neubert, E.A. Fisher, and M.J. Garabedian. 2008. Phosphorylation of liver X receptor alpha selectively regulates target gene expression in macrophages. *Molecular and cellular biology*. 28:2626–36.

Torres, R., A. Herrerias, M. Serra-Pagès, A. Marco, J. Plaza, C. Costa-Farré, M. Montoya, C. Picado, and F. de Mora. 2013. Locally administered prostaglandin E2 prevents aeroallergen-induced airway sensitization in mice through immunomodulatory mechanisms. *Pharmacological research : the official journal of the Italian Pharmacological Society*. 70:50–9.

Treuter, E., and N. Venteclef. 2011. Transcriptional control of metabolic and inflammatory pathways by nuclear receptor SUMOylation. *Biochimica et biophysica acta*. 1812:909–18.

Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annual review of immunology*. 13:251–76.

Valledor, A.F., F.E. Borràs, M. Cullell-Young, and A. Celada. 1998. Transcription factors that regulate monocyte/macrophage differentiation. *Journal of leukocyte biology*. 63:405–17.

Valledor, A.F., M. Comalada, J. Xaus, and A. Celada. 2000a. The differential time-course of extracellular-regulated kinase activity correlates with the macrophage response toward proliferation or activation. *The Journal of biological chemistry*. 275:7403–9.

Valledor, A.F., L.-C. Hsu, S. Ogawa, D. Sawka-Verhelle, M. Karin, and C.K. Glass. 2004. Activation of liver X receptors and retinoid X receptors prevents bacterial-induced macrophage apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*. 101:17813–8.

Valledor, A.F., J. Xaus, M. Comalada, C. Soler, and A. Celada. 2000b. Protein kinase C epsilon is required for the induction of mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *Journal of immunology (Baltimore, Md. : 1950)*. 164:29–37.

Varin, A., and S. Gordon. 2009. Alternative activation of macrophages: immune function and cellular biology. *Immunobiology*. 214:630–41.

Velazquez, L., M. Fellous, G.R. Stark, and S. Pellegrini. 1992. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. *Cell*. 70:313–22.

Venkateswaran, A., B.A. Laffitte, S.B. Joseph, P.A. Mak, D.C. Wilpitz, P.A. Edwards, and P. Tontonoz. 2000. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. *Proceedings of the National Academy of Sciences of the United States of America*. 97:12097–102.

Venteclef, N., T. Jakobsson, A. Ehrlund, A. Damdimopoulos, L. Mikkonen, E. Ellis, L.-M. Nilsson, P. Parini, O.A. Jänne, J.-A. Gustafsson, K.R. Steffensen, and E. Treuter. 2010. GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRbeta in the hepatic acute phase response. *Genes & development*. 24:381–95.

Venteclef, N., T. Jakobsson, K.R. Steffensen, and E. Treuter. 2011. Metabolic nuclear receptor signaling and the inflammatory acute phase response. *Trends in endocrinology and metabolism: TEM*. 22:333–43.

Wagner, B.L., A.F. Valledor, G. Shao, C.L. Daige, E.D. Bischoff, M. Petrowski, K. Jepsen, S.H. Baek, R.A. Heyman, M.G. Rosenfeld, I.G. Schulman, and C.K. Glass. 2003. Promoter-specific roles for liver X receptor/corepressor complexes in the regulation of ABCA1 and SREBP1 gene expression. *Molecular and cellular biology*. 23:5780–9.

Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Tumor necrosis factor signaling. *Cell death and differentiation*. 10:45–65.

Wang, Y., B. Kurdi-Haidar, and J.F. Oram. 2004. LXR-mediated activation of macrophage stearoyl-CoA desaturase generates unsaturated fatty acids that destabilize ABCA1. *Journal of lipid research*. 45:972–80.

Wen, Z., Z. Zhong, and J.E. Darnell. 1995. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell*. 82:241–50.

Whitney, K.D., M.A. Watson, B. Goodwin, C.M. Galardi, J.M. Maglich, J.G. Wilson, T.M. Willson, J.L. Collins, and S.A. Kliewer. 2001. Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. *The Journal of biological chemistry*. 276:43509–15.

Williams, L., L. Bradley, A. Smith, and B. Foxwell. 2004. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *Journal of immunology (Baltimore, Md. : 1950)*. 172:567–76.

Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T.Y. Neben, C.L. Karp, and D.D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science (New York, N.Y.)*. 282:2258–61.

Wirnsberger, G., D. Hebenstreit, G. Posselt, J. Horejs-Hoeck, and A. Duschl. 2006. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. *European journal of immunology*. 36:1882–91.

Woollard, K.J., and F. Geissmann. 2010. Monocytes in atherosclerosis: subsets and functions. *Nature reviews. Cardiology*. 7:77–86.

Wright, K.L., L.C. White, A. Kelly, S. Beck, J. Trowsdale, and J.P. Ting. 1995. Coordinate regulation of the human TAP1 and LMP2 genes from a shared bidirectional promoter. *The Journal of experimental medicine*. 181:1459–71.

Wright, S.D., R.A. Ramos, P.S. Tobias, R.J. Ulevitch, and J.C. Mathison. 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science (New York, N.Y.)*. 249:1431–3.

Wright, S.D., P.S. Tobias, R.J. Ulevitch, and R.A. Ramos. 1989. Lipopolysaccharide (LPS) binding protein opsonizes LPS-bearing particles for recognition by a novel receptor on macrophages. *The Journal of experimental medicine*. 170:1231–41.

Wynes, M.W., and D.W.H. Riches. 2003. Induction of macrophage insulin-like growth factor-I expression by the Th2 cytokines IL-4 and IL-13. *Journal of immunology (Baltimore, Md. : 1950)*. 171:3550–9.

Xaus, J., M. Cardó, A.F. Valledor, C. Soler, J. Lloberas, and A. Celada. 1999. Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. *Immunity*. 11:103–13.

Yadav, A., V. Saini, and S. Arora. 2010. MCP-1: chemoattractant with a role beyond immunity: a review. *Clinica chimica acta; international journal of clinical chemistry*. 411:1570–9.

Yamamoto, M., M. Okuyama, J.S. Ma, T. Kimura, N. Kamiyama, H. Saiga, J. Ohshima, M. Sasai, H. Kayama, T. Okamoto, D.C.S. Huang, D. Soldati-Favre, K. Horie, J. Takeda, and K. Takeda. 2012. A cluster of interferon-γ-inducible p65 GTPases plays a critical role in host defense against Toxoplasma gondii. *Immunity*. 37:302–13.

Yamamoto, T., H. Shimano, N. Inoue, Y. Nakagawa, T. Matsuzaka, A. Takahashi, N. Yahagi, H. Sone, H. Suzuki, H. Toyoshima, and N. Yamada. 2007. Protein kinase A suppresses sterol regulatory element-binding protein-1C expression via phosphorylation of liver X receptor in the liver. *The Journal of biological chemistry*. 282:11687–95.

Yang, D., S.G. Elner, Z.-M. Bian, G.O. Till, H.R. Petty, and V.M. Elner. 2007. Pro-inflammatory cytokines increase reactive oxygen species through mitochondria and NADPH oxidase in cultured RPE cells. *Experimental eye research*. 85:462–72.

Yoshida, A., Y. Koide, M. Uchijima, and T.O. Yoshida. 1994. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochemical and biophysical research communications*. 198:857–61.

Yoshikawa, T., H. Shimano, M. Amemiya-Kudo, N. Yahagi, A.H. Hasty, T. Matsuzaka, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, J. Osuga, K. Harada, T. Gotoda, S. Kimura, S. Ishibashi, and N. Yamada. 2001. Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Molecular and cellular biology*. 21:2991–3000.

Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W.E. Paul. 1995. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science (New York, N.Y.)*. 270:1845–7.

Zelcer, N., C. Hong, R. Boyadjian, and P. Tontonoz. 2009. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. *Science (New York, N.Y.)*. 325:100–4.

Zelcer, N., and P. Tontonoz. 2006. Liver X receptors as integrators of metabolic and inflammatory signaling. *The Journal of clinical investigation*. 116:607–14.

Resumen

Introducción

Los macrófagos son células de origen mieloide con un papel fundamental tanto en la respuesta inmunológica como en la homeostasis. En la respuesta inmunológica, los macrófagos son importantes efectores de la inmunidad innata y moduladores de la inmunidad adaptativa. Los macrófagos detectan señales de peligro, participan en la generación de un estado inflamatorio y fagocitan patógenos. A continuación, presentan antígenos a linfocitos T y modulan la activación de estos últimos a través de la secreción de citoquinas. Además, los macrófagos juegan un papel fundamental en la fase de resolución de la inflamación y reparación de los tejidos dañados (Stout and Suttles, 2004).

De forma general, los macrófagos se clasifican como M1 o clásicamente activados y M2 o alternativamente activados, aunque podemos hablar de un tercer tipo de macrófagos deactivados. Estos macrófagos polarizados difieren en la expresión de receptores, las funciones efectoras y la producción de citoquinas y quimioquinas. Los macrófagos M1 se caracterizan por altos niveles de expresión de citoquinas proinflamatorias, alta producción de especies reactivas de nitrógeno y oxigeno con actividad microbicida, fomento de la respuesta linfocitaria de tipo Th1 y una fuerte actividad tumoricida. En cambio, los macrófagos M2 están involucrados en la defensa contra parásitos, el fomento de la reparación tisular y la resolución de la inflamación (Sica and Mantovani, 2012).

Los Liver X Receptors (LXRα y LXRβ), son factores de transcripción miembros de la superfamilia de receptores nucleares que son regulados por formas oxidadas derivadas del colesterol (oxisteroles) (Janowski et al, 1996) y ciertos intermediarios de la biosíntesis del colesterol (Forman et al. 1997). Para unirse a elementos de respuesta específicos (LXREs) en los promotores de sus genes diana los LXRs forman heterodímeros obligatorios con los Retinoid X Receptors (RXRα, RXRβ y RXRγ), miembros también de la superfamilia de receptores nucleares que son activados por el 9-cis-ácido retinoico y ácidos grasos poli-insaturados de cadena larga (Forman et al. 1997). Gran parte de los genes diana de los LXR-RXR son reguladores del metabolismo del colesterol (Tontonoz y David J Mangelsdorf 2003). Algunos ligandos sintéticos de LXR se ha demostrado que reducen la aterosclerosis en modelos animales (Tangirala et

al, 2002; Joseph et al, 2002), así como también el tratamiento con ligandos de RXR produce cambios marcados en el balance de colesterol (Repa et al. 2000) y reduce el desarrollo de la aterosclerosis en ratones deficientes para la apoliproteína E (Claudel et al. 2001).

Los receptores nucleares son también capaces de reprimir la expresión de ciertos genes sin que sea necesaria la unión del receptor nuclear al DNA, un mecanismo que se denomina transrepresión. Los LXRs activados farmacológicamente pueden transreprimir la expresión de determinados genes inflamatorios (Joseph et al., 2003; Ghisletti et al., 2007, 2009), ejerciendo acciones anti-inflamatorias en macrófagos y linfocitos T, así como en modelos animales *in vivo* (revisado en A-Gonzalez et al. 2011). En nuestro laboratorio hemos visto que los ligandos de LXR transreprimen genes proinflamatorios en macrófagos en presencia de IFNγ (Pascual-García et al., 2013) o LPS. Uno de los objetivos de este trabajo es comparar la capacidad de transrepresión de LXR sobre genes de activación clásica en macrófagos primarios y en células espumosas (macrófagos cargados de lípidos) y profundizar en los mecanismos de tal represión.

Por otro lado, la resolución del proceso inflamatorio está mediada por un amplio rango de moléculas incluyendo citoquinas anti-inflamatorias como la IL-10, y TGFβ, lípidos bioactivos derivados de lipoxigenasa (lipoxinas, prostaglandinas, etc.) y factores de transcripción, así como por subgrupos de células inmunitarias como macrófagos activados alternativamente (macrófagos M2). Los LXRs en macrófagos enlazan dos mecanismos importantes de la resolución de la inflamación: la supresión de la producción de citoquinas inflamatorias, como se ha mencionado anteriormente, (Joseph et al., 2003; Pascual-García et al., 2013) y el potenciamiento de la eferocitosis a través de la inducción de al menos tres mediadores de fagocitosis: ABCA-1, transglutaminasa-2 y el receptor tirosina quinasa de la subfamilia Tyro-3 MER (MERTK), específico para el reconocimiento de células apoptóticas (Rébé et al. 2009, A-Gonzalez et al. 2009). Sin embargo, la relación entre los LXRs y las citoquinas de activación alternativa no se conoce, siendo ésta una parte del campo que hemos querido explorar en este trabajo.

Uno de los genes diana de LXR, *Idol*, ha sido descrito como una E3 ubiquitin ligasa y su actividad ha sido asociada a la regulación post-transcripcional del receptor de las LDL (Zelcer et al. 2009). Basándonos en la capacidad de Idol de afectar post-transcripcionalmente la expresión génica, en este trabajo decidimos explorar si esta proteína podría estar también facilitando la represión de genes proinflamatorios a través de un mecanismo indirecto que involucre la regulación de la degradación de otras proteínas. Adicionalmente, observaciones iniciales sobre Idol indicaban una potencial interacción de ésta con la *Myosin regulatory light chain protein* (MRLC) (Bornhauser et al., 2003b), de allí su previo nombre oficial Mylip (*Myosin regulatory light chain interacting protein*). Basándonos en estos estudios, así como en la implicación de la MRLC en los procesos de reestructuración del citoesqueleto durante la formación de fagosomas, en este trabajo también nos planteamos la hipótesis de que Idol podría estar jugando algún papel durante la fagocitosis en macrófagos.

Objetivos

1. Estudiar la transrepresión de genes proinflamatorios mediada por LXR en macrófagos primarios y células espumosas.

2. Explorar el papel del gen diana de LXR, Idol, en la transrepresión de genes pro-inflamatorios y en la eliminación de células apoptóticas por fagocitosis (eferocitosis).

3. Evaluar el efecto de los agonistas de LXR sobre la activación alternativa de macrófagos y en un modelo experimental de asma inducida por alérgeno. Estudiar los efectos recíprocos de las citoquinas de activación alternativa sobre la expresión de genes diana de LXR.

Resultados

1. **Estudio de la transrepresión de genes proinflamatorios mediada por LXR. Evaluación de los efectos de los agonistas de LXR en células espumosas versus macrófagos primarios**

A partir de estudios previos (Joseph et al., 2003; Ghisletti et al., 2007; Ogawa et al., 2005; Pascual-García et al., 2013), hemos determinado un conjunto de genes inducidos por IFNγ y LPS que son transreprimidos específicamente por agonistas de LXR en macrófagos. Para confirmar los efectos inhibitorios de GW3965 sobre la señalización de IFN-γ y LPS llevamos a cabo ensayos de transrepresion en macrófagos derivados de médula ósea (BMDM) pretratados durante 3h con el agonista de LXR y posteriormente estimulados con IFN-γ (5ng/ml) y LPS (5ng/ml) durante 6h. Hemos evaluado la expresión de genes involucrados en la respuesta inflamatoria como *Nos2, Ptgs2, Itgal, Cxcl10, Cxcl11, Mcp1, Ccl5*. Los resultados indican que la capacidad de transrepresión de LXR difiere dependiendo del estímulo inflamatorio. En general, la señal de IFN-γ es parcialmente inhibida por el tratamiento con GW3965 mientras que la señal de LPS se encuentra afectada robustamente por el mismo agonista.

A continuación, hemos evaluado los efectos anti-inflamatorios de LXR sobre macrófagos convertidos a células espumosas, uno de los tipos celulares mayoritarios en la placa aterosclerótica. Para generar las células espumosas, hemos incubado BMDM con AcLDL (10μg/ml) durante 24h. La confirmación de la carga de lípidos en estas células ha sido realizada mediante tinción con Oil Red O. A continuación, las células han sido estimuladas con el ligando de LXR, GW3965, y tratadas con IFN-γ (5ng/ml) o LPS (5ng/ml). De forma interesante, observamos que el tratamiento con AcLDLs inhibe significativamente la expresión de genes inducidos por LPS, pero no por IFN-γ. Por otro lado, el ligando de LXR es capaz de transreprimir la expresión de dichos genes en cualquiera de los dos contextos, tanto en las células espumosas expuestas a LPS como a IFN-γ, lo que apoya el valor terapéutico de los agonistas de LXR en la placa aterosclerótica. Dadas estas observaciones, el siguiente paso en este objetivo fue evaluar si la señalización por LPS está afectada por el tratamiento de macrófagos con AcLDL. Para esto, macrófagos previamente cargados durante 24h con AcLDL (10μg/ml) fueron estimulados con LPS durante 15, 30 y 60 minutos. Tras la estimulación con LPS

se realizó la extracción de proteína para el análisis por *western-blotting* de moléculas de la cascada de señalización de LPS. El patrón de fosforilación de los miembros de la familia de MAPKs, ERK y p38, y del inhibidor de NFκB, IκBα, (una medida indirecta de la activación de NFκB) fueron similares en las células espumosas y en las muestras control. Estos resultados indican que ni la activación de ERK, o de p38, así como de NFκB, son afectadas por el tratamiento de macrófagos con AcLDL.

Otra posibilidad mediante la cual la señalización por TLR puede estar siendo suprimida en las células espumosas es por el hecho de la acumulación de altos niveles de lípidos que son metabolizados y pueden dar lugar a la formación de ligandos naturales para otros receptores nucleares, que también tienen efectos antiinflamatorios como PPAR-γ. Teniendo esto en cuenta, decidimos investigar con más detalle el *crosstalk* entre LXR y PPAR-γ en la represión de genes inducidos por LPS. Para estos estudios de transrepresión hemos utilizado macrófagos peritoneales obtenidos de ratones C57Bl/6 *wild type* y deficientes para ambas isoformas de LXR (LXRα y β -/-), mediante la inyección con tioglicolato (3%), ya que estos macrófagos expresan niveles más altos de PPAR-γ (Ricote et al., 1998).

En colaboración con Daniel Alameda y Mercedes Ricote (CNIC, Madrid), utilizamos información de ensayos de perfil de expresión génica desarrollados por su grupo para seleccionar algunos genes inducidos por LPS que fueran transreprimidos por ambos receptores nucleares (LXR y PPAR-γ) o solo por uno de ellos en macrófagos primarios.

Hemos evaluado la expresión de numerosos genes inducidos por LPS. Observamos que con 18 horas de pre-estimulación con ligandos de LXR la mayoría de los genes se encontraron reprimidos entre un 30 y 50% con respecto al control (tratamiento con el vehículo, DMSO).

De los resultados obtenidos, comparando macrófagos *wild type* estimulados con los agonistas de los dos receptores nucleares con macrófagos deficientes para ambas isoformas de LXR, hemos podido identificar tres grupos de genes. Un gran grupo de genes que se transreprimen por los dos receptores nucleares, LXR y PPAR-γ, como *Nos2, Ptgs2* y *Ccl2,* entre otros. Otro grupo de genes que son transreprimidos únicamente por PPAR-γ, pero que no son afectados significativamente por LXR, que

incluye *Ccl17, Il12b* e *Il6.* Por último, hay otro grupo reducido de genes que son sólo sensibles al ligando de LXR, y no al ligando de PPAR-γ, incluyendo *Serpinb2, Pdpn* e *Il1b*. El uso de macrófagos deficientes para LXR revela que las acciones represivas del agonista de LXR son específicas bajo las condiciones usadas en estos experimentos. Más aún, los efectos inhibitorios del agonista de PPAR-γ son todavía observados en ausencia de LXR funcionales. Esto ocurre tanto para genes que son únicamente afectados por el agonista de PPAR-γ, pero también para genes que pueden ser reprimidos por ambos agonistas, lo cual sugiere que ambos receptores nucleares pueden transreprimir los mismos genes independientemente el uno del otro.

2. Análisis del posible papel de Idol en la regulación de genes pro-inflamatorios

Durante el desarrollo de estos estudios con macrófagos peritoneales hemos observado que el tratamiento prolongado con GW3965 tiene efectos más robustos sobre la transrepresión que tratamientos cortos. Aunque la transrepresión para algunos genes ya puede ser observada con 2h de pre-incubación con GW3965, la activación prolongada de la vía de LXR resultó en un aumento de la transrepresión en la mayoría de los genes estudiados.

Seguidamente, evaluamos si la cooperación con el compañero heterodimérico de LXR, RXR, contribuye al potencial de transrepresión por una activación prolongada de LXR. En colaboración con el grupo dirigido por la Dra. Mercedes Ricote en el CNIC, Madrid, hemos utilizado animales doble *knock out* para RXRα y RXRβ, en los que hemos determinado si LXR requiere de la presencia de RXR funcional para transreprimir algunos de estos genes. De forma interesante, nuestros resultados muestran que la expresión funcional de RXRs es necesaria para la transrepresión de al menos algunos de los genes sensibles al ligando de LXR, como por ejemplo *Ccl2* y *Ptgs2*. Un análisis más extenso de la contribución de la heterodimerización con RXR para la transrepresión mediada por LXR está siendo generado por Daniel Alameda en el grupo de la Dra. Ricote y será incluido en su trabajo de tesis.

Tomando estas observaciones en conjunto, quisimos evaluar la hipótesis de si la regulación transcripcional positiva de uno de los genes diana de LXR/RXR podría estar contribuyendo en el mecanismo de represión de la respuesta inflamatoria mediada por

LXR. Uno de los posibles candidatos para este estudio fue el gen diana de LXR Idol, una E3 ubiquitin ligasa cuya función se ha asociado a la regulación post-transcripcional de miembros de la familia del receptor de las LDL (Zelcer et al., 2009; Hong et al., 2010).

Para llevar a cabo este objetivo hemos clonado el gen de la proteína Idol en el vector de expresión pcDNA3.1 (Invitrogen), junto con un segmento 3x-flag procedente de un vector pCMV-3xflag (Sigma). Mediante selección en un medio con G418, generamos un clon de macrófagos Raw264.7 que sobre-expresa de manera estable la proteína Idol, así como un clon que expresa de manera estable el vector sin la proteína. Utilizando estas células hemos evaluado si Idol juega un papel tanto en montar una respuesta pro-inflamatoria o en la capacidad de LXR para mediar la transrepresión. Para ello hemos utilizado el modelo de inducción de genes pro-inflamatorios descrito en el apartado 1. En general, la sobre-expresión de Idol no interfirió negativamente en la inducción de genes pro-inflamatorios por LPS, tampoco en la transrepresión mediada por LXR de los diferentes genes pro-inflamatorios analizados. Sin embargo, una diferencia importante que hemos identificado en estas células que sobre-expresan *Idol* es la sobre-inducción del gen *Ccl2* (*Mcp1*), que codifica para la quimioquina CCL2, tanto a nivel basal como tras el tratamiento con LPS e IFN-γ. Análisis mediante ELISA para CCL2 realizados con los sobrenadantes de las células Raw264.7 que sobre-expresan *Idol* demuestran que esta sobreexpresión a nivel de mRNA se correlaciona con un aumento significativo de la cantidad de CCL2 secretada al medio por estas células. De forma interesante, la activación de LXR fue capaz de reprimir la secreción de CCL2 inducida por LPS, en correlación con los efectos inhibitorios ejercidos por este receptor nuclear sobre los niveles de mRNA de *Ccl2*, sugiriendo que LXR y Idol ejercen acciones opuestas sobre la producción de la quimioquina CCL2 en macrófagos. Junto con estas observaciones encontramos que la expresión basal de un gen inducido por CCL2, *monocyte chemotactic protein (MCP-1)-induced protein* (Mcpip1), está aumentada en las células que sobreexpresan *Idol*, posiblemente como consecuencia de la función autocrina de CCL2 sobre las propias células que lo producen.

Dado que *Idol* regula negativamente la vida media del receptor de LDL, nos preguntamos si el aumento en la expresión de *Ccl2* podría estar indirectamente mediado por la disminución en superficie del receptor de LDL. Para evaluar este

aspecto, hemos analizado la expresión de *Ccl2* en macrófagos deficientes para el receptor de LDL (LDLR -/-), y observamos que estas células expresan niveles similares de *Ccl2* que los macrófagos *wild type* en respuesta a LPS y a IFN-γ. Por tanto, el aumento de la expresión de CCL2 en las células que sobreexpresan Idol no se debe a una respuesta frente a la degradación aumentada del receptor de LDL. En conjunto, nuestros resultados sugieren que Idol regula positivamente la expresión de *Ccl2*, independientemente de sus efectos sobre el LDLR.

3. Evaluación del papel del Idol en promover la fagocitosis de células apoptóticas

Basándonos en observaciones iniciales que sugieren que Idol podría interactuar con la myosin regulatory light chain protein (MRLC) (Bornhauser et al., 2003b), y la implicación de la MRLC en la re-estructuración del citoesqueleto durante la formación del fagosoma, nos cuestionamos si Idol podría estar jugando un papel en la fagocitosis mediada por macrófagos.

Utilizando los macrófagos Raw264.7 transfectados establemente con *Idol*, hemos realizado ensayos de fagocitosis de timocitos apoptóticos (AT), marcados con CellTraker green (Molecular Probes). Los timocitos fueron obtenidos a partir del timo de ratones en periodo neonatal y la inducción de apoptosis se llevó a cabo mediante incubación con dexametasona durante 4h. Mediante contaje directo de las imágenes obtenidas por microscopía confocal, hemos observado diferencias a nivel de unión de cuerpos apoptóticos (timocitos) en la superficie de los macrófagos Raw264.7 que sobre-expresan *Idol*. Las células con sobre-expresión de Idol mostraron tener mayor número de timocitos adheridos por célula, así como un mayor porcentaje de timocitos adheridos en total comparado con las células control. A continuación, evaluamos si la sobre-expresión de Idol junto con la activación de LXR por el ligando GW3965 potencia la fagocitosis en macrófagos Raw264.7. La preincubación de la células durante 18h con el ligando de LXR, antes del tratamiento con AT, incrementó su capacidad tanto para adherir como para fagocitar los cuerpos apoptóticos en los diferentes clones evaluados. El % más alto de fagocitosis fue observado en las células Raw264.7-Idol estimuladas con el agonista de LXR, sugiriendo un potencial papel para Idol en la

eliminación de células apoptóticas. No obstante, futuros estudios deberán incluir un mayor número de experimentos para confirmar la significancia estadística de estos resultados.

4. Efectos mediados por LXR sobre la activación alternativa de macrófagos

Para evaluar el efecto de los ligandos de LXR sobre la expresión de genes de la activación alternativa, hemos realizado primero una cinética de estimulación con IL-4, preincubando los macrófagos durante 6h con los ligandos T0901317 y GW3965 (1µM), y posteriormente durante 12, 24 y 36h con IL-4 (10ng/ml). Observamos que la expresión de los principales genes asociados con la activación alternativa de macrófagos, entre ellos *Ym1*, *Arg1*, *Mgl1* y *Mrc1*, no fueron afectados por el tratamiento con el ligando de LXR. Sin embargo, la incubación de macrófagos con GW3965 inhibió significativamente la expresión de los genes que codifican para las quimioquinas *Ccl22* (MDC) y *Ccl17* (TARC).

Por otro lado, evaluamos si la presencia de LXR funcional es necesaria o afecta la inducción de genes marcadores de la activación alternativa y observamos que, en general, la ausencia de LXR no afecta el patrón de expresión de éstos genes. Sin embargo, observamos un aumento significativo en la inducción de *Ccl22* y *Ccl17* mediada por IL-4 en macrófagos de ratones deficientes para ambas isoformas de LXR (α y β -/-), con respecto a la inducción obtenida en macrófagos *wild type*. Así mismo, este aumento a nivel de expresión génica se correspondió con un aumento en la cantidad de quimioquina secretada al medio, determinado por ELISA a partir de los sobrenadantes de macrófagos deficientes para LXRs estimulados con IL-4.

A continuación, evaluamos si la activación de LXR tiene un efecto sobre la señalización de IL-4 mediante el estudio por *western blotting* de la fosforilación de STAT-6, factor de transcripción que media gran parte de los efectos transcripcionales de IL-4. Realizamos una estimulación con IL-4 a distintos tiempos en macrófagos previamente estimulados o no con GW3965, así como en macrófagos deficientes para LXR (α y β -/-). Encontramos que en macrófagos *wild type*, STAT-6 se observa fosforilado a los 30min, 3, 6, 12 y 24 horas tras la estimulación con IL-4 (la fosforilación disminuye con el tiempo y a 24 horas se observa muy poca señal). La presencia del agonista de LXR no

modifica este patrón de fosforilación. Tampoco observamos diferencias entre células *wild type* y células deficientes para LXR.

Otra manera en la que LXR podría estar afectando la expresión de los genes *Ccl17* y *Ccl22* es mediante interferencia sobre la unión de STAT-6 a sus elementos de respuesta específicos (elementos GAS) en las regiones reguladoras de estos genes. Para esto, hemos realizado ensayos de inmunoprecipitación de cromatina (ChIP) en macrófagos *wild type* y deficientes para LXRs, estimulados con IL-4, utilizando un anticuerpo anti-STAT6. A partir de las bases de datos que muestran resultados de ChIP-seq generados por otros grupos, elegimos analizar varias regiones cercanas a los genes *Ccl22* y *Ccl17* en las que potencialmente se une STAT-6. No obtuvimos enriquecimiento de ninguna de esas regiones en cromatina inmunoprecipitada con anticuerpo anti-STAT-6 en macrófagos *wild type* ni en macrófagos LXRαβ-/-, por lo que es necesario explorar otras regiones de posible interacción de STAT-6 en los promotores de *Ccl22* y *Ccl17*, que permitan definir si LXR ejerce algún tipo de interferencia sobre la unión de STAT-6 a estos genes.

- Papel de LXR en un modelo murino de asma alérgica

Dados los efectos observados sobre las quimioquinas CCL22 y CCL17, decidimos evaluar el papel de LXR en un modelo de inducción de asma por alérgeno, ya que estas quimioquinas, así como los procesos de activación alternativa de macrófagos podrían tener un papel en la evolución de enfermedades de hipersensibilidad como el asma (Bisset y col, 2005). En colaboración con el grupo dirigido por el Dr. De Mora (Universidad Autónoma de Barcelona) ratones *wild type* y deficientes para ambas isoformas de LXR fueron expuestos durante 10 días a un extracto de ácaros del polvo (HDM) por administración intranasal. Otro grupo de ratones *wild type* fue tratado mediante inyección intraperitoneal del ligando de LXR GW3965 24h antes y durante los 10 días de exposición al alérgeno. 24 horas tras la última exposición a HDM evaluamos la hiperreactividad bronquial (o resistencia pulmonar) mediante pletismografía y tomamos muestras de lavado broncoalveolar y de tejido pulmonar. De forma interesante, observamos que los ratones deficientes de LXR tienen mayor dificultad respiratoria que los *wild type* tras ser expuestos a HDM en las mismas

condiciones. Así mismo, los ratones que fueron tratados con GW3965 mostraron una resistencia pulmonar que permaneció cercana a la línea basal, similar a la respuesta de ratones no sensibilizados, sugiriendo que el tratamiento con el agonista de LXR provee de efectos beneficiosos para prevenir la deficiencia pulmonar en este modelo de asma inducida por alérgeno.

Evaluando los niveles de expresión de mRNA de distintos mediadores inflamatorios de la vía respiratoria observamos un incremento en la expresión de los genes *Ccl17, Ccl22* y *Ccl2* así como también de *Il4* y *Ccr8* (receptor de CCL1) en las muestras de tejido pulmonar de ratones sensibilizados. De manera interesante, el tratamiento con GW3965 disminuyó el patrón de expresión de estas citoquinas en los ratones tratados con el ligando GW3965. El grado de infiltración celular también fue evaluado en estos animales, observándose un número significativamente mayor de eosinófilos en las muestras de ratones expuestos a HDM en comparación con los no sensibilizados. De forma similar, en los ratones tratados con GW3965 observamos una tendencia hacia un número menor de eosinófilos y monocitos en comparación con los animales que fueron sensibilizados pero inyectados con vehículo.

Además del análisis de expresión génica y de infiltración celular en los tejidos pulmonares, las muestras de fluido bronqueoalveolar (BAL) y suero de todos los animales fueron analizadas mediante ELISA para determinar los niveles de las quimioquinas CCL22 y CCL17. Encontramos que los niveles de ambas citoquinas se encuentran aumentados en los ratones sensibilizados con respecto al control, sin embargo no se observan diferencias entre los grupos tratados con GW3965 y los no tratados, sugiriendo que la administración de GW3965 tiene un efecto local sobre la inflamación en los pulmones más que un efecto sobre la concentración de quimioquinas a nivel sistémico.

- Efectos recíprocos de la activación alternativa de macrófagos sobre la expresión de genes diana de LXR

Como complementación a estos estudios, también exploramos si la estimulación con IL-4 tiene un impacto recíproco sobre la expresión de genes diana de LXR. Para esto, hemos pretratado BMDM con esta citoquina durante 18 horas y posteriormente

hemos activado la vía de LXR añadiendo los agonistas T0901317 y GW3965 (1μM) durante 24 horas. En este conjunto de experimentos también comparamos los efectos de IL-4 con aquellos de las citoquinas de deactivación, IL-10 y TGFβ.

En los macrófagos previamente estimulados con las citoquinas de deactivación IL-10 y TGFβ, analizamos la expresión a nivel de mRNA de varios de los genes diana de LXR, y no observamos efectos significativos de dichas citoquinas. Sin embargo, el tratamiento con IL-4 resultó en una disminución significativa de la inducción de los genes *Aim*, *Apoe* y *Apoc2* a nivel basal y en respuesta a los ligandos de LXR. Además, utilizando macrófagos deficientes para Stat6, pudimos determinar que estos efectos son dependientes de este factor de transcripción.

Profundizando en el mecanismo de este efecto, quisimos determinar si la activación de la señal de IL-4 afecta la unión de LXR a un sitio de regulación o *enhancer* de uno de los genes diana de LXR, *Aim*, escogido mediante el análisis de datos de *ChIP-sequencing* reportados previamente. Para esto, utilizando macrófagos pretratados con IL-4 durante 12h previo a la estimulación con una combinación de ligandos para LXR y RXR durante 90 min, realizamos ensayos de inmunoprecipitación de cromatina con anticuerpos anti-LXR y anti-RXR y evaluamos, por qRT-PCR, el enriquecimiento relativo de una zona con un sitio de unión de LXR en el gen *Aim*. Los resultados obtenidos mostraron que la unión de LXR a esa región reguladora de *Aim* es aumentada por el tratamiento con la combinación de ligandos para LXR/RXR, sin embargo, la estimulación con IL-4 no afecta esta unión del receptor a la zona *enhancer*, sugiriendo que IL-4 es capaz de inhibir la expresión de *Aim*, sin afectar negativamente la unión de LXR a esta región reguladora del gen.

Discusión

En los últimos años se han acumulado fuertes evidencias que avalan el papel inhibitorio de LXR en el control de la inflamación (Revisado en A-González and Castrillo 2011). Múltiples estudios se han enfocado en la capacidad de los agonistas de LXR para reprimir la señalización inflamatoria en respuesta a LPS bacteriano (Joseph et al., 2003; Ghisletti et al., 2007, 2009). En nuestro laboratorio hemos observado que los ligandos de LXR tienen capacidad de transreprimir genes proinflamatorios inducidos por IFN-γ en macrófagos y microglía, afectando de esta manera la respuesta de tipo M1 o inflamatoria (Pascual-García et al., 2013).

En la primera parte de este estudio, hemos continuado explorando algunos aspectos de la represión de macrófagos activados clásicamente mediada por LXR, encontrando que la eficiencia de represión transcripcional por parte de los agonistas de LXR difiere dependiendo del estímulo inflamatorio. En BMDM la activación de LXR resultó en una represión parcial de los genes inflamatorios inducidos por IFN-γ mientras que en los genes inducidos por LPS se observan efectos inhibitorios más robustos, resultados que están en concordancia con reportes previos que sugieren que el/los mecanismo/s usados para la transrepresión difiere dependiendo del disparador inflamatorio (Ghisletti et al., 2007, 2009). De hecho, es probable que lo que conocemos colectivamente como transrepresión sea una combinación de mecanismos que son específicos de gen y señal.

De forma interesante, en nuestros estudios hemos observado que la conversión de macrófagos a células espumosas resulta en una inhibición dramática de la respuesta inflamatoria a LPS pero no a IFN-γ. Estos resultados apoyan la hipótesis de que la acumulación de colesterol suprime, antes que estimula, la activación dependiente de TLR de la expresión de genes inflamatorios en macrófagos, en línea con trabajos recientes en los que se observa que macrófagos peritoneales cargados de lípidos muestran un fenotipo 'de-activado', con una marcada supresión de los genes diana de TLR4 (Spann et al., 2012). Sin embargo, el hecho de que la respuesta inflamatoria a IFN-γ permanece sin alterarse después de la carga con colesterol es intrigante y podría ayudar a explicar por qué el fenotipo de macrófagos más abundante dentro de

lesiones ateroscleróticas es todavía el de clásicamente activados o M1 (Kadl et al., 2010).

El mecanismo que media esta drástica supresión de los genes inducidos por TLR4 en células espumosas inducidas por acLDL todavía no está claro. En nuestro sistema no hemos observado que el tratamiento con acLDL conduzca a cambios significativos en la capacidad del LPS para inducir la estimulación de las vías de p38 y ERK, así como tampoco en la degradación de IκB. Otra posibilidad que hemos considerado es que la carga de macrófagos con lípidos podría resultar en la generación de agonistas naturales para receptores nucleares, incluyendo LXR y quizás PPAR-γ, que pueden interferir con eventos transcripcionales que ocurren después de la activación de NFκB.

Estudios previos han demostrado que LXR y PPAR-γ pueden ambos transreprimir un gran número de genes inducidos por LPS en macrófagos (Ogawa et al., 2005). A través de un extenso estudio de análisis de expresión génica realizado en colaboración con el grupo de la Dra. Ricote en CNIC, Madrid, hemos caracterizado efectos específicos de los agonistas de LXR y de PPAR-γ en la transrepresión de genes inducidos por LPS. Encontramos que LXR y PPAR-γ comparten la capacidad de transreprimir ciertos genes como *Nos2*, *Ptgs2 (Cox2)* y *Ccl2 (Mcp1)*. Hay un conjunto de genes que son únicamente sensibles al agonista de PPAR-γ, y otro grupo reducido de genes que pueden ser sólo repimidos por el agonista de LXR.

De forma interesante, y también en colaboración con el grupo de la Dra. Ricote, hemos observado que la transrepresión mediada por LXR de algunos genes se ve disminuida significativamente en macrófagos deficientes para RXRα y β, sugiriendo que LXR y RXR cooperan para algunos de los efectos de represión mediados por LXR.

Por otra parte, en este trabajo hemos estudiado el posible papel del gen diana de LXR/RXR, la E3-ubiquitina ligasa Idol en la regulación de la expresión de genes inflamatorios. En nuestras manos, la sobre-expresión de Idol no inhibe la respuesta inflamatoria a LPS ni conduce a cambios en la capacidad transrepresora de LXR, lo que sugiere que la inducción de dicha E3-ubiquitina ligasa no ejerce ninguna función como mecanismo de transrepresión utilizado por LXR. Sin embargo, nuestros resultados sugieren un papel selectivo de Idol en el mecanismo de regulación positiva de la quimioquina CCL2, independiente de los efectos post-transcripcionales sobre el

receptor de LDLs e independiente también de los efectos represores que LXR ejerce sobre esta misma quimioquina.

Trabajos anteriores avalan un papel regulador de Idol sobre procesos de morfología y motilidad celular, al parecer a través de la regulación de la degradación de la Myosin regulatory light chain protein (MRLC) (P A Olsson et al. 1999; Bornhauser, Per-Anders Olsson, y Dan Lindholm 2003; Nagano et al. 2006) que afecta a la capacidad de reestructuración de los filamentos de actina y el resultado final depende del tipo celular y la señal inductora. En nuestros experimentos de fagocitosis de células apoptóticas con macrófagos que sobre-expresan establemente esta proteína (Raw264.7-Idol) observamos que éstas tienen una mayor capacidad para unir cuerpos apoptóticos en la superficie y además mostraron mayores niveles de fagocitosis cuando activamos LXR, en comparación con las células control. Sin embargo, aunque un mayor número de experimentos similares son necesarios para mostrar una significancia estadística, estos datos sugieren un nuevo papel para Idol en mediar la adherencia y fagocitosis de células apoptóticas.

En la segunda parte de este trabajo estudiamos el efecto de los LXRs sobre la expresión de genes de respuesta tipo M2 o de activación alternativa de macrófagos. A diferencia de lo que observamos para los genes pro-inflamatorios, la estimulación con ligandos de LXR no afecta la expresión de la mayoría de genes de la activación alternativa inducidos por IL-4. De alguna manera, LXR es capaz de interferir con mayor potencia sobre la inducción de genes mediada por STAT-1 (Pascual-García et al., 2013) que sobre la inducción de genes mediada por STAT-6.

Sin embargo, de forma interesante también observamos que LXR ejerce un efecto inhibitorio específico sobre la expresión inducida por IL-4 de las quimioquinas *Ccl22* y *Ccl17*. La regulación negativa de la expresión de *Ccl22* and *Ccl17* es mediada por ambas isoformas de LXR (α y β), ya que es en ensayos con macrófagos deficientes para las dos isoformas (LXRαβ DKO) cuando observamos que ambos genes se sobreinducen mayormente.

Ambas quimioquinas, CCL22 y CCL17, han sido implicadas en numerosas enfermedades incluyendo la inflamación pulmonar inducida por alérgeno, dermatitis atópica y linfoma (Jacobsen et al., 2008; Hirota et al., 2011; Chang et al., 2012). Nuestros resultados utilizando un modelo *in vivo* de inflamación pulmonar inducida por

alérgeno, sugieren que LXR podría tener un efecto protector dado que los animales *wild type* tratados con GW3965 muestran una tendencia a una mayor capacidad respiratoria ante la inducción de la respuesta asmática. Del mismo modo, los ratones deficientes para LXR muestran una función respiratoria significativamente empeorada con respecto a los ratones *wild type*. El análisis de la respuesta local en los tejidos pulmonares mostró que los niveles de *IL-4* y de las quimioquinas *Ccl17, Ccl22* y *Ccl2* son inducidos en los ratones asmáticos. De forma interesante, los niveles de estos mediadores de inflamación de las vías respiratorias disminuyeron en los animales tratados con GW3965, pese a que es necesario un número más elevado de animales por grupo para determinar si las diferencias son estadísticamente significantes. En conjunto, estos resultados sugieren que la modulación de la actividad de LXR ofrece un prospecto para nuevas aproximaciones terapéuticas en el tratamiento del asma y quizás otras enfermedades respiratorias, en línea con otros trabajos que muestran que LXR es capaz de suprimir respuestas inflamatorias en células de las vías respiratorias y en un modelo de inflamación respiratoria inducido por LPS (Birrell et al., 2007; Delvecchio et al., 2007)

Finalmente, hemos visto un efecto recíproco de la citoquina de activación alternativa IL-4 sobre la expresión de un subconjunto específico de genes diana de LXR como son *Aim*, *Apoe* y *Apoc2*, que es dependiente de STAT-6, revelando una relación negativa entre ambos factores de transcripción. No obstante, los efectos sobre la expresión de *Aim* no están mediados por una interferencia sobre la capacidad de unión de LXR a una región genómica reguladora *upstream* de este gen en presencia de IL-4. Un escenario similar ha sido recientemente descrito por nuestro grupo en el contexto de la activación de macrófagos con IFN-γ. LXR y STAT-1 establecen una comunicación negativa recíproca, y la activación de STAT-1 conduce a la inhibición selectiva de ciertos genes inducidos por LXR (por ejemplo, *Abca1*, *Srebp1c* y *Aim*) sin afectar la capacidad de unión de LXR y RXR a elementos de respuesta de dichos genes afectados (Pascual-García et al., 2013). Curiosamente, la activación de STAT-1 y STAT-6 no se traduce en la inhibición del mismo grupo de genes diana de LXR, lo que sugiere que la interferencia de estas dos vías sobre la capacidad transcripcional de LXR involucra mecanismos moleculares distintos que podrían operar de manera específica para cada gen.

Conclusiones

1. El tratamiento de macrófagos con acLDLs conduce a una fuerte represión de la inducción de genes de respuesta a LPS, sin interferir negativamente con la respuesta inducida por IFN-γ.

2. El tratamiento de macrófagos con acLDLs no afecta a la activación de las moléculas de señalización por LPS ERK1/2 y p38, ni tampoco a la degradación de IκBα.

3. PPAR-γ comparte con LXR la capacidad de reprimir un conjunto de genes inflamatorios común, sin embargo, la expresión de LXR funcional no es requerida para la transrepresión mediada por PPAR-γ.

4. LXR y RXR cooperan en la transrepresión inducida por GW3965.

5. La sobreexpresión del gen diana de LXR Idol no inhibe la transrepresión de genes pro-inflamatorios mediada por LXR, resulta en una expresión y secreción incrementada de CCL2 independientemente de sus efectos sobre el LDLR, y conlleva a una mayor adhesión de timocitos apoptóticos.

6. La activación de LXR inhibe selectivamente la expresión mediada por LXR de las quimioquinas CCL22 y CCL17 sin alterar el patrón de fosforilación de STAT-6.

7. El tratamiento con GW3965 tiene efectos inhibitorios sobre la reactividad de las vías respiratorias y la expresión de genes inflamatorios en pulmones en un modelo murino de asma inducida por alérgeno.

8. IL-4, vía la activación de STAT-6, interfiere recíprocamente con la expresión de un subconjunto selectivo de genes dependientes de LXR (*Apoc2*, *Apoe* y *Aim*). Al menos en la región regulatoria de *Aim*, este efecto no está mediado por una disminución del reclutamiento de LXR/RXR.

Annexes

Collaboration:

Liver X Receptors Inhibit Macrophage Proliferation through Downregulation of Cyclins D1 and B1 and Cyclin-Dependent Kinases 2 and 4

Mónica Pascual-García, Jose M. Carbó, Theresa León, Jonathan Matalonga, Ruud Out, Theo Van Berkel, Maria-Rosa Sarrias, Francisco Lozano, Antonio Celada and Annabel F. Valledor.

The student collaborated with the analysis of mRNA relative expression of the genes *Cdk2* and *Cdk4* by q-RT-PCR. These data is not shown in the thesis manuscript.

Collaboration:

Reciprocal Negative Cross-Talk between Liver X Receptors (LXRs) and STAT1: Effects on IFN-γ–Induced Inflammatory Responses and LXR-Dependent Gene Expression

Mónica Pascual-García, Laura Rue*, Theresa León*, Josep Julve, José María Carbó, Jonathan Matalonga, Herbert Auer, Antonio Celada, Joan Carles Escolà-Gil, Knut R. Steffensen, Esther Pérez-Navarro, Annabel F. Valledor

LR and TL* Equally contributed to this work.

The student collaborated with the experiments to analyze the transrepression of IFN-γ- and LPS- inducible genes mediated by LXR in acLDL-loaded macrophages, and also to the analysis of the expression of LXR target genes in macrophages treated with the cytokines IL-4, IL- 10 or TGF-β.