The Anti-Inflammatory Role of Nuclear Receptors in Dendritic Cells

A thesis submitted for the degree of Ph.D.

By

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March 2012.

Based on research carried out at

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Declaration

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ACKNOWLEDGEMENTS

There are so many people that I would like to thank and definitely not enough space to say exactly how grateful I am to you all. I have been lucky enough to work with an amazing group of people over the past few years. Firstly I would like to thank Christine for all your help, support, enthusiasm and patience – and for telling me not to do anymore of those p50 blots! I have thoroughly enjoyed working with you and learning from you over the last few years. To everyone in the Lab – you are the reason why I have such great memories when I look back at my time in DCU. Whenever I think of failed experiments, tough days and tears, there is always a great memory of you guys that goes along with it. Laura – there would have been a lot more tears and lot less laughs without you. I seriously considered adding the term "righting the world" to my Materials and Methods section! Ciara –thank you so much for all your help as I was finishing up. You were starting a new job and still always had time for my endless questions. Joey – thanks for giving me my most hardcore "memory" – 24hr drinking session!!!. I'm looking forward to celebrating with you soon Dr deCourcey. Fiona (and the little man ☺) the lab would definitely be a lot less cheerful without you in it. I don't know how you do it, but you always have time for everyone else and always have a smile to go with it ! To Croatia's most valuable export – Maja, you and Francis have been such great friends to me. Thank you for always listening. You always told me things I needed to hear and not always the things I wanted to hear at the time – the sign of a true friend. To Mark, thank you for your unwavering enthusiasm. It really is infectious (although I can't believe you haven't been broken yet!!). To Kathy and Iza – the newbies ☺ you are a breath of fresh air in the lab and I am slightly jealous of the the memories and experiences you have ahead of you. To Paul, Joey, Gillian and Carolyn – thank you for all your help with the animal studies – without which I could not have

done this research. Thank you to everyone in the BRS – all the trips and nights out are what kept me sane over the last few years.

To my family, I owe you the biggest thank you of all. Thank you for all your love, support and patience. Thank you for understanding and for always being there when I needed you. I know how lucky I am that even after missed birthdays, missed phonecalls and missed visits you were still always on my side supporting me.

Finally a very special thank you to Ze and Reja – for never letting me forget whats really important.

xxx

PUBLICATIONS

Canavan M, McCarthy C, Ben Larbi N, Dowling J, Kagechika H, Moynagh P, Melgar S, Loscher CE (2012)
Liver X Receptor suppresses the production of the IL-12 family of cytokines via NFκBp50
Submitted to the Journal of Immunology

Dowling J., McCoy C., Doyle S., BenLarbi N., Canavan M .,O'Neill L., Loscher CE (2012)
Conjugated linoleic acid suppresses IRF3 activation via modulation of CD14
J Nutr Biochem. 2012 Aug 15. [Epub ahead of print]

Draper E., Reynolds C. M., Canavan M., Mills K. H., Loscher C., Roche H. M. (2011)
Omega-3 fatty acids attenuate dendritic cell function via NFκB independent of PPARy.
Journal of Nutritional Biochemistry 2011 Aug;22 (8):784-90

Canavan M., deCourcey J., Ben Larbi N., Loscher CE. LXR suppresses IL-17 secretion from Th17 cells through an interaction with RXR. (Manuscript in preparation for submission to the Journal of Immunology and Cell Biology)

McCarthy C, Murphy C, Canavan M, Dowling JK, Guiry PJ, Loscher CE. A purified marine compound isolated from the marine sponge *Membranipora membranacea* suppresses macrophage function following exposure to bacterial but not viral ligands. (Manuscript in preparation)

ABSTRACT

The liver X receptor (LXR), peroxisome proliferator activated receptor γ (PPARγ) and retinoid X receptor (RXR) are members of the nuclear receptor superfamily. Once activated these receptors can form heterodimers with each other in order to control key processes within the cell. The role of these NRs in immune cells is relatively uncharacterized; therefore we examined the effects of their activation on cytokine production and cell surface marker expression on murine bone marrow-derived dendritic cells (BMDC). We demonstrated that these NR agonists can specifically modulate the IL-12 family of cytokines as well as altering the expression of co-stimulatory markers on the cell surface. We also show that the effect of LXR activation on these IL-12 family of cytokines is a result of heterodimerising with RXR. Furthermore, we found that expression of LXR was regulated during inflammatory disease. In order to determine the mechanism by which LXR exerts its anti-inflammatory effects we next examined its effects on DCs activated by a panel of TLR ligands. These results showed that the target of LXR was a common element of the TLR pathways; therefore we determined its effects on NFκB and IRF3 activation. Interestingly, we show that LXR: RXR heterodimers are important in NFκB inhibition but not IRF3. We next showed that LXR can specifically target the p35, p40 and EBI3 subunits of the IL-12 cytokine family. Given that these subunits are directly under the control of the NFκB subunit p50, we examined the effect of LXR activation on this p50 subunit and showed that LXR colocalises in DC with p50 and that LXR activation prevents translocation of the p50 subunit into the nucleus. In summary our study provides evidence for an anti-inflammatory role for LXR in DCs and highlights its potential as a therapeutic target for chronic inflammatory diseases.

ABBREVIATIONS

9cRA	9 cis Retinoic acid
ABCA1	ATP-binding cassette transporter 1
APC	Antigen presenting cell
APR	Acute phase response
AR	Androgen receptor
BAFF	B cell activating factor
BMDC	Bone marrow derived dendritic cell
CBP	CREB binding protein
CD	Crohns disease
CIA	Collagen induced arthritis
CNS	Central nervous system
CREB	cAMP response element-binding
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
CVD	Cardiovascular disease
DBD	DNA binding domain
DC	Dendritic cell
DLN	Draining lymph node
DSS	Dextran sodium sulfate
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein Barr induced virus 3
ER	Endoplasmic reticulum
FXR	Farnesoid X receptor
GRR	Glycine rich region
IBD	Inflammatory bowel disease
IFN	Interferon

IKK	IκB kinase
iNKT	Invariant natural killer T cell
IRAK	Interleukin-1 receptor–associated kinase
IRF3	Interferon regulatory factor 3
KO	Knock out
LBD	Ligand binding domain
LBP	LPS binding protein
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MDDC	Monocyte derived dendritic cell
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MyD88	Myeloid differentiation factor 88
NK	Natural Killer
Nor-1	Neuron-derived orphan receptor 1
Nurr1	Nuclear receptor related 1 protein
Nurr77	Nerve growth factor-induced clone B
OPC	Oligodendrocyte progenitor cell
PAMP	Pathogen associated molecular pattern
Poly:IC	Polyinosinic:polycytidylic acid
PPAR	Peroxisome proliferator activated receptor
PRR	Pattern recognition receptor
PXR	Pregnane X receptor
RA	Rheumatoid arthritis
RHD	Rel homology domain
RHIM	Rip homotypic interaction motif

RIP	Ribosome inactivating protein
RXR	Retinoid X receptor
SUMO	Small ubiquitin related modifier
TAK	Transforming growth factor-beta-activated kinase
TCR	T cell receptor
Th	T helper
TIR	Toll/Interleukin-1 receptor domain
TLR	Toll-like receptor
TR	Thyroid receptor
TRAF	TNF receptor-associated factors
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TZD	Thiazolidinedione
UC	Ulcerative colitis
VDR	Vitamin D receptor
VSV	Vesicular stomatitis virus

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CHAPTER 1

GENERAL

INTRODUCTION

1.0 INNATE IMMUNITY: AN OVERVIEW

The mammalian immune system is comprised of 3 branches: innate immunity, acquired immunity and physical and chemical barriers. Physical and chemical barriers are considered the first line of a host's defence and prevent harmful microbes from entering the body. These barriers consist of the skin, low stomach pH, lysozyme that hydrolyses the cell wall of bacteria, mucus that traps and removes the bacteria, IgM that also traps the invading organism and also secreted antimicrobial peptides that can kill some pathogens (Turvey, Broide 2010). If pathogens somehow penetrate these chemical and physical barriers, the innate immune system is alerted. Speed is a defining characteristic of this system – within minutes of pathogen exposure, the innate immune system generates an inflammatory response by activating both chemical and cellular elements(Basset et al. 2003).

The cellular element of this system comprises of non-hematopoietic and hematopoietic cells such as macrophages, dendritic cells mast cells, neutrophils, eosinophils and natural killer (NK) cells. The principal function of these immune cells is to eliminate harmful pathogens by phagocytosis. However they also release biologically active molecules such as cytokines, chemokines and chemotactic lipids which alert other circulating cells, causing them to migrate to the site of infection to aid in the destruction of the pathogen (Si-Tahar, Touqui & Chignard 2009). These active molecules represent the chemical element of the immune system and can also include proteins/peptides that can hydrolyse microbes. Immune cells such as DC also have a unique function in activating the adaptive immune response (Maldonado-Lopez et al. 1999).

1.1 OVERVIEW OF ADAPTIVE IMMUNITY

Unlike the innate immune system which takes minutes to take effect, the adaptive immune system can take between 3-5 days to mount an efficient response to an infection. This system is only alerted if the innate immune system is unable to effectively clear an infection. Although the innate immune system acts rapidly, it is unspecific and only recognizes conserved molecular patterns on pathogens (PAMPs) through an array of pattern recognition receptors (PRR) (Janeway, Medzhitov 2002). The adaptive immune system however is highly specific and includes T lymphocytes, which mature in the thymus and antibody producing B lymphocytes which arise in the bone marrow (Maldonado-Lopez et al. 1999, Mitchell, Miller 1968, Owen, Raff 1970). These cells have a diverse repertoire of antigen receptors with random specificities which are clonally expressed. Consequently the specificities of these cells are endless. However, because the adaptive immune system uses these randomly generated antigen receptors it cannot reliably distinguish between self and non – self antigens (Palm, Medzhitov 2009). Thus an important role of the innate immune system is to instruct the adaptive immune system as to the origin of the antigen, i.e. self or non self and what type of response, if any, it should mount to effectively clear the pathogen. Antigen presenting cells (APC) such as dendritic cells (DC) are central to this process and integrate information from the innate immune system and relay it to lymphocytes of the adaptive immune system (de Jong, Smits & Kapsenberg 2005).

1.2 DENDRITIC CELLS: LINKING INNATE AND ADAPTIVE IMMUNITY

DC are a heterogeneous family of cells of haematopoietic origin that act as professional APC. They link innate and adaptive immunity by capturing, processing and presenting antigens to T cells. They are found in most tissues such as heart, kidney and liver. However they are most abundant in those tissues that reside between both external and internal environments such as the skin and GI tract. Here, their job is to sample the environment and alert the immune system to the presence of infections and other harmful stimuli (Coquerelle, Moser 2010). DC express a large array of phagocytic receptors such as lectins and scavenger receptors which enable them to phagocytose pathogens. They also express a variety of Toll-like receptors (TLRs) and other PRR which enable them to respond appropriately to these specific pathogens (Palucka, Banchereau 1999). DC that sample antigen in the peripheral tissues are classified as immature DC or phagocytic DC. However once they have encountered a pathogen, or infected or apoptosing cells these immature DC undergo phenotypic and functional changes referred to as maturation. This includes induction of costimulatory molecules, antigen processing and increased major histocompatibility complex (MHC) expression. Activated DC presenting pathogen- derived antigens on MHC I or II molecules also migrate to the draining lymph node where they activate and instruct naïve T cells (Blanco et al. 2008).

T cells are essential for the clearance and elimination of pathogens from the body. They control the extent and duration of an immune response against a wide variety of pathogens and are also important mediators in immunological memory. $CD4^+$ T cells can be divided into 4 different cell populations T helper 1 (Th1), T helper 2 (Th2), T

helper 17 (Th17) and T regulatory cells (Treg) based on the different cytokines they secrete and thus the different functions they have in the adaptive immune response (Zhu, Yamane & Paul 2010).

Th1 cells are defined by the production of their signature cytokine interferon γ (IFNγ). Their primary role in the body is to protect the host against intracellular microbes and viruses. However if this Th1 response becomes dysregulated, it can contribute to inflammatory diseases such as inflammatory bowel disease (IBD), multiple sclerosis (MS) and rheumatoid arthritis (RA) through tissue damage and self reactivity (Gutcher, Becher 2007). Th2 cells are defined by the production of IL-4, IL-5, IL-9 and IL-13. This Th lineage is essential in protecting the host from extracellular parasites such as helminths and nematodes. These cells are also associated with humoral responses which utilize high levels of immunoglobulins to neutralize extracellular organisms. It is now known that chronic inflammatory airway diseases such as asthma and allergy are characterised by abnormally high levels of Th2 cells, therefore the regulation of these cells is crucial (Paul, Zhu 2010). $CD4^+$ T cells are classified as Th17 cells based on the secretion of their signature cytokine IL-17A, however these cells can also produce high levels of the pro-inflammatory cytokines IL-17F, IL-22 and IL-21. Th17 cells are critical for protection against a wide array of both Gram negative and Gram positive bacteria as well as certain fungi and parasites such as *Candida albicans* and *Toxoplasma gondii,* respectively. This protection is due to the recruitment of neutrophils and the induction of antimicrobial peptides (O'Connor, Zenewicz & Flavell 2010). Although Th17 cells have essential role in host protection, it has emerged that they can also become critical mediators of autoimmune diseases if the Th17 response becomes dysregulated. Treg cells, which predominantly secrete IL-10 and TGFβ, are immunosuppressive cells which dampen down the immune response after a pathogen

has been cleared thus preventing excessive inflammation and tissue damage. {{186 Mills,K.H. 2004}}.

In order for an effective T cell response to be initiated three important signals are required; costimulation, presentation of antigen as well as a specific cytokine milieu. Indeed the cytokine milieu present at the time of antigen encounter is said to be the most influential factor in deciding Th cell fate. Interestingly, each Th cell lineage can produce a cytokine that plays a role in promoting its own differentiation while simultaneously inhibiting the differentiation of other Th cell lineages (Zhu, Paul 2010). IL-12 and IL-27, which are produced by innate immune cells following infection, potently induce the differentiation of Th1 cells (Wan, Flavell 2009). IFNγ is another key cytokine that can regulate Th1 differentiation as well as playing a crucial role in phenotype stabilization. Th2 cells fail to produce IFNγ. Instead the signature cytokines associated with this subset are IL-4, IL-5 and IL-13. The presence of IL-2 and IL-4 is essential in driving the differentiation of these Th2 cells (Agnello et al. 2003). The presence of TGFβ and IL-6 are essential and non-redundant in driving a Th17 response while TGFβ in conjunction with the anti-inflammatory cytokine IL-10 are involved in the differentiation of Treg cells (Wan, Flavell 2009). In order for these Th responses to be generated however, costimulation and presentation of antigen to the T cell receptor (TCR) via MHC must also occur.

1.3 MHC PRESENTATION

APCs such as DC express MHC molecules I and II which allow antigens to be presented on their surface and are critical in communicating the type and source of an infection to adaptive immune cells such as T cells. Although structurally MHCI and

MHCII are very similar, notably their most striking difference is in the antigen peptide binding groove, which is closed on MHCI molecules but open at both ends on MHCII molecules. This structural difference allows only limited sized peptides (8-10 amino acids) to bind to class I molecules while much larger peptides (15-20 amino acids) are free to bind to class II MHC molecules. Primarily class I molecules present peptides from endogenous antigens that are found in the cytosol. These antigens are normally derived from viral proteins which are synthesized in any virally infected cell. Antigens loaded onto MHCI molecules are typically presented to $CD8^+$ cytotoxic T cells which results in cell death (Savina, Amigorena 2007). These molecules are present on all nucleated cells and thus provide the body with protection from viral infection. MHCII molecules however are only expressed on APCs and are induced by innate immune recognition receptors such as TLRs. These receptors lead to APC activation, further upregulation of MHCII and most importantly the phagocytosis of the pathogen in question (Villadangos, Schnorrer & Wilson 2005). These pathogens become degraded in endocytic vesicles however an important role for MHCII is to display peptides from these pathogens and present them to $CD4^+$ helper T cells. T cell receptors on the surface of these cells recognize the MHC – peptide complex and proliferate and differentiate into specific T cell subsets (Kapsenberg 2003).

1.5 COSTIMULATION

Costimulation is an essential process necessary to facilitate complete T cell activation and pathogen clearance. CD80 and CD86 are costimulatory markers that are also members of the B7 family of coreceptors. These coreceptors are expressed on APC and provide vital costimulatory signals to enhance and maintain a T cell response via an

interaction with CD28 (Sharpe, Freeman 2002). CD28 is a type I transmembrane glycoprotein constitutively expressed on naive T cells that promotes T cell activation and proliferation through interacting with these B7 molecules (Stuart, Racke 2002). Although both CD80 and CD86 bind to the same receptor on T cells, there are slight differences in their function. CD80 is almost absent from immature DC whereas CD86 is expressed in low amounts (Orabona et al. 2004). CD80 is thought to play a role in Th1 differentiation mainly through the induction of IL-2 and IFNγ production, whereas CD86 promotes Th2 differentiation through the induction of IL-4 and IL-10. CD86 is also upregulated on APCs earlier than CD80 in response to activation signals (Stuart, Racke 2002). Cytotoxic T lymphocyte –associated Antigen 4 (CTLA 4) is also expressed on T cells and can bind to the B7 molecules with higher affinity than that of CD28. However unlike CD28 which is a positive regulator of T cell function CTLA 4 ligation downregulates the T cell response by interfering with T cell receptor (TCR) signals and thus is a negative regulator of T cell activation (Sharpe, Freeman 2002).

CD40 is another essential costimulatory marker that is expressed on DC, macrophages, Langerhan cells and B cells and is upregulated following exposure to activation signals. It binds to CD40L (CD154), a member of the TNF receptor family found on activated T cells and leads to the upregulation of CD80 and CD86 as well as inducing IL-12 production from the APC (Watford et al. 2003). It is therefore essential in maintaining T cell activation and differentiation. Costimulation is therefore a vital mechanism present in the adaptive immune response that allows the host to efficiently mount specific T cell responses against an invading organism while simultaneously putting in place regulatory mechanisms that can terminate an immune response if needed. This process is essential in maintaining immunological homeostasis and preventing autoimmunity.

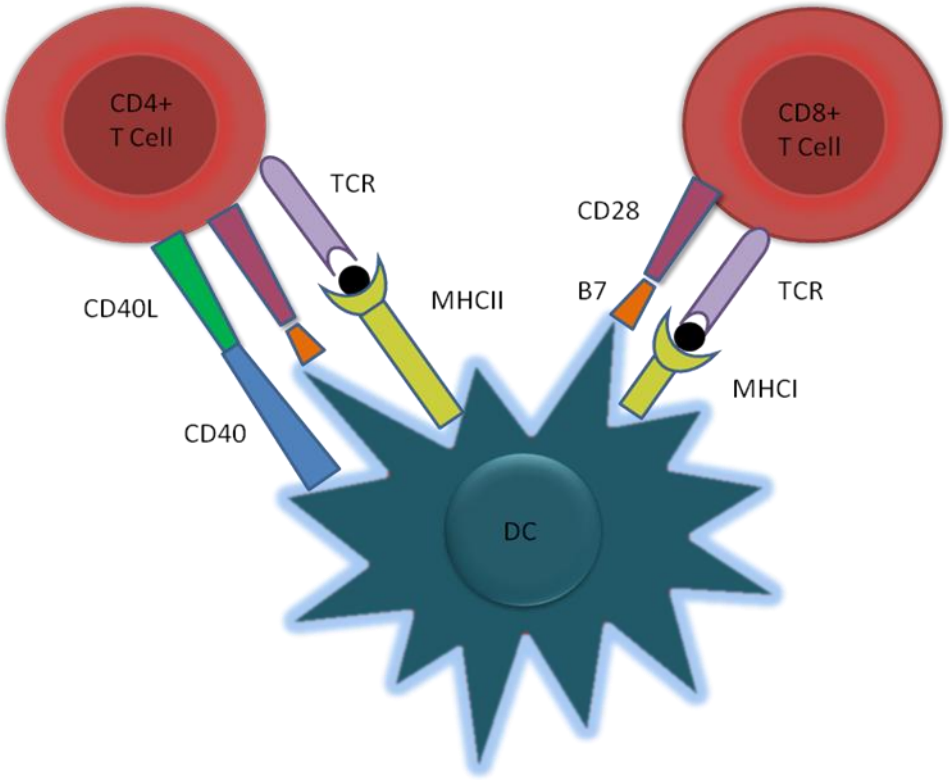

Fig 1.1: Signals required for effective T cells activation [adapted from (Bayry et al. 2004)].

1.6 CYTOKINES

Cytokines are chemical immunomodulators that are released by cells following infection, stress or trauma. They initiate intracellular signalling cascades which ultimately results in cellular development, homeostasis and immunity. Typically, cytokines act in an autocrine and paracrine fashion and are mostly small polypeptides or glycoproteins between 6-30kDa (Zidek, Anzenbacher & Kmonickova 2009). Cytokines that are produced by leukocytes and primarily act on other leukocytes are termed interleukins, while those that inhibit viral replication are known as IFNs. Cytokines that cause differentiation and proliferation of stem cells are called colony stimulatory factors whereas those that have chemoattractant capabilities are termed chemokines (Parkin,

Cohen 2001). IL-6, TNFα and IL-1β are examples of pro-inflammatory cytokines released by DC in response to a stimulus. These cytokines aid in the recruitment of phagocytes, induce fever and induce the production of C-reactive protein and enzymes such as ceruloplasmin and metallothionine that all aid in the elimination of the infection (Basset et al. 2003). Cytokines can also be released in response to injury, whereby their production recruits neutrophils and macrophages to the site of injury to remove damaged or dead cells. However overproduction of these cytokines and subsequent overactivation of these cells can lead to the further tissue damage, excessive inflammation and ultimately contribute to the pathogenesis of autoimmune and inflammatory diseases (Feldmann, Brennan & Maini 1998). Therefore the production of IL-10 an anti-inflammatory cytokine is essential in maintaining immunological homeostasis within the cell. IL-10 downregulates the production of cytokines from T cells, and inhibits NK cell, $CD8^+$ and macrophage function (Conti et al. 2003).

1.6.1 THE IL-12 FAMILY

The IL-12 family of cytokines consists of 4 members, IL-12, IL-23 IL-27 and the newly identified IL-35. These cytokines are unique in that they are heterodimeric proteins composed of both an alpha chain and beta chain that are structurally related to each other. IL-12 for example is a 70kDa protein that is composed of a disulphide linked p35 alpha chain and a p40 beta chain that are expressed on different chromosomes. IL-23 another pro-inflammatory IL-12 related cytokine, is also composed of a beta p40 chain which is linked to the alpha p19 chain, an IL-12p35 – related molecule (Collison, Vignali 2008). Indeed there is a great deal of redundancy between the components of these cytokines. IL-27 is composed of Epstein – Barr virus-induced gene 3 (EBI-3) , a

p40 related molecule and p28, a p35 related molecule, and finally IL-35 is composed of EBI-3 and the IL-12 alpha chain p35 (Yoshida, Miyazaki 2008). IL-12, IL-23 and IL-27 are all secreted by APCs such as macrophages and DC whereas IL-35 secretion has only been described in Treg cells. It is possible that IL-35 given its overlapping structure with IL-27 and IL-12 may also be produced under certain circumstances by APC however this remains to be proven (Collison, Vignali 2008).

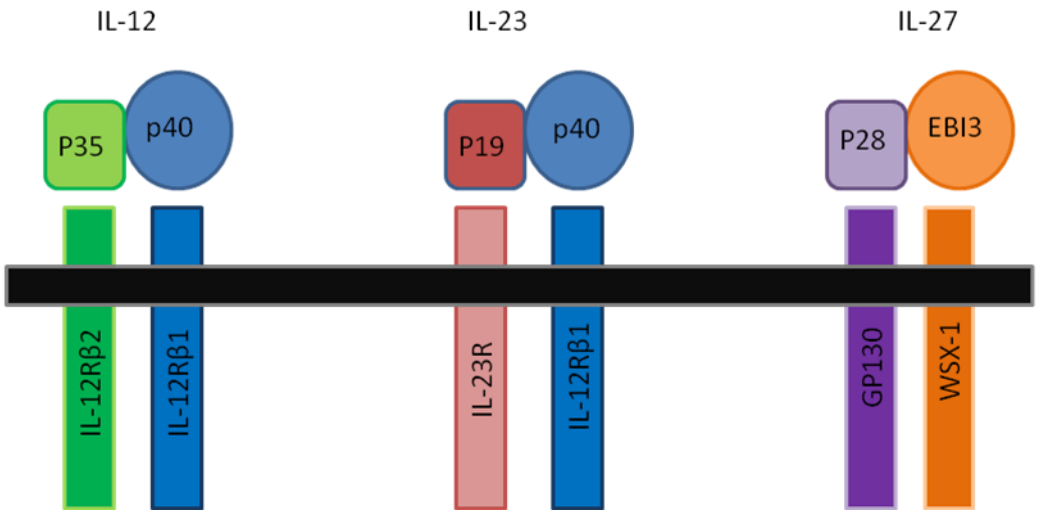

Figure: 1.2 The IL-12 family of cytokines and their shared or unique cytokine/cytokine receptor subunits [adapted from(Collison, Vignali 2008)].

These cytokines have important implications for innate immunity by activating and recruiting nearby cells however they can also determine the type and duration of the adaptive immune response by driving specific T cell responses. They can also play a role in NK cell activation as well regulating antibody production from B cells (Trinchieri 2003).

1.6.1.1 IL-12

IL-12, which was the first heterodimeric cytokine to be discovered is a potent inducer of IFNγ from T cells and NK cells and is also essential for the differentiation of the Th1 response. This cytokine also plays an essential role in T cell trafficking and migration through the induction of adhesion molecules such as P and E selectin. Interestingly, IL-12 only induces the expression of these adhesion molecules on Th1 cells and not other T helper cell subsets thus recruiting these cells to sites where only a Th1 response is needed (Hamza, Barnett & Li 2010). IL-12p40 is produced predominantly by innate immune cells such as monocytes, macrophages, neutrophils and DC whereas p35 transcripts are found in many cell types. However, because IL-12 is a heterodimeric cytokine and both the alpha and beta chains need to be coexpressed in the same cell, its production thus becomes limited due to the low expression of p35 compared with p40 (Goriely, Neurath & Goldman 2008).

These cytokines, like most cytokines are synthesized and then released following PAMP recognition via PRRs on the surface of immune cells. The effects of IL-12, IL-23, IL-27 and IL-35 are all mediated through their interactions with their specific receptors. This interaction initiates the JAK/STAT pathway which ultimately leads to the expression of a profile of genes that is typical of the given cytokine (O'Shea, Murray 2008). In order for effective IL-12 signalling both IL-12Rβ1 and IL-12Rβ2 receptors are required. These receptors are conserved amongst mouse and human with 68% amino acid sequence homology in the IL-12Rb2 protein and 54% homology in the IL-12Rb1 protein. IL-12Rβ1 is required for the high affinity binding of IL-12 and it is associated with the Jak family member Tyk2 while IL-12Rβ2 mediates signal transduction via 3 tyrosine residues that act as a docking site for STAT proteins (Hamza, Barnett & Li 2010). Following IL-12 occupancy of its receptor, Jak2 and Tyk2 proteins are

phosphorylated leading to the activation of STAT1, STAT3, STAT4 and STAT5. STAT4 however is the only STAT protein that is indispensable in IL-12 signalling. Similarly p38, a member of the mitogen-activated protein kinase pathway (MAPK) has also been shown to be essential in order for IL-12 to exert its cellular effects (Collison, Vignali 2008).

As outlined above, there are numerous immunomodulatory benefits to IL-12 production in the body however if this production becomes dysregulated, it has the potential to contribute to inflammatory and autoimmune diseases. More specifically, in a mouse model of rheumatoid arthritis, collagen induced arthritis (CIA) and multiple sclerosis, experimental autoimmune encephalomyelitis (EAE) IL-12 has been implicated as a major contributor to host pathology (Hamza, Barnett & Li 2010). In humans a study in Spanish IBD patients has shown an association with IL-12Rb and IL-23 R polymorphisms and disease severity (Marquez et al. 2008). Increased expression and activation of IL-12 induced STAT4 signalling has also been identified in the mucosa of ulcerative colitis (UC) patients (Pang et al. 2007). IL-12 and IL-23 are highly expressed in the gut of humans and mice with IBD and anti p40 antibody treatment can reduce inflammation and disease severity (McGovern, Powrie 2007).

1.6.1.2 IL-23

IL-23 is composed of the IL-12 subunit p40 and the newly identified p35 related subunit, p19. It is important for immunity against fungal infections and similar to IL-12, IL-23 is a proinflammatory cytokine that can induce IFNγ production and promote a Th1 response. However in the absence of IL-23, IFNγ production and Th1 differentiation are normal suggesting that IL-23 can synergize with IL-12 to induce

these Th1 cells but is not an absolute requirement for their differentiation (Wang et al. 2011). IL-23 is however essential for the survival and maintenance of Th17 cells. Originally thought as an essential cytokine for Th17 polarization along with TGF-β, IL-6 and IL-21, it is now generally accepted that IL-23 does not act on naive cells to induce Th17 differentiation. Instead it believed that IL-23 is involved in the expansion, maturation and maintenance of these cells (Stritesky, Yeh & Kaplan 2008).

The IL-23 receptor is composed of the IL-12Rβ1, which is required for high affinity binding and the novel IL-23R chain which is involved in signal transduction. The IL-23R gene is on human chromosome 1 within 150kb of IL-12Rβ2 (Watford et al. 2003). It is weakly expressed on NK cells, monocytes and DC and although it is not expressed on naive T cells it is highly upregulated on Th17. This is consistent with the central role for IL-23 in the maintenance and homeostasis of Th17 effector cells. Although a number of STAT proteins are activated in response to IL-23 only STAT3 appears to be indispensable in IL-23 signalling (Yang et al. 2007).

IL-23 has also been shown to drive local intestinal inflammation and in animal models of colitis is essential for maintaining chronic disease. IL-23 is also responsible for T-cell mediated inflammation, as seen in animal models of MS. Studies have shown that p19 and p40 knockout (KO) mice are resistant to EAE however upon the addition of exogenous IL-23, mice become susceptible to disease again (Gee et al. 2009). IL-23 can thus be seen as a cytokine that, when functioning normally has a protective role in the host, however if its production becomes dysregulated inflammatory disorders can be induced.

1.6.1.3 IL-27

As mentioned earlier, IL-27 is composed of two subunits the alpha subunit - p28 and the beta subunit - EBI3. EBI3 harbours its name from its initial discovery in B lymphocytes infected with the Epstein-Barr virus. P28 is normally expressed in activated innate immune cells where it is non-covalently linked to EBI3 (Carl, Bai 2008). However IL-27 has also been shown to be expressed in other cell types such as cells of the central nervous system (CNS) including astrocytes and microglia (Fitzgerald, Rostami 2009). IL-27 is strongly induced in response to Gram positive and Gram negative bacteria (through recognition via TLR2 and TLR4 respectively) and in response to viral DNA (TLR9). Following TLR stimulation, the transcription factor nuclear factor κ B (NFκB) binds to a promoter region of the EBI3 gene (Yoshida, Nakaya & Miyazaki 2009). Unlike other IL-12 related cytokines, IL-27 can exert both pro and anti-inflammatory effects by promoting Th1 differentiation while also inhibiting Th17 differentiation. IL-27 upregulates T-bet, the master regulator of Th1 differentiation which subsequently leads to the upregulation of the IL-12Rβ2. These cells then become more responsive to IL-12 stimulation and thus are driven into a Th1 phenotype (Fitzgerald, Rostami 2009).

IL-27 signals through the gp130 and WSX-1 receptors. Gp130 is ubiquitously expressed and is a component of receptors for many cytokines including IL-6 and IL-11 (Villarino, Huang & Hunter 2004). The WSX-1 receptor, more commonly known and the IL-27Rα is primarily expressed on T cells however it also expressed to a lesser extent on naive and memory B cells as well as alternatively activated macrophages (Carl, Bai 2008). The signalling pathway initiated by IL-27R engagement activates JAK1 and JAK2 which subsequently results in the phosphorylation of STAT1, STAT2, STAT3 and STAT5. Only STAT1 and STAT3 are critical to IL-27 signalling however,

as demonstrated by the loss of IL-27 activity in STAT1 and STAT3 knockout mice (Villarino, Huang & Hunter 2004).

IL-27 has thus proven to be an exciting target in the treatment of Th1 mediated inflammatory diseases given its role as a differentiating factor in the differentiation of these Th cells. Indeed the severity of adjuvant induced arthritis in rats and mice can be ameliorated using IL-27 specific antibodies (Villarino, Huang & Hunter 2004). IL-27p28 and EBI3 transcripts were significantly elevated in active CD highlighting the diverse role that this cytokine can play in disease (Rogler, Andus 1998). It is clear that despite their similarities, IL-12, IL23, IL-27 and IL-35 have unique and distinct functions within the immune system

1.7 PATTERN RECOGNITION RECEPTORS

Cells of the innate immune system express a series of germline encoded receptors known as PRR. These receptors can sense the presence of an infection via recognition of conserved microbial PAMPs (Palm, Medzhitov 2009). Given that invading pathogens can have large variability and molecular heterogeneity amongst each other, it is essential that these PRRs have specific characteristics to recognize all of these pathogens (Medzhitov, Janeway 1997). PRRs must recognize PAMPs that are unique to pathogens and are not found on any eukaryotic cells, thus ensuring that the innate immune system is only alerted during an infection. PAMPs must also be common to a vast amount of pathogens to ensure that a limited number of PRRs can detect all infections. Finally, it is vital that these PAMPs are essential for the survival or pathogenicity of the micro organism and therefore are difficult to alter (Medzhitov, Janeway 1997). PRRs can be divided into 3 types which include humoral proteins that circulate in the plasma, endocytic receptors that are expressed on the cell surface and

signalling receptors that can be expressed either on the cell surface or intracellularly. TLRs are an example of the latter and are the receptors that are relevant to this study.

1.7.1 THE TLR FAMILY

TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich repeat motifs and a cytoplasmic signalling domain homologous to that of IL-1R, termed the Toll/IL-1R homology (TIR) domain (O'Neill, Bowie 2007). These TLRs are found on a wide range of cells such as mast cells, macrophages, dendritic cells and epithelial cells and to date 12 members have been identified - 9 of which are conserved amongst human and mouse (Mogensen 2009).

TLR4 is undoubtedly the most extensively studied TLR to date. It was the first member of the family to be discovered and was identified as the receptor for bacterial lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria. TLR4 is not the only receptor involved in LPS recognition. At the plasma membrane, LPS binding protein (LBP) is thought to transfer LPS monomers to CD14, a GPI-linked cell surface protein. A small protein known as MD2 also binds to the extracellular region of TLR4 and aids in LPS-recognition (Janeway, Medzhitov 2002). In addition to binding LPS, TLR4 is also involved in the recognition of respiratory syncytial virus fusion proteins, mouse mammary tumour virus envelope proteins, *Streptococcus pneumonia* pneumolysin and the plant-derived cytostatic drug paclitaxel (Hennessy, Parker & O'Neill 2010). In addition to TL4, TLR2, 6 and 5 are also expressed on the cell surface and typically recognize bacterial and fungal components. TLR2 does not recognize these PAMPs independently, but instead functions by forming heterodimers with either

TLR1 or TLR6 (Janeway, Medzhitov 2002). Specifically TLR2-TLR1 recognizes triacylated lipopeptides from Gram-negative bacteria and mycoplasma, whereas the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram positive bacteria and mycoplasma. TLR5 recognizes flagellin, the protein subunits that make up bacterial flagella. Flagellin is highly conserved at the N and C terminal and it is in this region that TLR5 is thought to bind (Kawai, Akira 2010). TLR 3, 7, 8 and 9 are found in intracellular compartments such as endosomes lysosomes and endoplasmic reticulum (ER). Here they play a role in microbial and viral nucleic acid recognition. TLR 9 recognizes unmethylated CpG motifs present in the bacterial genome. Since mammalian DNA is methylated and bacteria lack CpG methylation enzymes this is an effective way to ensure that only bacterial DNA and not host DNA is recognised (Janeway, Medzhitov 2002). TLR7 recognizes imidazoquindine derivatives such as resiquimad and imiquimod and guanine analogs such as loxoribine as well as recognizing ssRNA viruses such as human HIV and vesicular stomatitis virus (VSV). TLR7 can also recognize synthetic poly (U) RNA and certain small interfering RNAs. TLR3 specifically recognizes dsRNA from dsRNA viruses such as neovirus as well as the synthetic analogue polyinosinic-polycytidylic (PolyIC). Stimulation of TLR3 induces anti-viral immune responses by promoting the production of both type I interferons as well as inflammatory cytokines (Blasius, Beutler 2010).

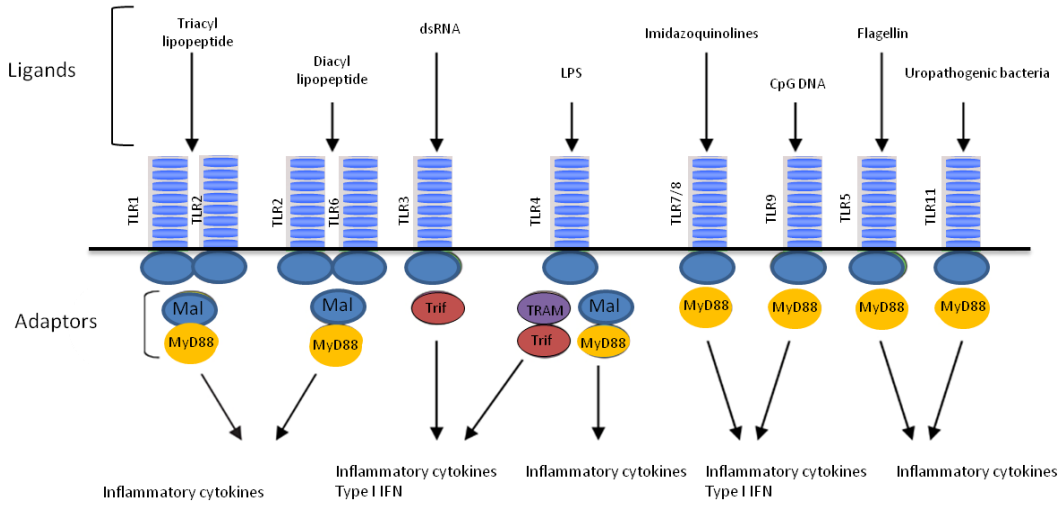

Figure 1.3: TLR receptors, ligands and adaptor molecules [adapted from (Kawai, Akira 2006)].

1.7.2 TLR SIGNALLING – MyD88 DEPENDENT PATHWAY

Upon recognition of their ligands, TLRs induce the expression of a variety of defence genes that are necessary for fighting invading pathogens. These genes include inflammatory cytokines, chemokines, antimicrobial peptides, MHC and co-stimulatory molecules (Janeway, Medzhitov 2002). Recognition of PAMPs by TLRs leads to the recruitment of a unique set of intracellular TIR-domain-containing adaptors including myeloid differentiation factor 88 (MyD88), Mal, TIR-domain-containing adaptor inducing interferon-β (TRIF) (Akira, Uematsu & Takeuchi 2006),(Oshiumi et al. 2003) Trif-related adaptor molecule (TRAM) (Oshiumi et al. 2003). All TLRs with the important exception of TLR3 depend, to some degree on MyD88 in order to signal (Fig 1.3). MyD88 contains a C-terminal TIR domain that mediates its interaction with the TLR and an N-terminal death domain that interacts with its downstream target, the IL-1R associated kinases (IRAKs) (Wesche et al. 1997). Ligand binding to the TLR causes

MyD88 to associate with the cytoplasmic portion of the receptor leading to the recruitment of IRAK-4 and IRAK-1. These IRAK proteins, once phosphorylated dissociate from MyD88 and interact with TNF receptor associated factor 6 (TRAF6) – and E3 ubiquitin ligase (Kawai, Akira 2007). TRAF6 promotes the synthesis of polyubiquitin chains which activate TGFβ activated kinase 1 (TAK1) (Chen 2005). TAK1 in combination with TAK1 binding proteins TAB1 and TAB2 activate downstream pathways involving IκB kinase (IKK) (discussed further in section 1.8.2) and the MAPK family. This interaction ultimately leads to the induction of NFκB and MAPK.

1.7.3 TRIF- DEPENDENT/ MYD88 INDEPENDENT PATHWAY

The TRIF dependent pathway results in activation of both interferon regulatory factor 3 (IRF3) and NFκB and is utilised by TLR3 and TLR4 activation (Kawai, Akira 2008). TLR4 is unique in that it has two arms to its signalling pathway – a MyD88 dependent component that promotes fast activation of NFκB activation and induction of proinflammatory molecules and also a MyD88-independent pathway (also used by TLR3) that results in the slow activation of NFκB and IRF3. Ultimately IRF3 activation leads to the production of type-1 interferons, IFN-α and IFN-β, other IFN inducible genes and co-stimulatory molecules (Moynagh 2005).

TRIF interacts with the TIR domain of TLR3 to mediate downstream signalling, whereas another adaptor molecule TRAM acts as a bridging receptor between TLR4 and TRIF (Fig 2.4) in order to signal (Oshiumi et al. 2003). The N- terminal and C-terminal regions of TRIF have two distinct functions following TLR3 or TLR4 ligation. The N-terminal of TRIF recruits IKKs, which phosphorylate the C-terminal region of IRF3 (Sharma et al. 2003). Phosphorylated IRF3 subsequently forms a dimer, which

translocates to the nucleus to induce expression of target genes. The C-terminal region of TRIF however contains a Rip homotypic interaction motif (RHIM), which mediates its interaction with RIP1, a member of the ribosome inactivating protein (RIP) family involved in NFκB activation (Meylan et al. 2004). RIP1 is polyubiquitinated and forms a complex with TRAF6 which ultimately leads to NFκB activation (Kawai, Akira 2007).

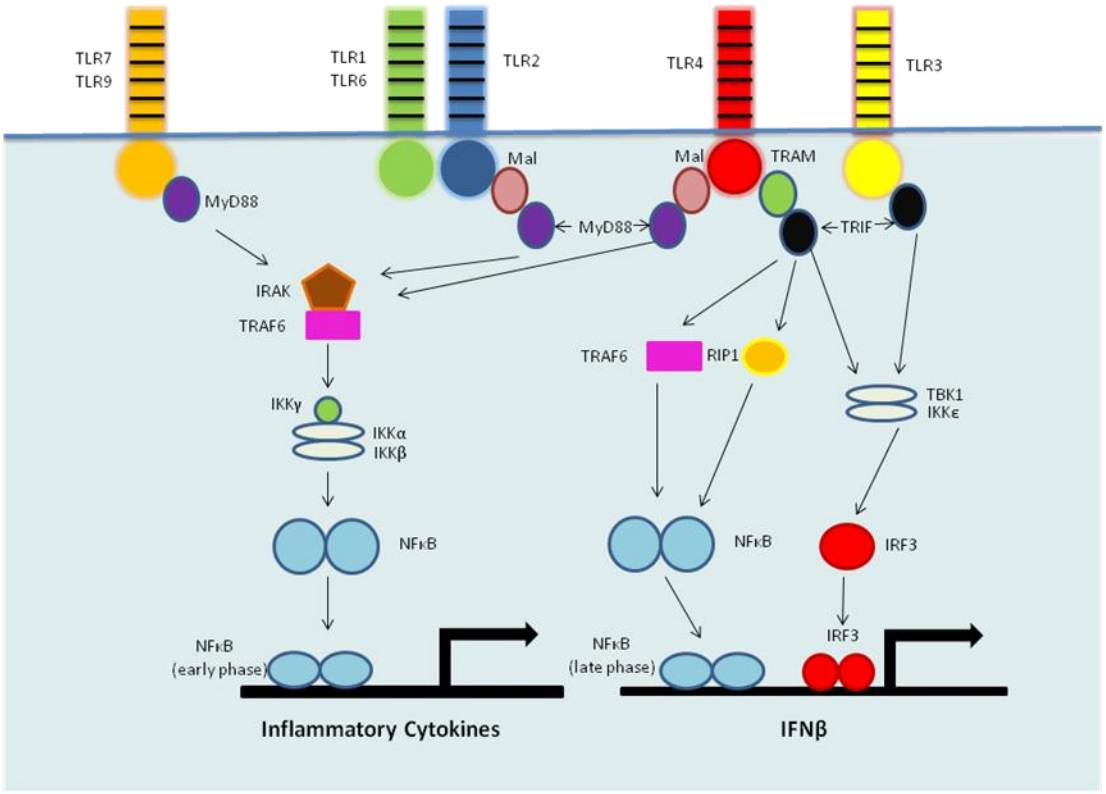

Fig 1.4: The MyD88 dependent and independent TLR pathways [adapted from (Takeda, Akira 2005).

1.8 NFκB

NFκB is a eukaryotic transcription factor that exists in virtually all cell types (Ghosh, May & Kopp 1998). It can be activated by a wide range of various stimuli, including cytokines, bacteria, viruses, viral proteins, double-stranded RNA and physical and chemical stresses (Siebenlist, Franzoso & Brown 1994). NFκB responsive sites (known as κB sites) have been identified in the promoters of numerous genes encoding proteins involved in the acute phase response, cytokines, immunoreceptors and cell adhesion molecules (Schmid, Adler 2000).

NFκB consists of five family members which form homo and heterodimeric complexes with each other in other to function. Each family member contains an N-terminal 300 amino acid conserved region known as the Rel homology domain (RHD) which mediates DNA binding, nuclear localisation and dimerisation between subunits (Beinke, Ley 2004). These NFκB members include NFκB1, NFκB2, Rel A (p65), RelB and c-Rel. On the basis of their structure, these proteins can be further divided into 2 subclasses. The first consists of NFκB1 and NFκB2 which are both produced as precursor proteins of 105 and 100 kilodaltons respectively. These proteins are then processed to form functionally mature NFκB proteins known as NFκBp50 and NFκBp52. The second class of NFκB proteins consists of the Rel proteins – Rel A (p65), RelB and c-Rel which are all synthesised as mature proteins and contain transactivation domains in their C-termini (Schmid, Adler 2000). Most of these proteins are transcritpionally active; however some combinations are thought to act as repressive complexes. Thus p50/p65, p65/p65, p65/c-Rel and p50/c-Rel are all transcriptionally active whereas p50 homodimers and p52 homodimers are transcritpionally repressive (Ghosh, May & Kopp 1998).

1.8.1 NFκB1 & NFκB2 PROCESSING

Both NFκB1 and NFκB2 encode precursor proteins that are much larger than the mature functional products, p50 and p52. NFκB1 and NFκB2 must therefore be processed first to generate their mature by-products. Although the exact mechanism of NFκB processing is not known, there are some aspects of its mechanism that have been widely accepted. Similar to IκB, these NFκB precursors are phosphorylated by IKK, where p105 processing is associated with IKKβ and p100 processing is associated with phosphorylation by IKKα. Numerous studies have also suggested the need for post-translational processing via the ubiquitin-proteasome system however as of yet the ubiquitin enzymes required for p50 and p52 generation have not been identified. Interestingly, ubiquitin regulated proteolysis is usually a progressive event that yields small peptides but not partial protein fragments (Chen 2005). Therefore the generation of p50 and p52 are exceptional. The mechanism involved is unclear however the identification of a 23 amino acid glycine rich region (GRR) within the N terminal of p105 is believed to play an important role in this process. It is believed that processing is initiated by ubiquitination which is followed by protosomal degradation of the p105 precursor from the C terminal end. Once the proteasome hits the GRR, it stops processing p105 and mature p50 is released (Ben-Neriah 2002).

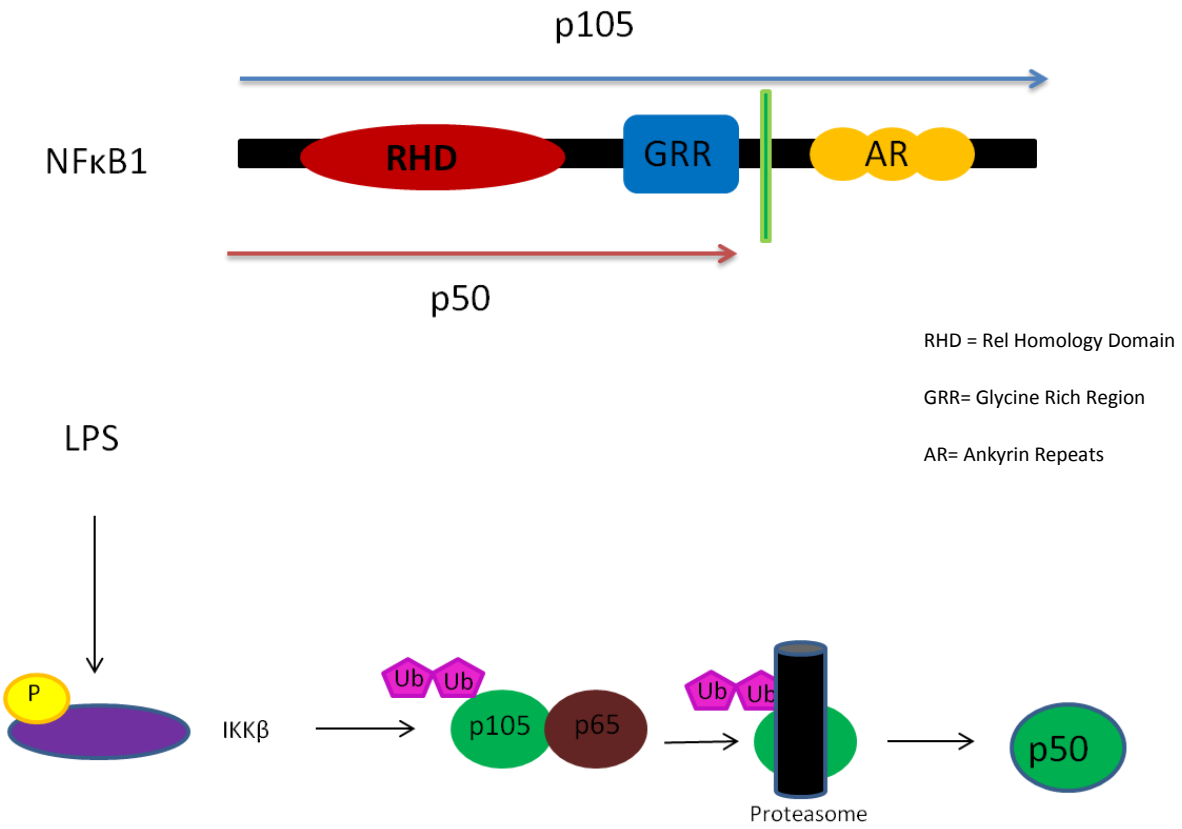

Figure 1.5: Structure of immature p105 and mature p50; Processing of p105 to p50

1.8.2 NFκB ACTIVATION PATHWAYS

In the cell, two main NFκB pathways exist – the canonical and non- canonical pathways. The canonical pathway is induced by signalling through cytokine receptors, antigen receptors and pattern recognition receptors and relies on the degradation of IκB proteins. IκB's are inhibitory proteins that sequester NFκB in the cytoplasm in the absence of activating signals. However, in the presence of an appropriate activating stimulus, IκB is degraded, releasing NFκB and enabling it to translocate to the nucleus where it can initiate the transcription of specific target genes (Beinke, Ley 2004). Degradation of IκB is mediated through its phosphorylation by the IκB kinase complex (IKK) which targets the proteins for ubiquitination and subsequent proteasomal degradation (Oeckinghaus, Hayden & Ghosh 2011). In contrast, the non-canonical or

alternative pathway relies on the processing of p100 instead of the degradation of IκB and involves the formation of RelB/p52 complexes (Sun 2011). This pathway is induced by specific members of the TNF cytokine family, such as CD40 ligand, B cell activating factor (BAFF) and lymphotoxin β. Therefore in this pathway, the processing of p100 serves two purposes. Processing of p100 generates mature p52 which is needed to complex with RelB in order to signal. However p100 also functions as an IκB-like molecule, preventing the translocation of RelB in the absence of an appropriate activating signal (Oeckinghaus, Hayden & Ghosh 2011).

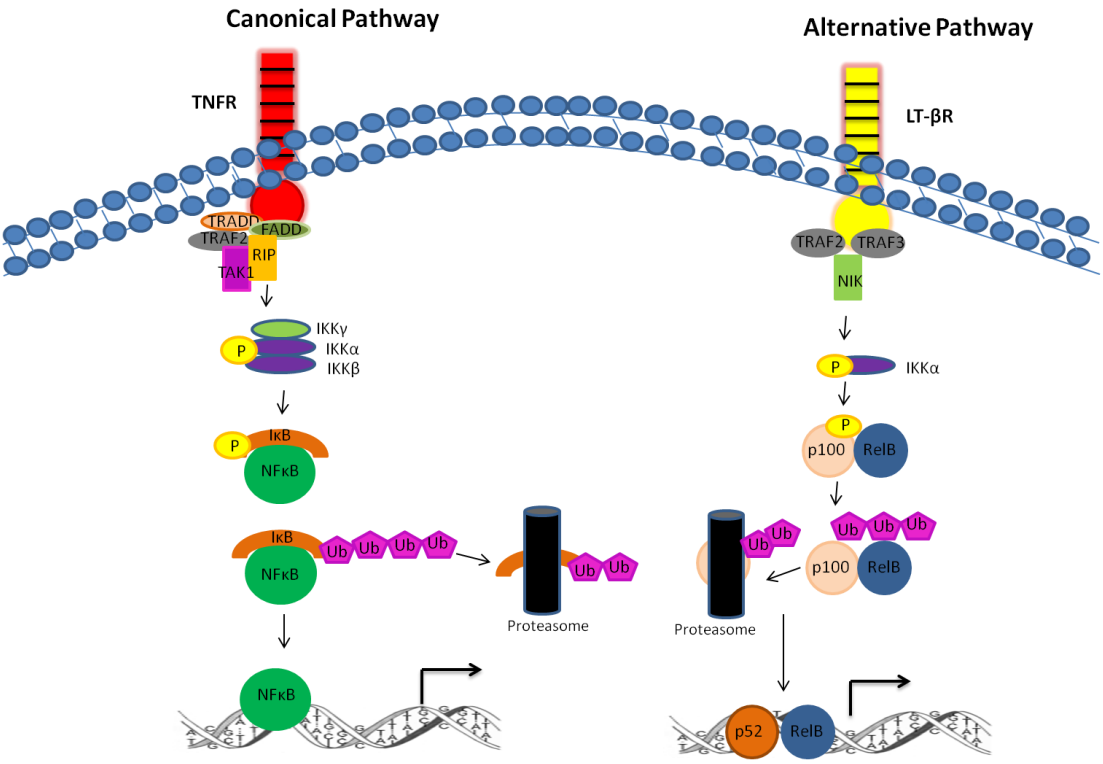

Figure 1.6: The canonical and alternative pathways of NFκB activation (adapted from (Oeckinghaus, Ghosh 2009))

1.8.3 NFκB AND DISEASE

Dysregulated cytokine production and signalling mechanisms by a variety of immune cells has been implicated in the pathogenesis of inflammatory diseases such as RA and IBD. NFκB is a major regulator of these processes and is therefore likely to contribute to these diseases. Indeed, enhanced NFκB activation has been reported in the synovium of RA patients (Jimi, Ghosh 2005). Furthermore, in transgenic mice expressing a dominant negative form of IκBα which repressed NFκB activation, disease severity in response to CIA was decreased compared to wildtype (WT) (Brown, Claudio & Siebenlist 2008).

The expression and activation of NFκB is also strongly induced in the inflamed gut of IBD patients (Atreya, Atreya & Neurath 2008). The amount of activated NFκB was also reported to correlate with the severity of intestinal inflammation (Rogler et al. 1998). Many of the established immunosuppressive drugs used to treat IBD currently, such as corticosteroids, sulfasalazine, methotrexate and anti-TNFα antibodies are known to mediate their antiinflammatory effects partly via NFκB inhibition (Auphan et al. 1995, Guidi et al. 2005, Thiele et al. 1999). Therefore, NFκB may prove to be a promising therapeutic target for the treatment of these diseases in the future.

1.9 NUCLEAR RECEPTORS

Nuclear receptors are a large family of ligand activated transcription factors, consisting of 48 members. Depending on their DNA and ligand binding properties these receptors can then be subdivided into three categories. The steroid and thyroid hormone receptors such as the Vitamin D Receptor (VDR) are one class of nuclear receptor and are the most extensively studied nuclear receptors to date. Indeed the VDR has proven to be a promising drug target in the treatment of certain cancers, autoimmune diseases,

infections, cardiovascular disease (CVD) as well as bone and mineral disorders. The orphan nuclear receptors are structurally similar to the steroid and thyroid receptors however their physiological ligands *in vivo* are still unknown (Szatmari, Nagy 2008). The NR4a receptors are an example of a subfamily of orphan nuclear receptors consisting of nerve growth factor-induced clone B (Nur77), nuclear receptor related 1 (Nurr1) and neuron derived orphan receptor 1 (NOR-1). Much work has been done in this area to discover the natural ligands for these receptors however it is also thought that these receptors may possess ligand independent transcriptional activities as their activity is tightly controlled at the level of expression (Benoit et al. 2006). The third classes of nuclear receptors are known as the "adopted" orphan nuclear receptors and until recently their natural ligands and physiological role had yet to be defined. These receptors form heterodimers with the Retinoid X Receptor (RXR) and can be activated by either or both of the heterodimers ligands, referred to as dual ligand permissiveness and synergism (Son, Lee 2010). Other adopted orphan receptors that have been the focus of intense research recently are the Liver X receptors (LXRα and LXRβ), peroxisome proliferator activated receptor γ (PPARγ) and the Farnesoid X Receptor (FXR). The nuclear receptors in general, upon activation control target genes involved in a number of key cellular processes such as lipid metabolism, differentiation, and energy homeostasis (Huang, Glass 2010). The nuclear receptors that were of particular interest to us in this study were LXR, RXR and PPARγ – members of the adopted orphan nuclear receptor family.

1.9.1 GENERAL STRUCTURE OF NUCLEAR RECEPTORS

Nuclear receptors are organized into four functional domains, two of which are well characterised both structurally and functionally and are well conserved throughout evolution (Waku et al. 2009). The DNA binding domain (DBD) (C domain) is the most

highly conserved region of all the functional domains. This domain contains two zinc finger like motifs that initiate the binding of the receptor to the promoter of its target genes. Besides these zinc fingers, there are also amino acid motifs that determine DNA:Receptor recognition sites. A large part of this domain is also responsible for dimerising (Waku et al. 2009). Studies have also shown that this domain encodes both nuclear localization signals as well as nuclear export signals (Franco, Li & Wei 2003). The C terminal hydrophobic ligand binding domain (LBD) is the largest domain (E/F domain) and is responsible for the binding of a specific ligand to the receptor. This region also contains a ligand dependent activation function (AF-2) which recruits co-activators to the receptor complex in order to aid transcription (Hall, Quignodon & Desvergne 2008). The other two domains are variable in both length and sequence. The hinge region (domain D) is less conserved than the other domains and contains between approximately 30-50 amino acids that links the DBD to the LBD. This domain is also a docking site for cofactors. When phosphorylated it is also correlated with increased transcriptional activity (Leibovitz, Schiffrin 2007). Finally there is the N terminal A/B domain whose size ranges from 20 amino acids in the VDR, 140 in PPARγ, to over 600 in the steroid ligand subfamily. In some cases these A/B regions can act as transcriptional activators, provide sites of protein phosphorylation or form direct interactions with other receptor domains or regulatory proteins. This domain also contains a ligand independent function (AF-1) which shows weak conservation across the entire nuclear receptor family (<15%). Although this domain can act as a ligand independent transcriptional activator, it is also known to synergise with the AF-2 domain (Waku et al. 2009).

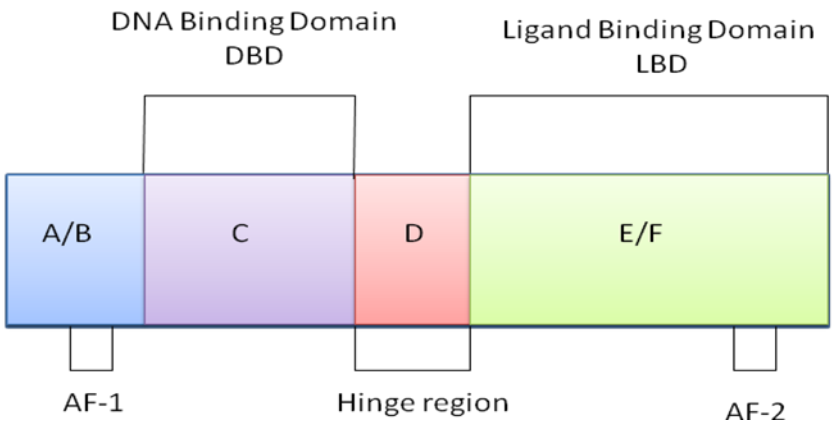

Figure 1.7: General structure of nuclear receptors [adapted from (Bain et al. 2007)].

1.9.2 MECHANISM OF ACTION

Although all three classes of nuclear receptors have a similar overall structure, there are subtle differences in the mechanism by which they carry out gene expression. Typically, members of the steroid/thyroid nuclear receptor group are located in the cytoplasm and upon receptor occupancy with its ligand, release heat shock proteins, translocate to the nucleus and bind as homodimers in a head to head orientation to target gene sequences (palindromic) (Leibovitz, Schiffrin 2007). As mentioned earlier both the orphan and adopted nuclear receptors, unlike the steroid receptors, typically function as heterodimers and bind in a head-to-tail orientation. These heterodimers can be located in the nucleus even in the absence of a ligand and are bound to their response elements. The presence of specific and potent co-repressors prohibits the transcription of these target genes. However activation of these receptors in the presence of their ligands promotes the release of the co-repressors while simultaneously encouraging the recruitment of co-activators which ultimately leads to transcription. The co-repressor complex is replaced by a chromatin remodelling complex that includes the ATP-

coupled SWI/SNF complex. This facilitates the recruitment of another complex containing histone acetylases (SRC/p160), histone methylases, members of the basal transcription machinery & RNA polymerase II (Gelman et al. 2006). These co-activators contain LxxLL motifs that dock to the LBDs. Most co-activator complexes initiate transcription by these three mechanisms i.e. interacting with the basal transcription machinery (e.g. TRAP/DRIP), by inducing histone protein modifications or by local chromatin remodelling. Most of the 300 nuclear receptor co-activators that have been identified to date have enzymatic activities that result in phosphorylation, acetylation, methylation, ubiquitination and SUMOylation of target proteins. Acetylation of histones is one of the best studied mechanism in which these co-activators can initiate transcription. The conserved lysine residue in the N terminal of histones is acetylated and this results in the loosening of the nucleosome structure, making the DNA more accessible to transcription factors (Szeles, Torocsik & Nagy 2007). The co-activators recruited to the response elements may also have a specific preference for either or both partners of the heterodimer e.g. The TRAP220/DRIP205 complex of co-activators contains two LxxLL motifs with one of the motifs displaying preference for RXR, whereas the other motif interacts with either the TR or the VDR. So far, the expression of these co-activators is not thought to be limited to a particular tissue or subset of cells but instead, is believed to be ubiquitously expressed (Berger, Moller 2002). Co-repressors, on the other hand can inhibit transcription when the NR is in an unligand state, by directly interacting with the hinge / ligand binding domain. These co-repressors may also act by deacetylating histones which compacts nucleosomes into tight and inaccessible structures and thus prevents the initiation of transcription (Jenster 1998).

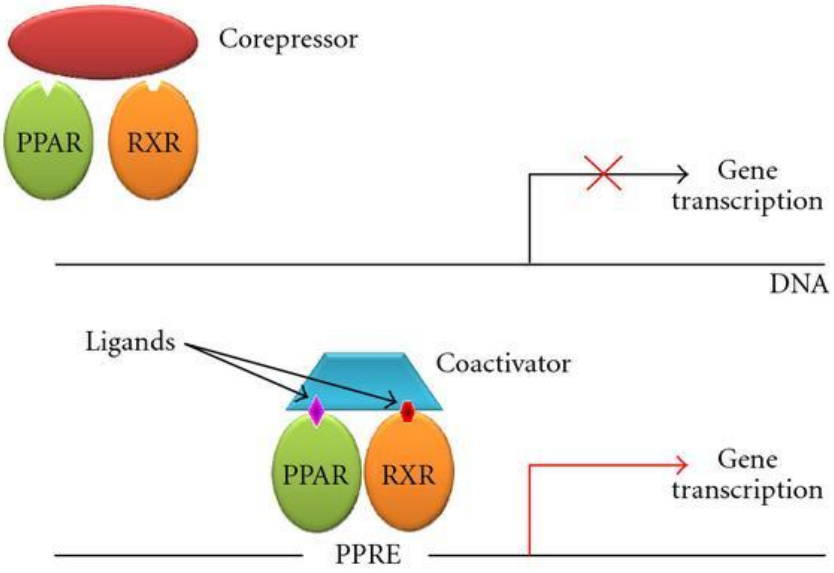

Figure 1.8: Mechanism of action of nuclear receptors [adapted from (Berger, Moller 2002)]

1.9.2.1 TRANSREPRESSION

Described above, is the most common mechanism in which nuclear receptors can exert their effects. This mechanism, which is also known as transactivation, usually results in the alteration of metabolic pathways or components within the cell. However these receptors can also carry out their functions in a ligand dependent transcriptional independent manner. This is more commonly known as transrepression and is different to active repression in that, although it antagonises gene expression it does not involve sequence specific binding by the nuclear receptor DBD. Instead it involves protein-protein interactions between ligand bound nuclear receptors and transcription factors or co-regulatory complexes located at the promoter elements of target genes (Straus, Glass 2007). Transrepression mainly results in anti-inflammatory effects by directly interfering with inflammatory response pathways and transcription factors such as NFκB, AP-1, NFAT and STATs. Indeed molecular studies have shown that PPARγ can

interfere with NFκB by physically interacting with the p50 and p65 subunits (Pascual, Glass 2006).

Although it is understood that a ligand bound NR can physically interact with TFs or coregulatory complexes and repress gene expression, the exact mechanism in which this occurs is not fully understood or indeed may vary depending on the cell type, ligand or type of stimulus applied. Some of the models that have been proposed thus far suggest that NRs may compete with NFκB /AP-1 for coactivators that are essential for their signalling since the coactivators required to drive these inflammatory responses overlap with those needed to initiate transcription of nuclear receptor target genes (Lee, Kim & Baek 2008). Indeed, it has been shown that the GR, a member of the steroid/thyroid class of nuclear receptors can negatively affect AP-1 signalling by sequestering the cAMP response element (CREB) binding protein CBP/p300, a coactivator that is necessary for this signalling (Martens et al. 2005). This has also since been proven to be the case for RXR and the androgen receptor (AR) (DiSepio et al. 1999, Aarnisalo, Palvimo & Janne 1998). It has also been proposed that NRs can alter coactivator use as well as regulating components of signal transduction pathways involved in NFkB and AP-1. In addition, these receptors may also physically interact with NFκB and AP-1, sequestering it from its transcription target site (Berger, Moller 2002).

It has been described recently by a number of independent groups that SUMOylation may be a key mechanism in which NRs can actively transrepress other inflammatory transcription factors. SUMOylation of transcription factors has previously been linked with both increased and decreased transcriptional activity (Pascual et al. 2005). SUMO or small ubiquitin-related modifier is a family of proteins consisting of SUMO1, 2 and 3 that carry out post translational modifications within a cell. Pascual *et al* showed that SUMOylation of a single lysine residue in the PPARγ LBD resulted in its adherence to

the NcoR corepressor complex on the *inos* promoter, preventing the clearance of the complex upon exposure to inflammatory stimulus and thus resulting in the *inos* gene remaining repressed (Pascual et al. 2005).

Similarly Ghisletti *et al* demonstrated that LXR transrepression also uses SUMOylation and an NcoR-dependent pathway. However in contrast to PPARγ, LXRs are SUMOylated by SUMO2 and SUMO3 rather than SUMO1, as is the case for PPARγ. These studies provide increasing evidence that NR can transrepress inflammatory genes by distinct and parallel pathways that ultimately effect corepressors associated with target genes or indeed the transcription factors that induce their transcription (Ghisletti et al. 2007).

1.10 PPARγ

PPARγ is a subtype of the peroxisome proliferator activated receptor family in which two other isoforms PPARα and PPARβ also exist. As members of the adopted nuclear orphan receptor superfamily they form obligate heterodimers with the RXR and are activated endogenously by fatty acids, fatty acid metabolites/derivatives, lipophilic hormones and also synthetically by the Type 2 diabetes class of drug known as the glitazones. PPARγ was originally thought of as regulator of nutrient metabolism and energy homeostasis due to its presence in high concentrations in adipocytes (Berger, Akiyama & Meinke 2005, Berger, Akiyama & Meinke 2005). However after its discovery in monocytes and macrophages a role in the immune system and inflammatory disease began to emerge. It has since been found in a number of immune cells including B cells, T cells and DC. PPARγ activated DC differ from conventional mature DC in that they have diminished migratory capabilities, increased phagocytic

abilities, altered differentiated phenotype and the ability to induce T cell tolerance. Murine splenic $CD11c^+$ DC express PPARγ and it has also been detected in bone marrow derived DC (Szatmari, Nagy 2008). The receptor itself is highly and acutely unregulated during DC differentiation. In addition more than 1000 transcripts are regulated by PPARγ during DC differentiation, most of which represent genes involved in lipid metabolism and transport and alterations in immune function appear to be secondary to these changes in gene expression (Szatmari et al. 2007). PPARγ instructed DC differentiate into less stimulatory DC compared to untreated counterparts as seen by an altered cell surface expression pattern of costimulatory molecules i.e. they express less CD80, TLR4 and CD36. These PPAR instructed DC also have an altered cytokine and chemokine profile as reported by a decrease in production of IL-12 and TNFα as well as reduced secretion of MCP2, IP-10 and RANTES (Szatmari, Rajnavolgyi & Nagy 2006). Alteration in cell surface marker expression and cytokine production undoubtedly affects signals 2 and 3 which are needed for effective T cell priming by DC. As expected, PPAR activated DC severely impaired $CD4^+$ T cell priming both *in vitro* and *in vivo* (Klotz et al. 2009). These PPAR ligand treated DC reportedly skew the differentiation of these APCs to a special DC subset that has a reduced Th1 capacity but an enhanced Th2 profile (Gosset et al. 2001). Thus activation of PPARγ in DC has consequences not only for its differentiation but also for adaptive immunity. In addition PPARγ activated DC express less CD1a but more CD1d, which results in enhanced capacity to activate a CD1d dependent cell type, the iNKT – invariant Natural killer T cell (Szatmari et al. 2004). Recent studies have shown that PPARγ strongly enhanced the expression of the co-inhibitory molecule B7H1 in DC (Klotz et al. 2009). Therefore, not only do PPARγ instructed DC have decreased signalling via signals 1 and 2 but increased co-inhibitory signals via B7H1 ultimately leading to diminished $CD8^+$ and

$CD4^+$ T cell responses. The receptor has also been discovered in murine Langerhan cells and its activation inhibited TNFα induced migration of these cells from the epidermis to the draining lymph node (DLN) (Angeli et al. 2003). Since it has also been reported that PPARγ prevents the expression of CCR7, a key chemokine receptor involved in DC motility, it seems likely that PPARγ activation may affect DC migration and their subsequent accumulation in lymphoid organs (Hanley et al. 2010).

1.11 LXR

The Liver X Receptors consists of two subtypes, LXRα and LXRβ which are 77% structurally identical to each other (Repa, Mangelsdorf 2000). However their expression pattern differs greatly. While LXRβ is expressed ubiquitously, the expression of LXRα is primarily in liver, intestine, kidney, adipose tissue and certain immune cells such as macrophages and DC (Zelcer, Tontonoz 2006). LXR plays a key role in cholesterol homeostasis. In the intestine, LXR controls the reabsorption of cholesterols via the transporter proteins ABCG5 and ABCG8 (Repa, Mangelsdorf 2000). Furthermore activation of these receptors *in vivo* leads to increased HDL levels, reduced cholesterol levels and reduced risk of atherosclerosis (Bradley et al. 2007). Unlike PPARγ whose role in DC has been well described, the role liver X receptors may play in DC function still remains somewhat elusive. LXR activation in mature human DC results in a significant decrease in proinflammatory cytokines IL-12p40, IL-12p70 as well as an increase in IL-10 secretion (Geyeregger et al. 2007). Interestingly, fascin an actin-bundling protein that is strongly related to dendrite formation, was shown to be abolished in mature human DC when treated with an LXR synthetic agonist. Lack of fascin in these cells prevented adequate immunological synapse formation between DC and T cells, thus showing the potential role LXRα may play in both innate and adaptive immunity (Geyeregger et al. 2007). It has also recently been shown that both human and

mouse tumours produce endogenous LXR ligands and that in the presence of these ligands and synthetic LXR agonists, CCR7 expression on maturing DC is inhibited. CCR7 acts as a homing receptor, directing mature dendritic cells to the lymph node to present antigen to naïve T and B cells. Inhibition of CCR7 results in impaired migration of DC to the draining lymph nodes thus affecting the initiation of the adaptive immune response (Villablanca et al. 2010). This could have important consequences for immunotherapy and cancer biology.

1.12 RXR

The Retinoid X receptor consists of 3 members – RXRα, RXRβ and RXRγ that are encoded on chromosome 9, 6 and 1 respectively. RXRα is predominantly expressed in the liver, kidney, skin and intestine whereas RXRβ is widely distributed and can be detected in almost every tissue (Dolle et al. 1994). The expression of RXRγ is restricted to the muscle and certain parts of the brain (Haugen et al. 1997). As mentioned previously, these subtypes can form homodimers or heterodimers with a large number of other nuclear receptors including the vitamin D receptor (VDR), FXR, the PPARs, LXR and thyroid hormone receptor (TR) (Mangelsdorf et al. 1992). Therefore, due to RXR's wide distribution pattern and its ability to heterodimerise with a number of other receptors, it can control a large number of genes in developmental, metabolic and immune processes (Germain et al. 2006). All three RXRs are activated endogenously by the vitamin A metabolite 9-cis retinoic acid (9CRA). Synthetic compounds known as rexinoids have also been developed to activate RXR however similar to 9CRA, these compounds activate all three RXR subsets. To date, no RXR agonist with particular subtype specificity has been reported (Kagechika, Shudo 2005). RXR is also expressed to varying degrees in immune cells such as macrophages, T cells, Langerhan cells and dendritic cells. RXRα is the most abundantly expressed RXR subtype in DC and has

been shown to have anti-inflammatory properties (Szeles et al. 2010). Activation of this receptor *in vitro* decreased the expression of CCR7 on maturing DC which as mentioned earlier, aids in the migration of DC to the draining lymph nodes where they can interact and instruct naive T cells (Villablanca et al. 2008). Chemotaxis of DC towards the CCR7 ligand MIP3β was also decreased in the presence of an RXR agonist (Zapata-Gonzalez et al. 2007). Taken together, these reports highlight the anti-inflammatory potential RXR may exhibit *in vivo.*

1.13 REGULATION OF NUCLEAR RECEPTORS BY MicroRNAs

Although the benefits of nuclear receptor activation are vast, ranging from anti-inflammatory, anti-atherosclerotic and anti-diabetic effects, dysregulated expression or activation of these receptors can result in unwanted side effects such as weight gain, increased risk of congestive heart failure and fatty liver disease (Osman 2012, Gregoire, Kersten & Harrington 2007). Therefore the regulation of these receptors is essential in order to maintain homesostasis. MicroRNAs represent a newly explored avenue in which these nuclear receptors are regulated. MicrRNAs are a family of short (21-25 nucleotides), non-coding RNAs that control gene expression at the post-transcriptional level. miRNAs are initially transcribed as long primary transcripts (pri-miRNA) which are subsequently cleaved by two ribonuclease (RNase) III endonucleases (Droshe and Dicer) to yield mature miRNA. These mature miRNAs can negatively regulate gene expression by base pairing to partially complementary segments in the 3' UTR (miRNA response element MREs) of target mRNAs. This leads to cleavage of the target mRNA and/or translational inhibition thus ultimately leading to the negative regulation in target gene expression (Osman 2012). Nuclear receptors are negatively regulated by miRNAs but can also positively regulate miRNA expression themselves. Upon activation with its synthetic ligand GW3965, LXR can induce the expression of mature hsa-miR-613 in

primary human hepatocytes. This positive regulation of hsa-miR-613 by LXR is mediated by the sterol regulatory element binding protein (SREBP)-1c, a known LXR target gene. Hsa-miR-613 can subsequently target the 3'UTR of LXRα, inhibiting its translation thus ensuring the tight regulation of this nuclear receptor (Ou et al. 2011).Recent work by Sun *et al* also identified miR-26 as an LXR suppressed gene. Mir-26 inhibits the expression of ABCA1 thus negatively effecting cholesterol efflux. However LXR once activated can inhibit miR-26 to further enhance cholesterol efflux and control metabolic regulation (Sun et al. 2012). Targeting these miRNAs may also be beneficial in maximising the effects of these receptors in response to their respective ligands. High doses of PPARγ agonists for example are used clinically to exert their therapeutic effects. However such high doses of these drugs often leads to unwanted side effects. It has therefore been proposed that targeting miR-27b, a microRNA that negatively regulates PPARγ, could increase its expression and responsiveness to its ligands (Karbiener et al. 2009). Targeting miRNA that regulate these receptrors may prove beneficial in the treatment of disease, however previous work has also highlighted that targeting these nuclear receptors directly may also be beneficial in a clinical setting.

1.14 TARGETING NUCLEAR RECEPTORS IN DISEASE

The Thiazolidinedione's (TZD) are a class of insulin sensitizing drugs known to potently activate PPARγ. These drugs are FDA approved for the treatment of type 2 diabetes and represent an excellent example of the benefits to targeting nuclear receptors in disease (Lehmann et al. 1995). Although PPARγ is predominantly expressed in liver and adipose tissue, its expression in a number of immune cells and in the intestinal epithelium led to the hypothesis that targeting PPARγ in inflammatory disorders may be beneficial. Indeed PPARγ expression has previously been associated with IBD, where patients with UC, but not Crohns disease (CD) showed reduced

PPARγ expression in colonic epithelial cells compared to healthy controls (Dubuquoy et al. 2003). 5-aminosaliclylic acid (5-ASA), a drug currently used to treat IBD, ameliorates colitis in WT mice but not heterozygous PPARγ$^{+/-}$ mice, suggesting that drugs currently used to treat IBD can mediate their effects via PPARγ (Rousseaux et al. 2005). Furthermore, in mouse models of chemically induced colitis, colonic inflammation was significantly reduced following treatment with PPARγ agonists (Su et al. 1999). A study undertaken by Desreumaux *et al* also highlighted RXR as a potential therapeutic target in the treatment of IBD. RXRα$^{+/-}$ mice displayed significantly increased susceptibility to chemically induced colitis compared to WT mice. Interestingly, by administering low doses of both PPARγ and RXR agonists, chemically induced colitis was significantly reduced (Desreumaux et al. 2001). This suggests that targeting a specific nuclear receptor heterodimer rather than individual receptors could be beneficial in treating disease. Lower doses of agonists could be used to activate both receptors therefore reducing off target and undesirable side effects in patients. Although the possible anti-inflammatory properties of LXR have been relatively unexplored, a link between the receptor and IBD has already been reported. Work undertaken by Anderson *et al* concluded that polymorphisms in LXR were strongly associated with an individual's susceptibility to UC (Andersen et al. 2011).

MS is generally viewed as an autoimmune inflammatory disease that is characterised by the destruction of CNS myelin due to persistent inflammation in the brain and spinal cord (Lassmann, Bruck & Lucchinetti 2007). Due to the expression of nuclear receptors in immune cells and their proven ability to control inflammatory processes, it is likely that a therapeutic link between them and disease exists. In an experimental mouse model of MS (known as EAE), PPARγ$^{+/-}$ mice developed an exacerbated form of disease compared to WT mice and the treatment of both WT and PPARγ$^{+/-}$ mice with a

specific PPARγ antagonist increased both the severity and duration of disease in these mice (Natarajan et al. 2003)(Raikwar et al. 2005). Administering PPARγ agonists to EAE mice also reduced the incidence and severity of disease (Feinstein et al. 2002). Oligodendrocyte precursor cells (OPC) are a population of adult stem cells that are involved in remyelination of the CNS. However during the course of MS, the population of these cells declines and symptoms become more pronounced. Huang *et al* demonstrated that RXR agonists were positive regulators of OPC differentiation and survival whereas RXR antagonists several impaired OPC differentiation indicating a possible role for RXR in MS recovery (Huang, Franklin 2011). Indeed, RXR agonists administered in conjunction with PPARγ agonists decreased proinflammatory cytokine production from primary mouse astrocytes – a cell type implicated in the pathology of MS (Xu et al. 2006).

RA is a final example of an autoimmune inflammatory disease. It is characterised by progressive joint destruction and infiltration to the synovium of $CD4^+$ T cells, macrophages and B cells (Firestein 2003). The expression of PPARγ was significantly increased at both the protein and mRNA level in macrophages, fibroblasts and endothelial cells in RA patients compared to healthy controls (Jiang et al. 2008). It was also reported that PPARγ agonists inhibited the release of proinflammatory cytokines from RA synovial membrane cells (Sumariwalla et al. 2009). Similarly in a model of rat adjuvant induced arthritis, PPARγ agonists reduced bone erosions and therefore reduced disease severity (Koufany et al. 2008). Interestingly, the role of LXR or RXR in RA has not been extensively explored however given the antiinflammatory effects they have displayed this far it seems likely that their activation could ameliorate disease.

AIMS AND OBJECTIVES

In this study we examined three prominent nuclear receptors; PPARγ which has already been well characterised and LXR and RXR which are less well studied. As the study progressed a greater focus was placed on LXR. Our overall objectives were as follows:

- To assess the effects of nuclear receptor activation in DC. In particular we focused on parameters important for the generation of Th cell responses i.e. costimulatory marker expression, MHC expression and cytokine secretion. We also examined particular nuclear receptor heterodimers in order to identify which partnerships were important for the anti-inflammatory effects of nuclear receptors in DC.

- To determine if nuclear receptors exerted their effects on a common element within the TLR signalling pathway.

- To determine the intracellular molecular target of LXR.

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CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

TISSUE CULTURE MATERIALS/REAGENTS

Materials	Source
Tissue culture flasks T-75 cm^2/T-175cm^2	Nunc™
Sterile Petri Dishes	Nunc™
6, 24, 96-well tissue culture plates	Nunc™
96 round bottom plates	Sarstedt
Dimethyl sulphoxide (DMSO)	Sigma®
GMCSF	J558 GMCSF producing cell line
Trypan blue (0.4% v/v)	Sigma®
CellTiter 96® Aqueous One Solution	Pierce
RPMI-1640	Invitrogen™
Foetal Calf Serum (FCS)	Invitrogen™
Penicillin Streptomycin	Invitrogen™
LPS (*E.Coli* serotype R515)	Alexis Biochemicals
PGN	Invitrogen™
PamC_3S_4	Invitrogen™
Poly:IC	Invitrogen™
Zymosan	Invitrogen™
Flagellin	Invitrogen™
CPG	Invitrogen™
DMEM	Invitrogen™
Hygrogold	Invitrogen™
Blasticidin	Invitrogen™

TABLE 2.1: All tissue culture materials/reagents and corresponding sources.

PROTEIN PURIFICATION REAGENTS

Materials	Source
BCA Protein Assay	Pierce
Potassium Chloride (KCl)	Sigma®
Sodium phosphate dibasic (Na_2PHO_4)	Sigma®
Dithiothreitol (DTT)	Sigma®
Potassium phosphate (KH_2PO_4)	Sigma®
Glycerol 99.99 %	Sigma®
Trizma Base	Sigma®
Sodium dodecylsulphate (SDS)	Sigma®
Tween® 20	Sigma®
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma®
Ammonium persulphate (APS)	Sigma®
Phenylmethanesulfonyl fluoride (PMSF)	Sigma®
Propan-2-ol (isopropanol)	VWR International Ltd.
30 % (w/v) Acrylamide/Bis solution	Bio-Rad
Sodium Orthovanidate	Sigma®
Leupeptin	Sigma®
Aprotinin	Sigma®
Benzamidine	Sigma®
Trypsin Inhibitor	Sigma®
Immobilon Western HRP Substrate	Millipore
Re-Blot Plus Solution (10 X)	Millipore
Ponceau S Solution	Sigma®
Nitrocellulose membranes	Biosciences
Fuji SuperRX film	FujiFilm Ireland Ltd.
Precision Plus Protein™ Dual Color Standard	Bio-Rad

TABLE 2.2: All protein purification reagents and corresponding sources.

WESTERN BLOTTING/ CO-IMMUNOPRECIPITATION REAGENTS

Antibody	Source	Clone
Anti-Phospho NFκB-p65	Cell Signalling	
Anti-NFκBp105/p50	Merck Millipore	
Anti- Total NFκBp65	Cell Signalling	
Anti-β actin	Sigma®	
Anti-LXR	Sigma®	
Anti-mouse IgG peroxidase	Sigma®	
Anti-rabbit IgG peroxidase	Sigma®	
Anti- goat IgG peroxidase	Sigma®	
Protein A/G Agarose beads	Santa Cruz Biotechnology Inc	

TABLE 2.3: Antibodies and reagents used for immunoblotting and immunoprecipitation experiments.

NUCLEAR RECEPTOR AGONISTS/ANTAGONISTS

Materials	Source
Rosiglitazone (RSG)	Sigma®
GW9662	Sigma®
T0901317	Sigma®

TABLE 2.4: All commercially available nuclear receptor agonists/antagonists and sources

ELISAS

Materials	Source
96-well microtitre plate	Nunc™
3,3',5,5'-tetramethyl-benzidine (TMB)	Sigma®

Tween® 20	Sigma®
Bovine serum albumin (BSA)	Sigma®
Sodium Azide (NaN_3)	Sigma®
DuoSet ELISA kits	R&D Systems®
1X PBS	Biosciences

TABLE 2.5: All ELISA materials/reagents and corresponding sources.

FLOW CYTOMETRY

Antibody	**Fluorochrome**	**Source**	**Isotype Control**	**Concentration/ 10^6 cells**
TLR4-MD-2	PE	BD	RatIgG2a	0.5 µg
CD11c	APC	BD	Ham IgG1	0.5 µg
CD40	FITC/PE	BD	Ham IgM	0.5 µg
CD80	PE	BD	Ham IgG	0.5 µg
CD86	FITC	BD	Rat IgG2a	0.5 µg
MHCII	FITC/PE	BD	Rat IgG2a	0.5 µg

TABLE 2.6: Antibodies used for FACS analysis of cell surface markers; suppliers and concentrations used.

FACS MACHINE/PREPARATION FACS MACHINE/PREPARATION

Materials	Source
FACS Flow	BD
FACSRinse	BD
FACSClean	BD
37% (v/v) paraformaldehyde	Sigma®

TABLE 2.7: Materials/reagents used for flow cytometry and FACS preparation.

DNA MANIPULATION AND LUCIFERASE ASSAYS

Materials	Source
geneJuice® Transfection Reagent	Novagen®
10 X Passive Lysis buffer	Promega
Colentrazine	Argus Fine Chemicals
Luciferin	Sigma®
Coenzyme A	Sigma®
ATP disodium salt hydrate	Sigma®
Ethylenediaminetetraacetic acid (EDTA)	Sigma®
Magnesium Sulphate ($MgSO_{4)}$	Sigma®
Dithiothreitol (DTT)	Sigma®

TABLE 2.8: Materials used for manipulation of DNA plasmids in transient transfections.

PLASMIDS

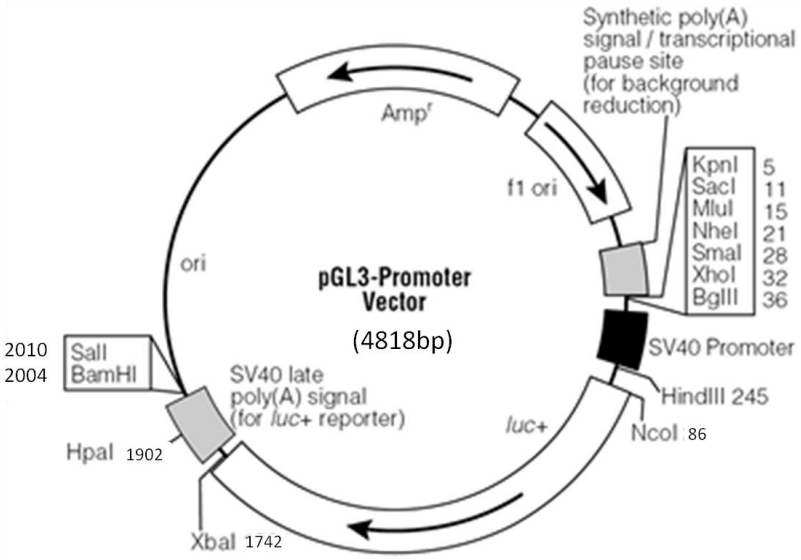


Figure 2.1: *NFκB vector map*

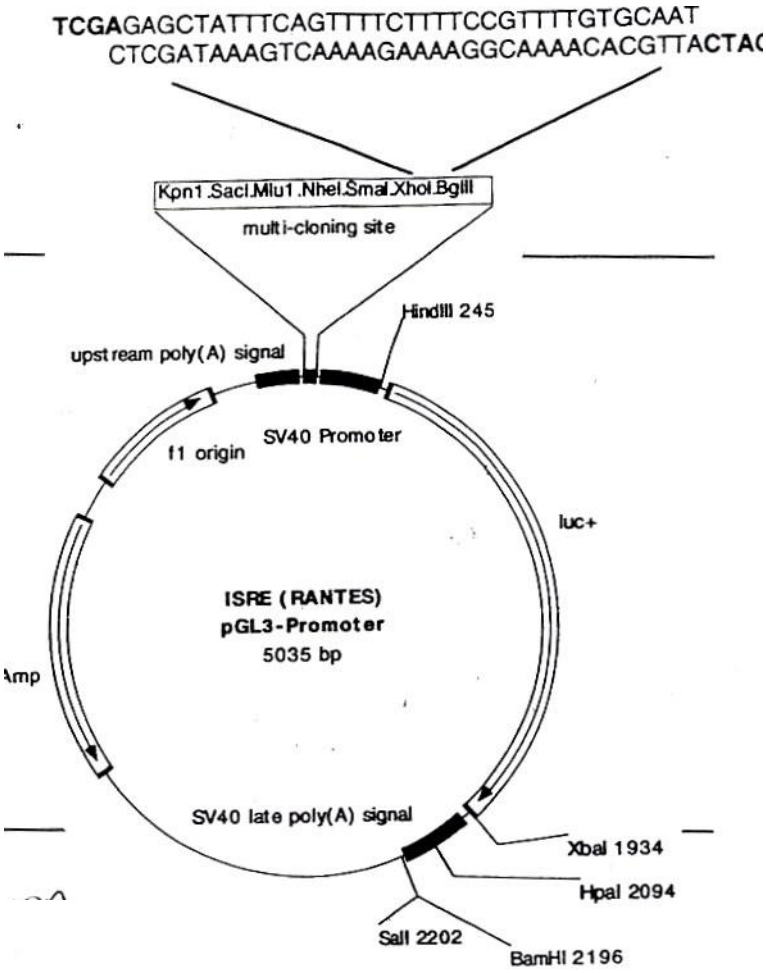


***Figure 2.2**: ISRE vector map*

RNA ISOLATION AND cDNA SYNTHESIS

Materials	Source
Nucleospin RNA II Columns	Fisher Scientific®
DEPC treated water	Invitrogen
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems

TABLE 2.9: Materials used for mRNA isolation from cells

RT-PCR

Materials	Source
TaqMan® Universal Mastermix	Applied Biosystems
MicroAmp® Optical 96-well plate	Applied Biosystems
MicroAmp® Optical Adhesive Film	Applied Biosystems
TaqMan® Gene Expression Assays	Applied Biosystems

TABLE 2.10: Primers and Mastermix used for RT-PCR reactions.

CONFOCAL MICROSCOPY

Materials	Source
AlexaFluor® 488/ 546 2^{o} Antibody	Invitrogen
Propidium Iodide	Milltenyi
Mounting Media	Dako

TABLE 2.11 Materials used to prepare confocal slides

2.2 METHODS

2.2.1 PREPARATION OF MAIN BUFFERS/ ELECTROPHORESIS GELS

Buffer	Composition
1 X Phosphate Buffered Saline (1 X PBS)	8 mM Na_2HPO_4, 1.5 M KH_2PO_4, 137 Mm NaCl, 2.7 mM KCL, pH 7.4
PBS-Tween (PBS-T)	1 X PBS with 0.05% Tween® 20
1 X Tris Buffered Saline (10 X TBS)	20 mM Trizma, 150 mM NaCl pH 7.2 – 7.4
TBS-Tween (TBS-T)	1 X TBS with 0.05% Tween® 20
TAE Running Buffer (1X)	40mM Tris Base, 0.35% Glacial Acetic Acid, 50mM EDTA

TABLE 2.12: Composition of most commonly used buffers.

2.2.2 PREPARATION OF NUCLEAR RECEPTOR AGONISTS AND ANTAGONISTS

RXR agonists/antagonists were a kind gift from Prof Hiroyuki Kagechika in Tokyo Medical and Dental University. Upon arrival, compounds were dissolved in sterile filtered DMSO to a stock concentration of 10mM and sterile filtered again. Aliquots of 20µl were stored at 4^{O}C for subsequent experimental use. Further stocks of agonists/antagonists received throughout these studies were tested for reproducibility. RSG and T0901317 were also dissolved in sterile filtered DMSO to a stock concentration of 100mM and sterile filtered again. Aliquots of 20µl were stored at 4^{O}C and -20^{O}C respectively for subsequent experimental use. The PPARγ antagonist GW9662 was dissolved in the same way to a final concentration of 10mM, aliquoted and stored for future use at 4^{O}C.

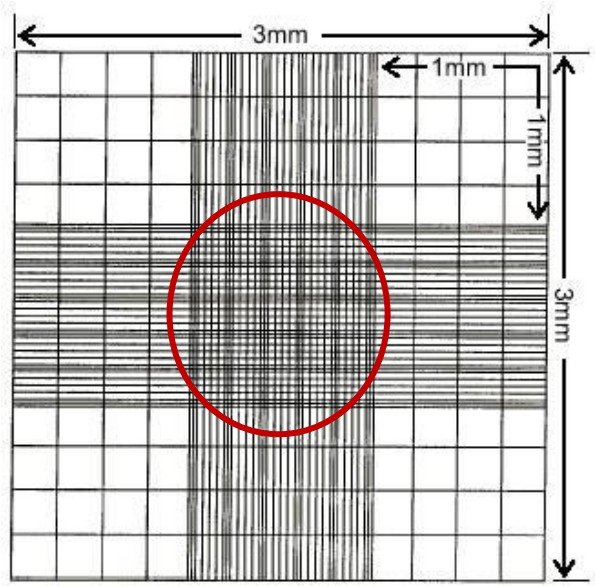
2.3 CELL CULTURE

All tissue culture was carried out using aseptic techniques in a class II laminar airflow unit (Holten 2010- ThermoElectron Corporation, OH, and USA). Cells were maintained in a 37°C incubator with 5% CO_2 and 95% humidified air (Model381- ThermoElectron Corporation, OH, and USA). Cells were grown in complete RPMI-1640 medium (cRPMI) [**see Appendix**]. FCS was heat inactivated to order to inactivate complement and then aliquoted for storage at -20°C. Supplemented medium was stored at 4°C.

2.3.1 CELL ENUMERATION AND VIABILITY ASSESSMENT

Cell viability was assessed using the Trypan blue exclusion method. This method is based on the principle that dead or dying cells which do not have an intact cell membrane will take up the dye and subsequently be stained blue. 100µl of cell suspension was mixed with 150µl PBS and 250µl of trypan blue solution (0.4% (v/v)).

After ~2 min cells were applied to a brightline haemocytometer (Sigma®) and examined under high-power magnification (×40) using an inverted microscope (Olympus CKX31, Olympus Corporation, Tokyo, Japan). Cells that were inside the central grid of 25 squares were counted.



A viable cell count was achieved by using the following formula:

$$\text{Cells/ml} = N \times 5 \times 10^4$$

Where N = total number of cells counted, 5 = dilution factor and 10^4 = constant.

***Figure 2.3**: Cell enumeration using the haemocytometer*

2.3.2 HUMAN EMBRYONIC KIDNEY CELL LINES HEK293

Human embryonic kidney cell line HEK293 stably transfected with TLR4, CD14 and MD-2, (HEK293-MTC) were a kind gift from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. The HEK293-MTC cell line was cultured with appropriately supplemented complete DMEM media **[see Appendix]**. Cells were cultured in 175cm^2 flasks as follows; complete DMEM supplemented with 50µg/ml Hygrogold and 1µg/ml Blasticidin to maintain expression of TLR4, CD14 and MD-2. Cells were passaged every 3 to 4 days based on confluency. For subculture, the media was removed from the flasks and cells washed twice with 5ml ice cold sterile PBS (Invitrogen™). Following this cells were removed from the surface of the flasks by

incubating them with 2ml of 1 X Trypsin solution (Sigma®) for 5 min at 37 °C. 10ml of complete media was then added to resuspend cells and cells were spun at 1200rpm for 5min. Finally cells were resuspended in media, subcultured or counted for experiments.

2.3.3 PREPARATION OF CELL STOCKS

Cells were grown to a state of sub-confluency and were harvested and counted as previously described in section 2.3.1-2.3.2. Cells were removed from culture and resuspended in 1ml cryoprotectant (10 % (v/v) dimethylsulphoxide (DMSO), 40 % (v/v) FCS and 50% RPMI) and transferred to labelled and dated cryovials (Nalgene®, Cryoware). These aliquots were placed at -20ºC for 2hrs and then at -80ºC for 3hrs before being stored in a liquid nitrogen vessel.

2.3.4 REVIVAL OF FROZEN STOCKS

Cryovials were carefully removed from the liquid nitrogen tank and quickly thawed in a 37 °C water bath. Thawed cells were transferred to 10ml RPMI on ice and carefully resuspended. Cells were spun at 1200rpm for 5min to remove excess DMSO. Following this, supernatant was discarded and cells resuspended in 10 ml of room temp RPMI. Cells were spun again and a third wash carried out using 37 °C RPMI. After the final wash cells were resuspended in 1ml of the appropriate medium and transferred to a 75cm^2 flask with appropriately supplemented media **[see Appendix]**.

2.3.5 ISOLATION AND CULTURE OF MURINE BONE MARROW DERIVED DENDRITIC CELLS

2.3.5.1 DAY 1 – HARVESTING CELLS

DC were harvested from Balb/c mice (Charles River). Bone marrow from the tibia and femurs was extracted by flushing cRPMI through the bones using a 27.5g needle into a sterile Petri dish. The bone marrow was broken up gently using a 19.5g needle and transferred to a sterile falcon tube and centrifuged for 5min at 1200rpm. Supernatant was then removed and the remaining pellet was resuspended in sufficient cRPMI to allow for 1ml of cells per Petri dish required. A cell count was performed using the trypan blue exclusion method to ensure an adequate number of viable cells have been harvested. 9ml of cRPMI and the growth factor GMCSF (40ng/ml) and 1ml of cells were added to each dish and the appropriate amount of agonists/antagonists or vehicle control was added.

2.3.5.2 DAY 4 – FEEDING CELLS

Petri dishes were tipped slightly in order to easily see the monolayer of cells adhered to the dish. Using a transfer pipette 6mls of media was removed gently without disturbing the layer of cells. 10mls of prewarmed cRPMI with GMCSF was added to the culture with the appropriate agonist/antagonist or vehicle control. Cells were incubated at $37^{O}C$.

2.3.5.3 DAY 7 – COUNTING AND PLATING CELLS

To remove semi-detached and therefore immature dendritic cells, cells were scraped gently using cell scrapers and transferred to a falcon tube. Each Petri dish was examined under a microscope to ensure ~ 90% of cells were recovered. Cells were centrifuged at 1200rpm for 5min, resuspended and counted using the trypan blue exclusion method.

The cell concentration was adjusted with cRPMI – generally cells were plated at 1 x10^6cells/ml. The appropriate agonists/antagonists or vehicle control were readded before cells were plated.

2.3.6 CULTURE OF THE J558 GMCSF- SECRETING CELL LINE

The murine gene for GMCSF was cloned into a mammalian expression vector (Karasuyama) and transfected into the plasmacytoma line X63-AgS. Cell stocks were kindly donated by Professor Kingston Mills (Trinity College, Dublin). After removal from liquid nitrogen and rapid thawing, cells were washed in 30mls cRPMI, and then resuspended in 5mls of selection medium consisting of 1mg/ml G418 Geneticin (GibcoBRL) in complete RPMI for 2 passages. Cells were seeded at 1x10^6 cells/ml each time and culture flasks were left in the upright position in the incubator. After the second passage cells were washed twice in cRPMI, counted and seeded in cRPMI at 1 x 10^6 cells/ml. When cells reached a medium density they were seeded at 2.5x10^5 cells/ml at each passage. Supernatant was collected from J558 from the first 9 passages and the amount of secreted GMCSF was quantified by ELISA (R&D). In order to differentiate bone marrow cells into CD11c positive BMDC, GMCSF was used at a concentration of 40ng/ml.

2.3.7 ADDITION OF NUCLEAR RECEPTOR AGONISTS/ ANTAGONISTS

Cells were cultured for 7 days in the presence of the vehicle control DMSO, and nuclear receptor agonists/antagonists. Addition of nuclear receptor agonists/antagonists from Day 1 of BMDC harvest was necessary in order to alter the differentiation process of the DC and concurs with previous reports published within this field (Klotz et al. 2007)

(Zapata-Gonzalez et al. 2007, Torocsik et al. 2010). RSG was added at a concentration of 10μM, T0901317 at a concentration of 2μM and RXR agonists PA024 and HX630 at a concentration of 1μM. Nuclear receptor antagonists were added where appropriate, 2hrs prior to agonist treatment at a concentration of 1μM.

2.3.8 TOLL-LIKE RECEPTOR ACTIVATION

Cells were activated with TLR ligands which are outlined in **Table 2.13**, and incubated for 24hrs before being used in the relevant assays.

TLR	LIGAND	STOCK CONCENTRATION	WORKING CONCENTRATION
2/1	**Pam3CsK4**	1mg/ml	1μg/ml
2/6	**Zymosan**	1mg/ml	10μg/ml
3	**Poly:(IC)**	1mg/ml	10μg/ml
4	**LPS**	1mg/ml	100ng/ml
5	**Flagellin**	100μg/ml	5μg/ml
7	**Loxoribine**	10mM	1mM
9	**CpG**	500μM	2μM

TABLE 2.13: *Concentrations of the TLR ligands used for the maturation of dendritic cells*

2.3.9 CYTOTOXICITY ASSAY FOR AGONIST/ANTAGONIST TREATED BMDC

The Cell Titer 96 Aqueous One Solution (Promega) is a colourimetric method for determining the number of viable cells in a sample. It contains an MTS terazolium compound that is bioreduced by cells into a soluble coloured formazan product. The

quantity of this formazan product is measured at an absorbance reading of 490nm and is directly proportional to the number of living cells in the culture.

BMDC were cultured for 7 days in the presence of specific agonists and antagonists at different concentrations. Compounds were added on day 1 of culture. After 7 days cells were collected, counted and plated (100µl per well at $1x10^6$ cells/ml) in a 96-well plate and stimulated with LPS (100ng/ml) for 24hrs. 20µl of CellTiter 96 Aqueous One solution was then added to each well and plates were incubated for 2 hours at 37^OC in 5% CO_2. After this time the absorbance was read at 490nm. The cell viability of each sample was calculated by treating the absorbance of the vehicle control (DMSO) as 100% and comparing the remaining samples to this and expressing these values as percentage viability.

2.4 ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

ELISA can be used to quantify cytokine secretion from a particular sample. The principles of a sandwich ELISA are illustrated below. In general, a 96 well plate is coated with a suitable capture antibody. These antibodies are usually diluted in a buffer such as PBS and incubated overnight at room temperature. A blocking buffer such as BSA is then added to block any remaining protein- binding sites on the plate. The sample which contains unknown concentrations of the cytokine along with a series of standards of known concentrations is then added to the plate and incubated overnight at 4^OC to allow the antigen to bind to the specific capture antibody. The following day a biotinylated detection antibody for the cytokine of interest is added and incubated for 2hrs at room temperature. Horseradish peroxidase conjugated to streptavidin is then added to the plate. Streptavidin binds biotin with high affinity so when the substrate

TMB is added, the enzyme HRP catalyzes the TMB substrate to form a blue compound. The blue colour that is formed increases in intensity with increasing concentrations of cytokine. This reaction is then stopped by adding sulphuric acid which converts the blue colour to a yellow colour that can be detected at 450nm on a spectrophotometer. Thus the intensity of the colour formed is proportional to the amount of cytokine present. Throughout this study the concentrations of specific cytokines were determined using ELISA Duoset kits from R & D systems according to manufacturer's instructions.

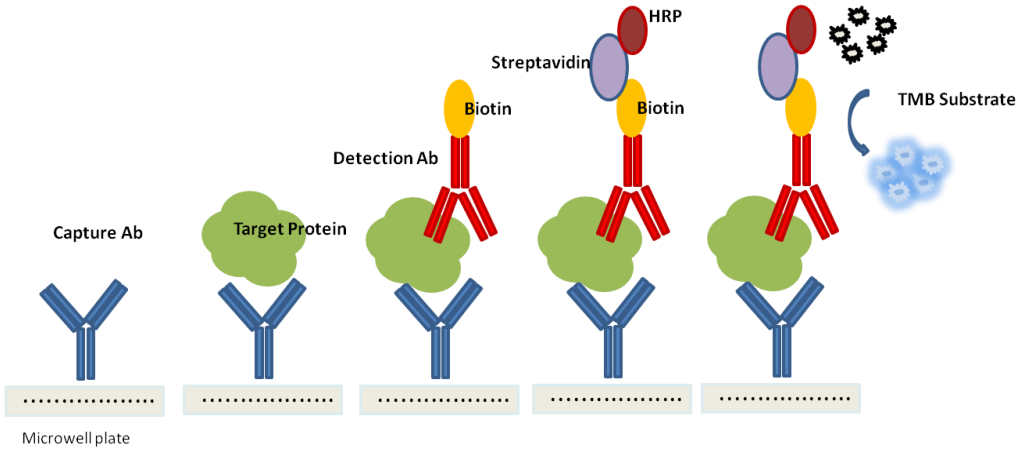


Figure 2.4 *The principle of a sandwich ELISA [adapted from* http://www.epitomics.com/products/product_info/6111-1]

2.4.1 DETECTION OF IL-12p40, IL-12p70, IL-23, IL-27, IL-10, TNFα AND IL-6

96-well Nunc™ microtitre plates were coated with 100µl of the relevant capture antibody diluted to working concentration in PBS see **Table 2.14** and incubated overnight at room temperature. After washing plates x 3 with wash buffer (PBS/0.05% Tween® 20), wells were blocked with 300µl of reagent diluent (1% w/v BSA/PBS) for at least 1 h at room temperature. After repeating the washing step, 50µl of reagent diluent and 50µl of

supernatant or serially diluted standards (top standard serially diluted in reagent diluent – see **Table 2.14**) were added to wells in duplicate. Plates were incubated overnight at 4^{o}C. The following day plates were washed x 3 with wash buffer. 100µl of the relevant biotinylated detection antibody, diluted in reagent diluent (1:180 dilution), was added to each well and plates were incubated for 2hrs at room temperature. Plates were washed x 3 with wash buffer and 100µl of streptavidin-HRP (1:200 dilution in reagent diluent) was added to each well. Plates were incubated for 20min in the dark at room temperature. Finally, wells were washed x 3 with wash buffer and 100µl of TMB was added to each well. Plates were incubated in the dark until colour developed. The reaction was stopped by adding 50µl 2N H_2SO_4 per well. Optical densities were read immediately at 450nm on VERSA Amax microplate reader (Molecular devices, CA, USA). Cytokine concentrations in supernatants were determined from standard curves.

2.4.1.1 IL-12p40, IL-6

Samples were diluted 1:100 in reagent diluent and 50µl of diluted samples and undiluted standards were added to the plates in duplicate. Concentrations of samples were multiplied by the dilution factor once calculated from the standard curve.

2.4.1.2 TNF-α

Samples were diluted 1:10 in reagent diluent and 50µl of diluted sample and undiluted standards were added to the plates in duplicate. Concentrations of samples were multiplied by the dilution factor once calculated from the standard curve.

2.4.2 DETECTION OF IL-1β

The method above was followed with two variations:

Blocking buffer used was 1% (w/v) BSA/PBS + 0.05% (w/v) NaN_3 and the reagent diluent was 0.1% (w/v) BSA/TBS + 0.05% (w/v) Tween.

CYTOKINE	CAPTURE ANTIBODY (µg/ml)	DETECTION ANTIBODY (ng/ml)	TOP STANDARD
IL-12p40	4.0	400	2000
IL-12p70	4.0	400	2500
IL-23	4.0	200	2500
IL-27	4.0	400	1000
IL-10	4.0	500	2000
TNFα	0.8	75	2000
IL-1β	4.0	400	1000
IL-6	2.0	200	1000

TABLE 2.14: *Concentration of standards, capture and detection antibodies used in sandwich ELISA assays.*

2.5 FLOW CYTOMETRY

2.5.1 BASIC PRINCIPLES OF FLOW CYTOMETRY

Flow Cytometry is a process by which the size, granularity and expression of protein markers can be identified on a particular cell. Each cell is suspended within a stream of

liquid and in a very short space of time is passed through a light source to be individually examined.

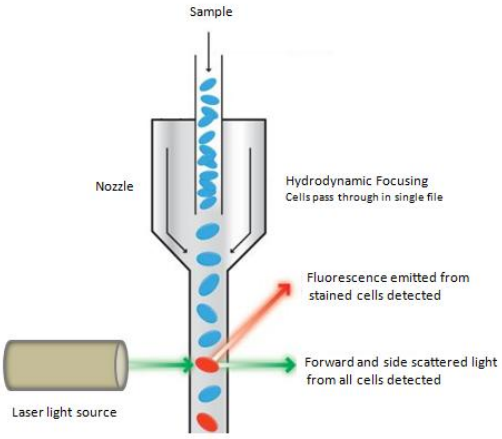


Figure 2.5 *Examination of individual cells by flow cytometry [adapted from* http://www.sonyinsider.com/2010/02/12/sony-acquires-icyt-and-officially-enters-flow-cytometry-business/]

In order to examine the expression of a particular protein, cells are incubated with a specific monoclonal antibody designed to target the protein of interest. These antibodies are conjugated to fluorochromes which emit light at various wavelengths once they have been excited by the laser. Throughout this study three main fluorochromes were used:

1) FITC - Fluorescein isothiocyanate which is excited at 495nm and has a maximum emission intensity of 520nm is detected using the FL1 channel.

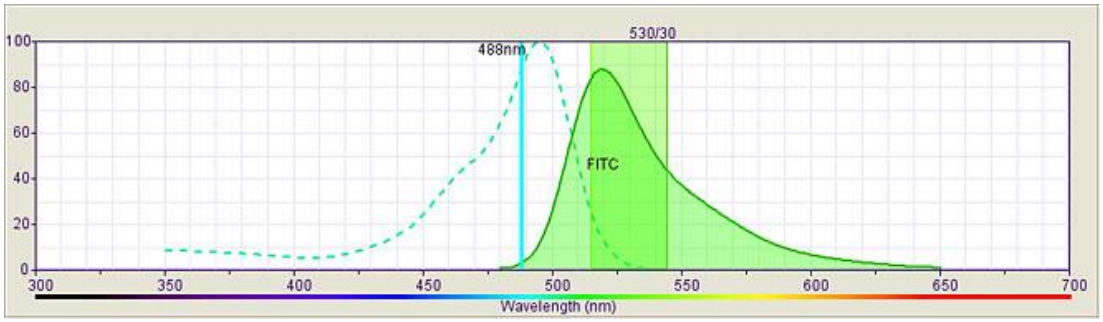


Figure 2.6 *Excitation and emission spectrum of FITC*

2) PE- Phycoerythrin which is excited at 495nm and has a maximum emission intensity of 578nm is detected using the FL2 channel.

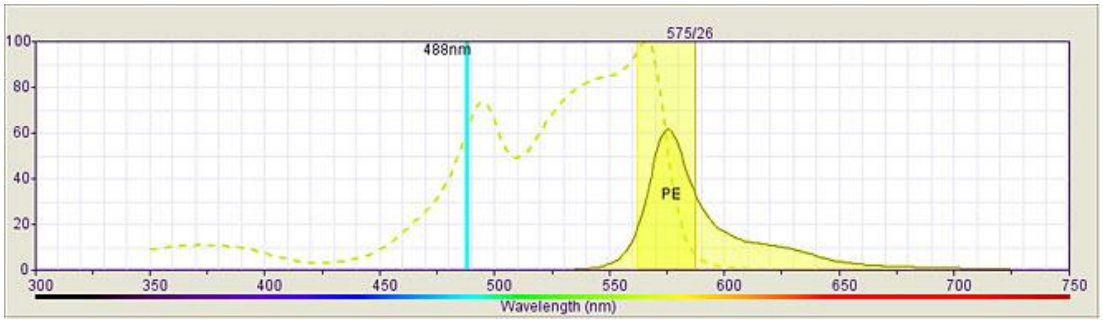


Figure 2.7 *Excitation and emission spectrum of PE*

3) APC- allophycocyanin which is excited at 635nm and has a maximum emission intensity of 660nm is detected using the FL4 channel.

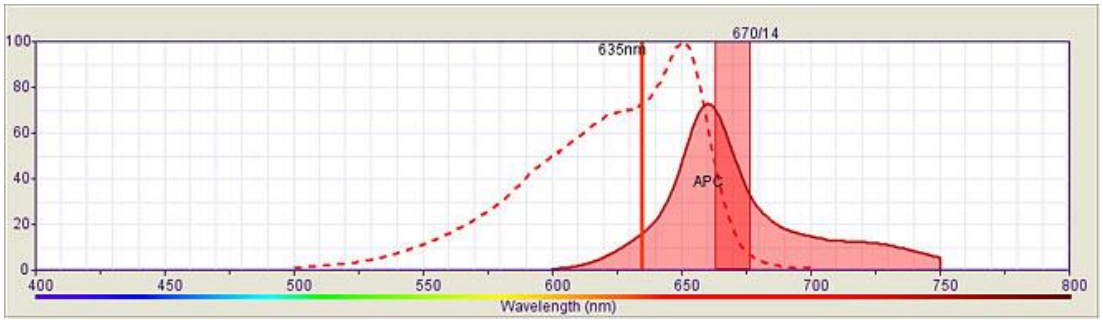


Figure 2.8 *Excitation and emission spectrum of APC [2.4 -2.6 adapted from* http://www.bdbiosciences.com/research/multicolor/spectrumguide/index.jsp]

2.5.2 CELL SURFACE MARKER STAINING

BMDC were cultured for 7 days in the presence of specific agonists/antagonists or vehicle control. On day 7 cells were plated on a 6 well plate at 2 x 10^6 cells per well (in 2mls) and stimulated for 24hrs. Cells were then carefully scraped from the wells and incubated in a falcon with equal amount FCS for 15 mins to reduce non specific binding. Cells were then centrifuged for 5 mins at 1200rpm and the resulting pellet was resuspended in FACS buffer. 200µl of cells were then plated on a 96 well round bottom plate giving approximately 400,000 cells per well. 1 well per treatment group (i.e. RXR agonist +/- LPS) was allocated for each antibody group plus 1 well for each corresponding isotype control group. Plates were spun at 2000rpm for 10min and supernatant carefully removed from the wells. 100µl of the relevant antibody or isotype mixture was added to the appropriate wells. Plates were incubated in the dark at 4 ^{o}C for 30min. Following incubation, plates were spun at 2000rpm for 10min at 4ºC. Cells were washed by resuspending in 200µl FACS buffer twice. Cells were then fixed in 200µl 4% (v/v) formaldehyde/PBS before being transferred to labelled FACS tubes. Samples were acquired immediately or left overnight in the dark at 4 ºC. 30,000 events were acquired per sample using a 4-colour Becton Dickinson (BD) FACSCalibur (fluorescence activated cell sorter). CD11c positive cells were gated using FlowJo software and subsequent analysis was carried out on this population.

2.6 DNA MANIPULATION

2.6.1 LUCIFERASE ASSAY BY TRANSIENT TRANSFECTION OF HEK293 CELLS

The ISRE luciferase plasmid, NFκB luciferase plasmid, *Renilla* luciferase plasmid and empty pcDNA3.1 vector along with luciferase assay reagents were kind gifts from Prof. Luke O'Neill, School of Biochemistry, Trinity College Dublin. All transfections were performed in 24-well tissue culture plates with a total volume of 500µl/well. Cells were seeded as follows at 4 x 10^5 cell/ml. Cells were incubated overnight and transfected the following morning using geneJuice® transfection reagent according to the manufacturers' instructions. GeneJuice® (Novagene) transfection reagent is a liposomal based transfection reagent. For ISRE/NFκB luciferase assays, 75 ng of ISRE/NFκB luciferase plasmid, 30 ng of *Renilla* luciferase, and 115 ng empty pcDNA3.1 vector made up to a total of 220ng of DNA were transfected into each well of a 24-well plate. For both ISRE and NFκB luciferase assays cells were left to rest for 24h after transfection before stimulating with 100 ng/ml LPS for 6h. Following stimulation media was aspirated from each well and cells were lysed in 100µl of 1 X passive lysis buffer (Promega, Southampton, UK) for 15min. Firefly luciferase activity was assayed by the addition of 40µl of luciferase assay mix (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 $MgSO_4$, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP) to 20µl of the lysed sample. In addition, *Renilla* luciferase was read by the addition of 40µl of a 1:1000 dilution of Coelentrazine (Argus Fine Chemicals) in PBS to 20µl of lysed sample. Luminescence was read using a Reporter microplate luminometer (Turner Designs). The *Renilla* luciferase plasmid was used to normalize for transfection efficiency in all experiments.

2.7 RNA ANALYSIS

2.7.1 RNA EXTRACTION

BMDC were plated at 1 x10^6 cells/ml (in 2mls) and left to rest overnight before being stimulated over a course of 24hrs with 100ng/ml LPS. After this time total RNA was isolated using the Nucleospin® RNA II spin columns according to manufacturer's instructions. Cells were lysed in a solution containing large amounts of chaotropic ions. RNA and DNA then bind to the silica membrane within the column and the contaminating DNA is removed by using a rDNase solution. The columns were then washed using a number of different buffers to remove salts, metabolites and macromolecular cellular components and finally the pure RNA was then eluted from the columns into RNAse free water. RNA was then quantified using the Nanodrop 1000 spectrophotometer (Thermo Scientific). DNA contamination was assessed by measuring the absorption at 260nm and 280nm and calculating the ratio between them. Ratio's that fell between 1.8 and 2.1 were considered pure RNA samples.

2.7.2 RNA ANALYSIS BY GEL ELECTROPHORESIS

To check RNA integrity, samples were run on a 2% (w/v) agarose SYBR® safe gel in order to visualise clear and unsmeared 18S and 26S ribosomal bands. The appropriate amount of agarose was dissolved in 100mls 1X TAE buffer by boiling for 1 min. After cooling 10µl of SYBR® safe (10,000X concentration) was added to gel mix, poured, protected from light and subsequently allowed to set. The RNA samples (1µl) were prepared for electrophoresis by adding to 2µl RNA sample buffer (Sigma®). Samples were heated for 10 mins at 65^{O}C before being loaded onto the gel. The gel was run in 1

X TAE buffer **[see Appendix]** at 100V for 1 hr before being visualised using the G-Box Gel Imaging system (Syngene).

2.7.3 cDNA SYNTHESIS

In order to convert total RNA to single stranded cDNA the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used. Using this assay the enzyme reverse transcriptase was used on a single strand of mRNA to generate complementary DNA, based on the pairing of RNA base pairs i.e. A, U, G and C to their DNA complements T, A, C and G respectively. Random primers were also used to produce pieces of cDNA from all over the mRNA. Up to 2µg of total RNA was converted to cDNA as follows:

Component	Volume/Reaction µl
10X RT Buffer	2µl
25X dNTP Mix	0.8µl
10X Random Primers	2µl
Reverse Transcriptase	1µl
Nuclease Free H_20/ RNA	14.2µl
TOTAL PER REACTION	20µl

TABLE 2.15: *Components of cDNA Reaction*

Reverse transcription was then performed using the following settings on a Thermacycler

	STEP 1	STEP 2	STEP 3	STEP 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

TABLE 2.16: *Thermocycler settings for cDNA synthesis*

2.7.4 QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (qRT-PCR)

2.7.4.1 OPTIMIZATION OF RT-PCR ASSAY

In order to ensure our assays had high reproducibility, consistency and accuracy our reactions were tested for the following:

Linear standard curve ($R^2 > 0.980$)

High amplification efficiency (90–105%)

Consistency across replicate reactions

In order to test these parameters and ensure each assay was optimized we ran a serial dilution of template cDNA and used these results to generate a standard curve for each primer. The standard curve was constructed by plotting the log of the dilution factor against the Ct value obtained during amplification of each dilution. The equation of the line, along with the coefficient of determination (R^2), was then used to evaluate whether

the assay was optimized. The slope of the line should then fall between 3.1 and 3.3 thus showing that the amplification between each serial dilution is evenly spaced. Similarly an R^2 value >0.980 represents data that is linear.

Amplification efficiency, E, was then calculated from the slope of the standard curve using the following formula:

$$E = 10^{\wedge (-1/slope)} - 1 \times 100$$

Assays that generated efficiencies between 90 – 105% were used for subsequent relative RT quantification experiments.

2.7.4.2 RELATIVE QUANTIFICATION USING RT-PCR

RT-PCR was then carried out using TaqMan Gene expression assays (Applied Biosystems). These assays consisted of two unlabeled primers for amplifying the sequence of interest (final concentration of 900nM each) and one dual- labelled TaqMan MGB probe (6-FAM dye and TAMRA –labelled) for the sequence of interest (final concentration of 250nM). These reactions exploit the 5'-3' nuclease activity of DNA polymerase to cleave the TaqMan probe during PCR as illustrated in **Figure 2.9**. The PCR reaction mix was prepared for each sample in triplicate by addition of the reagents listed in table 2.13 to individual wells of a 96 well reaction plate (Applied Biosystems). The plate was covered with an optical adhesive cover and centrifuged at 2,000rpm for 2 mins to eliminate air bubbles. Plates were then run on the ABI Prism 7500 sequence detection system using the conditions highlighted in **Table 2.18**

Component	Volume/Reaction (µl)
TaqMan Mastermix	10µl
Primer/Probe	1µl
DEPC H_2O	5µl
cDNA	4µl
TOTAL	20µl

***TABLE 2.17**: Components of RT reaction*

	STEP 1	STEP 2	STEP 3 (a)	STEP 3 (b)
Temperature (^oC)	50	95	95	60
Time	2 min	10 min	15 sec*	1 min*
			* Repeat for 40 cycles	

***TABLE 2.18:** Thermocycler settings for RT-PCR*

The reporter dye signal was measured against the internal reference dye (ROX) signal to normalise for non-PCR-related fluorescence occurring from well to well. The relative levels of different transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control cells was adjusted to 1 and served as the basal reference value throughout experiments

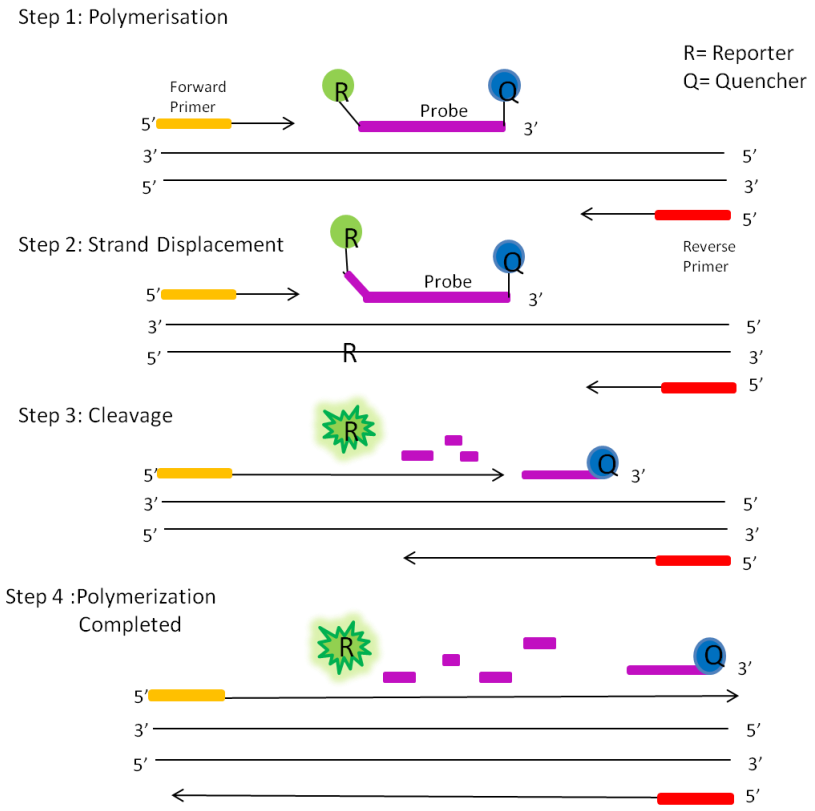


***Figure 2.9**: Principle of TaqMan Gene Expression Assays [adapted from http://www3.appliedbiosystems.com]*

In addition to sequence specific primers, TaqMan Gene Expression Assays also contain fluorescently labelled oligonucleotide probes called the TaqMan probe. The probe contains a fluorescent reporter at the 5' end and a quencher at the 3' end. When intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. During the combined annealing/extension step reaction, the probe hybridizes to the target and 5'—>3' exonuclease activity of Taq polymerase cleaves off the reporter. As a result, the reporter is separated from the quencher, and the resulting fluorescence signal is proportional to the amount of amplified product in the sample. (Adapted from Applied Biosystems TaqMan Gene Expression Assays http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf)

2.8 PROTEIN ANALYSIS

2.8.1 PREPARATION OF WHOLE CELL LYSATES

Cells were seeded at 1 x 10^6 cell/ml in a 6-well plate (2 ml/well) with/without relative treatments and left overnight to rest. Cells were then stimulated with LPS (100ng/ml) at the times indicated. Following activation, cells were washed with PBS and scraped in 100µl of lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% (w/v) igepal and 50 mM NaF, with 1 mM $Na3VO_4$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (leupeptin (25 µg/ml), aprotinin (25 µg/ml), benzamidine (1 mM), trypsin inhibitor (10 µg/ml). Protease and phophatase inhibitor were added just before use. Cells were then incubated with gently agitation at $4^{O}C$ for 30 mins. Following this incubation period samples were scraped into prechilled tubes and centrifuged at 12,000 x g for 10 mins at 4ºC. The protein concentration of the samples was then determined using the BCA assay and aliquots containing equal amounts of protein were mixed with 4X SDS sample buffer, boiled at $100^{O}C$ for 10 min and separated using SDS denaturing polyacrylamide gel electrophoresis.

2.8.2 SDS DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS- PAGE)

Proteins were separated by SDS denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Acrylamide gels (12 % (w/v)) **[see Appendix]** were cast between two glass plates and affixed to the electrophoresis unit using spring clamps. Electrode running buffer **[see Appendix]** was added to the upper and lower reservoirs. 10µl of prepared samples were loaded into the wells and run at 30 mA per gel for approximately 45 mins.

Pre-stained protein molecular weight markers (Bio-Rad laboratories) ranging from 10 – 250 kDa were added to the first lane in each gel.

2.8.3 PROTEIN TRANSFER

Proteins were quantitatively transferred to nitrocellulose membranes using the iBlot® Dry Blotting System (Invitrogen™). The iBlot® efficiently and reliably blots proteins from polyacrylamide gels in 7min without the need for additional buffers or an external power supply in a self-contained unit. Following transfer, the nitrocellulose membrane was removed and processed for immunoblotting.

2.8.4 IMMNUNOBLOTTING AND DETECTION

Following transfer, non-specific sites on the membrane were blocked with freshly prepared blocking buffer, 5 % (w/v) dried skimmed milk/TBS-T for 1hr on a slow rocker at room temperature. Membranes were then washed x 3 with TBS-T (wash buffer) and incubated with appropriate primary antibodies. Incubation details including reagent diluent and the concentration of antibodies used are listed in **Table 2.19**. Membranes were gently agitated with the primary antibodies overnight at 4 ºC. Following overnight incubation, membranes were washed eight times for 2min in wash buffer. Membranes were then incubated with the relevant secondary antibodies (horseradish peroxidase (HRP) conjugated secondary antibody) [see **Table 2.19**] and incubated with gentle agitation at room temperature for 1hr. Following incubation with secondary antibody, membranes were washed eight times for 2min with washing buffer.

HRP-labelled antibody complexes were visualised using the enhanced chemiluminescence (ECL) method. Membranes were incubated for 5 minutes in 3 ml of Immobilon Western HRP Substrate (Millipore). Excess substrate was decanted and the membrane placed between acetate sheets and immediately exposed to FujiFilm

SuperRX film in a dark room under red light. The film was developed using a film Hyperprocessor (Amersham Pharmacia Biotech). Exposure times varied depending on the concentration of protein used and the intensity of signals obtained. In general exposure times varied between 30sec to 5min. The density of resultant bands was calculated using the densitometry program on the Syngene gel analysis and documentation system (Syngene NJ USA).

2.8.5 STRIPPING AND RE-PROBING MEMBRANES

To reprobe membranes, antibody complexes were removed by incubating membranes in 10 ml 1 X Re-Blot Plus Solution (made according to manufacturer instructions) for 15min with gentle agitation. Following this membranes were washed in 5 ml of blocking buffer twice for 5min to remove excess stripping solution. At this point membranes were either re-probed with antibodies or stored in TBS-T at $4^{O}C$.

1° Antibody	Dilution	2° Antibody	Dilution
NFκBp105/p50	1:1000	Anti rabbit IgG	1:8000
NFκBp65	1:1000	Anti rabbit IgG	1:2000
Total NFκBp65	1:1000	Anti rabbit IgG	1:2000
B Actin	1:10,000	Anti mouse IgG	1:20,000
LXR	1:1000	Anti goat IgG	1:5000

Table 2.19 *Concentrations of antibodies used for western blotting*

2.9 CO-IMMUNOPRECIPITATION

Cell extracts were generated on ice or at 4^{o}C according to the following experimental strategy. Cells were first washed with pre-chilled 1X PBS (ml) then lysed with pre-chilled CoIP lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5% (w/v) igepal and 50 mM NaF, with 1 mM Na3VO_4, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (leupeptin (25 μg/ml), aprotinin (25 μg/ml), benzamidine (1 mM), trypsin inhibitor (10 μg/ml)) for 30 min on a rocker at 4^{o}C. Lysates were scraped into pre-chilled 1.5 ml eppendorf tubes and centrifuged at 12,000 g for 10min at 4^{o}C. Supernatants were removed to fresh tubes (a sample retained for whole cell lysate analysis) and incubated overnight with primary antibody (2μg). The following day Protein A/G agarose beads (30-40 μl) were added to each sample and they were again incubated at 4^{o}C overnight. The subsequent day samples were centrifuged at 16,000 g for 1min. The beads were washed with CoIP lysis buffer (600μl) and subject to re-centrifugation. This step was repeated four times. The 2X sample buffer (0.125 M Tris-HCl, pH 6.8, containing 20% (w/v) glycerol, 4% (w/v) SDS, 1.4 M β-mercaptoethanol and 0.0025% (w/v) bromophenol blue) was added to the beads for 30min at RT. Samples were boiled at 100^{o}C for 10min and analyzed using SDS polyacrylamide gel electrophoresis and western blotting as described in section 2.8.2 to 2.8.4. Light chain specific secondary antibodies were used to detect immunoprecipitated proteins.

2.10 CONFOCAL MICROSCOPY

BMDC were cultured for 7 days in the presence or absence of agonists/antagonists as described in section 2.3.5 and 2.3.7. Glass coverslips were sterilised in 100% ethanol, passed through a flame and inserted into 6 well culture plates. Plates were further

sterilized under an ultraviolet lamp for 30 min. Cells were harvested from culture and counted. Cells were plated at 2 x 10^5 cell/ml (2ml/well) and left to rest overnight. Agonists and antagonists were readded at appropriate times as previously described. For the investigation of NFκBp50 and NFκBp65 localization and LXR:p50 or p65 colocalization cells were stimulated for 15min with 100ng/ml LPS. Following stimulation media was removed and cells were washed on ice three time with ice cold PBS. Cells were fixed by incubating with 2% (v/v) paraformaldehyde/PBS, pH 7.4 for 30min on ice. Slides were then blocked for 1hr at room temperature using 100µl of blocking buffer (Filtered PBS/Glycine/Fish gelatin). Following this, slides were washed 3 times for 5 min each with 50µl filtered PBS. Slides were then incubated at 4^{O}C overnight in a humidified chamber with the relevant specific primary antibody. Slides were then washed 3 times for 5 minutes with filtered PBS before being incubated at room temperature with species specific appropriate secondary antibodies. After this time, slides were washed a further 2 times before being incubated with Propidium Iodide (PI) nuclear stain for 10 min (for nuclear translocation experiments). After a further 2 more washes, coverslips were mounted onto slides using fluorescent mounting media (Dako) and sealed with varnish. Cell preparations were analysed using the Zeiss LSM 710 confocal microscope

2.11 STATISICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance ($p<0.05$), post-hoc Student-Newmann-Keul test was used to determine which conditions were significantly different from each other.

CHAPTER 3

NUCLEAR RECEPTOR ACTIVATION MODULATES DENDRITIC CELL FUNCTION *IN VITRO*

3.1 INTRODUCTION

The nuclear receptor family of transcription factors consists of 48 members which, upon activation, play pivotal roles in reproduction, development and overall homeostasis within an organism. They can heterodimerise with other members of the nuclear receptor superfamily to carry out these effects. Functions such as these are carried out by the receptors ability to both positively and negatively regulate gene expression (Ogawa et al. 2005). However a key emerging feature of these nuclear receptors is their novel anti-inflammatory properties.

PPARγ, for example, which is activated endogenously by fatty acids, fatty acid metabolites/derivatives and lipophilic hormones was originally thought to be a regulator of nutrient metabolism and energy homeostasis due to its presence in high concentrations in adipocytes (Berger, Akiyama & Meinke 2005). However after its discovery in a number of innate and adaptive immune cells including B cells, T cells dendritic cells monocytes and macrophages a role in the immune system and potentially in inflammatory disease began to emerge. Similarly, the discovery of LXR and RXR in immune cells led to the conclusion that these receptors could have potential immunomodulatory properties (Berger, Moller 2002). Although expression of these receptors has been documented in dendritic cells, their effects on DC maturation and function have not yet been extensively explored. Indeed the role of these receptors in macrophage biology and lipid metabolism has dominated this area of research in the past (Castrillo, Tontonoz 2004).

DC are essential antigen presenting cells that efficiently link the innate immune system with the adaptive immune system. Without these cells, an appropriate and highly specific T cell response cannot be generated. DC are strategically positioned at sites of

potential pathogen entry, where they remain in an immature state and constantly patrol the environment for invading pathogens. These cells are equipped with a set of germ-line encoded PRR such as the TLRs which recognise conserved PAMPs shared by a large group of pathogens. Following a DC encountering a pathogen and subsequent TLR engagement, a program of DC maturation is initiated. Only after this DC maturation is complete can the mature effector DC drive the development and differentiation of T helper cells from naive precursors (de Jong, Smits & Kapsenberg 2005).

Specifically, the process of DC maturation involves the upregulation of the costimulatory markers CD80 and CD86. These accessory molecules can then interact with counter receptors on T cells in order to sustain T cell activation and initiate T helper cell polarization. CD40 is another important costimulatory marker expressed on immature DC and highly upregulated on mature DC. Ligation of CD40 to CD40L on T cells further promotes the DC maturation process by upregulating the expression of CD80 and CD86 as well as initiating the secretion of cytokines from DC (Palucka, Banchereau 1999).

High levels of both pro and anti-inflammatory cytokines are typically secreted from DC exposed to a maturation stimulus. These cytokines strongly influence the type and duration of a T helper cell response. IL-12, for example, is essential in driving a Th1 response while the IL-12 related cytokine IL-23 is responsible for maintaining a Th17 response (Hunter 2005). Since these T cell responses have also been implicated in several debilitating autoimmune diseases (notably IBD, RA and MS) any means to control these subsets and therefore ameliorate disease has therapeutic potential. For this reason we first investigated the effects of nuclear receptor activation on DC maturation and function to assess if LXR, RXR or PPARγ could alter cytokine secretion or surface

marker expression on mature DC. In order to effectively do this, we used potent synthetic nuclear receptor agonists to activate the nuclear receptor in question. PPARγ is highly activated upon exposure to the Type II diabetes drug, Rosiglitazone and LXR can be activated synthetically with the use of a benzenesulfonamide compound known as T0901317. Specific RXR agonists are to date, unavailable commercially however we received two selective RXR agonists as a kind gift from Prof Hiroyuki Kagechika, Tokyo Medical and Dental School. PA024 and HX630 are two dibenzodiazepine compounds which have the unique ability to selectively activate specific heterodimer pairs. Specifically PA024 activates RXR when it is associated to LXR whereas HX630 can activate RXR when it is associated with LXR or PPARγ (Kagechika, Shudo 2005). The importance of these differential affects between the two compounds will be highlighted further in Chapter 4.

We also examined the expression of these nuclear receptors and specific pro-inflammatory cytokines in two mouse models of IBD. The dextran sulfate sodium (DSS) model of IBD is a chemically induced model in which mice develop acute and chronic colonic inflammation. *Citrobacter rodentium* is an infection induced model of IBD whereby this gram negative pathogen is administered to mice and subsequently results in acute colonic inflammation (Mundy et al. 2005, Melgar et al. 2007). It was our aim to investigate if these nuclear receptors and proinflammatory cytokines were altered during disease.

3.2 RESULTS

3.2.1 INCREASING CONCENTRATIONS OF THE LXRα AGONIST T0901317 DOES NOT ALTER CELL VIABILITY IN BMDC

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or T0901317 at varying concentrations (20μM, 10μM and 2μM) over 7 days. Viability was assessed using the Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. The concentrations of agonist used i.e. 20μM, 10μM and 2μM were not found to be cytotoxic to the cells and subsequently do not affect DC viability. **[Figure 3.1]**

3.2.2 INCREASING DOSES OF THE LXR AGONIST T0901317 MODULATES CYTOKINE PRODUCTION FROM BMDC

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or T0901317 at varying concentrations (20μM, 10μM and 2μM) over 7 days. DC were then stimulated with LPS (100ng/ml; E.Coli serotype R515) for 24hr, supernatants were removed and levels of the DC representative cytokines IL-10, IL-12p40 and TNFα **[Figure 3.2]** were assessed using ELISA. All concentrations of T0901317 used (20μM, 10μM and 2μM) significantly decreased the production of the pro-inflammatory cytokines IL-12p40 and TNFα ($p<0.001$) compared to DMSO vehicle control. In contrast the levels of the anti-inflammatory cytokine IL-10 were significantly increased in T0901317 treated cells (20μM, $p<0.001$, 10μM $p<0.05$ and 2μM $p< 0.01$) compared to DMSO control **[Figure 3.2].**

3.2.3 LONG TERM EXPOSURE TO T0901317 MODULATES CYTOKINE PRODUCTION IN DC MORE EFFICIENTLY THAN SHORT TERM EXPOSURE.

Data obtained from the MTS assay and T0901317 dose response experiments allowed us to determine the optimum dose of LXR agonist to use for future experiments. A 2μM dose was selected and used for all future LXR work. We next compared short term exposure versus long term exposure to T0901317. BMDC were harvested as previously described and treated with T0901317 2μM for 7 days (long term exposure) or 1hr prior to LPS stimulation (short term exposure). There was a significant decrease in IL-12p70, IL-12p40 and IL-23 cytokine production following long term exposure with T0901317 ($p<0.001$, $p<0.05$) **[Figure 3.3].** However there is no significant decrease in these cytokines following short term treatment with T0901317, however a significant increase in IL-23 production was observed in short term treated cells ($p<0.01$) **[Figure 3.4].**

Therefore for all subsequent experiments we used 2μM T0901317 over a long term (7 day) treatment period.

3.2.4 THE LXR AGONIST T0901317 MODULATES LPS - INDUCED CYTOKINE PRODUCTION IN BMDC

Following confirmation of the optimum dose and exposure time to T0901317, we next examined a wider range of cytokines. BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or T0901317 for 7 days. DC were then stimulated with LPS (100ng/ml; E.Coli serotype R515) for 24hr, supernatants were removed and levels of IL-12p40, IL-12p70, IL-27 and IL-23 **[Figure 3.5]**, TNFα, IL-6, IL-1β and IL-10 **[Figure 3.6]** were assessed using ELISA.

T0901317 had a significant affect on the IL-12 family of cytokines as seen by a significant decrease in IL-12p40 ($p<0.001$), IL-12p70 ($p<0.01$), IL-27 ($p<0.001$) and IL-23 ($p<0.01$) production **[Figure 3.5]**. This decrease was not seen with TNFα or IL-6 **[Figure 3.6],** however a significant increase ($p<0.001$) in IL-1β was observed in T0901317 treated cells compared to DMSO vehicle control **[Figure 3.6].**

3.2.5 THE LXRα AGONIST T0901317 MODULATES CELL SURFACE MARKER EXPRESSION IN LPS MATURED BMDC.

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or T0901317 for 7 days. Cells were then stimulated with 100ng/ml LPS for 24hr before being washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80, CD86, and TLR4). Cells were then gated on the CD11c positive population.

LPS induced the upregulation of the surface markers CD86, CD80, CD40 and TLR4 **[Figure 3.7]** compared to unstimulated cells. The upregulation of these markers is typical following DC exposure to LPS. Mean Fluorescent Intensity values for each group are represented in the associated group colour and are displayed on the top corners of each histogram.

Following treatment of DC with T0901317, the increase in expression of CD40 by LPS was significantly decreased **[Figure 3.8].** While T0901317 treatment had the most potent effect on CD40 expression, it also decreased the expression of the costimulatory markers CD86, CD80 and TLR4 following LPS stimulation **[Figure 3.8].**

3.2.6 INCREASING CONCENTRATIONS OF THE PPARγ AGONIST ROSIGLITAZONE (RSG) AND PPARγ ANTAGONIST GW9662 DO NOT ALTER CELL VIABILITY IN BMDC

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control), the PPARγ agonist RSG (10μM) or the PPARγ antagonist GW9662 (2.5μM) for 7 days. Viability was then assessed using the Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. The concentration of agonist used i.e. 10μM and concentration of antagonist used (2.5μM) were not found to be cytotoxic to the cells and consequently do not affect DC viability. **[Figure 3.9]**

3.2.7 INCREASING DOSES OF THE PPARγ AGONIST RSG MODULATES CYTOKINE PRODUCTION FROM BMDC

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or varying concentrations of RSG (10μM, 1μM and 100nM) over 7 days. DC were then stimulated with LPS (100ng/ml) for 24hrs, supernatants were removed and levels of the DC representative cytokines IL-10, IL-12p40 and TNFα **[Figure 3.10]** were assessed using ELISA. There was a significant decrease in IL-12p40 production in RSG treated cells across all concentrations of agonist used ($p<0.001$). PPARγ activation also significantly decreases TNFα production following exposure to 10μM RSG ($p<0.001$), 1μM RSG and 100nM RSG ($p<0.01$) **[Figure 3.10]**. RSG treatment also significantly decreases the production of the anti-inflammatory cytokine IL-10 **[Figure 3.10].**

3.2.8 THE PPARγ AGONIST RSG MODULATES LPS- INDUCED CYTOKINE PRODUCTION IN BMDC

The results of the previous experiment highlighted the potent effect a 10μM dose of RSG can have on DC cytokine production. Therefore for all subsequent PPARγ related experiments we used this dose. We next examined its effects on a wider range of cytokines.

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or RSG (10μM) over 7 days. DC were then stimulated with LPS (100ng/ml) for 24hrs, supernatants were removed and levels of IL-12p40, IL-12p70, IL-23 and IL-27 **[Figure 3.11]** TNFα, IL-6, IL-1β and IL-10 **[Figure 3.12]** were assessed using ELISA.

Treatment of BMDC with RSG decreased the production of all the IL-12 family cytokines i.e. IL-12p40, IL-12p70, IL-23 and IL-27 ($p<0.001$) [**Figure 3.11]** in response to LPS. A significant decrease in the anti –inflammatory cytokine IL-10 ($p<0.001$) as well other proinflammatory cytokines TNFα and IL-6 ($p<0.01$) was also seen in RSG treated cells [**Figure 3.12].**

3.2.9 THE PPARγ AGONIST RSG MODULATES CELL SURFACE MARKER EXPRESSION IN LPS MATURED BMDC.

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or RSG (10μM) over 7 days. Cells were then stimulated with 100ng/ml LPS for 24hr before being washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80, CD86, and TLR4). Cells were then gated on the CD11c positive population.

As expected LPS upregulated the expression of CD86, CD80, CD40 and TLR4 **[Figure 3.13].** Treatment of cells with RSG resulted in suppression of CD80, CD86 and CD40 following LPS stimulation as seen by a significant downregulation in their expression compared to DMSO control cells **[Figure 3.14].** RSG treatment also decreased TLR4 expression but not to the same extent as CD80, CD86 and CD40 **[Figure 3.14].**

3.2.10 INCREASING CONCENTRATIONS OF THE RXR AGONIST HX630 AND LOW CONCENTRATIONS OF THE SPECIFIC RXR AGONIST PA024 DO NOT ALTER CELL VIABILITY IN BMDC

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured in the presence of the RXR agonist PA024 or HX630 over 7 days. Viability was then assessed using the Cell Titer 96 Aqueous One Solution (Promega, WI, USA) according to the manufacturer's instructions. 10μM PA024 decreased cell viability by 40% however lower concentrations i.e. 1μM and 100nM were not cytotoxic to the cells and subsequently did not affect DC viability. All doses of HX630 tested (10μM, 1μM and 100nM) also did not affect DC viability **[Figure 3.15]**

3.2.11 INCREASING DOSES OF THE RXR AGONISTS PA024 AND HX630 MODULATE CYTOKINE PRODUCTION FROM BMDC

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or varying concentrations of PA024 or HX630 (10μM, 1μM and 100nM) over 7 days. DC were then stimulated with LPS for 24hr and supernatants were analysed for the DC representative cytokines IL-10, IL-12p40 and TNFα **[Figure 3.16 & Figure 3.17]** using ELISA. Both RXR agonists had similar effects on cytokine production from DC with

both compounds significantly decreasing IL-12p40 production (p<0.001). All three doses of PA024 tested significantly increased the production of the anti-inflammatory cytokine IL-10 in a dose dependent manner **[Figure 3.16].** 1μM of HX630 significantly increased the production of IL-10 (p<0.01) however the higher (10μM) and lower (100nM) dose do not affect IL-10 production in DC **[Figure 3.17]**. RXR activation in these cells does not significantly alter the production of TNFα **[Figure 3.16 & Figure 3.17].**

3.2.12 LONG TERM AND SHORT TERM EXPOSURE TO RXR AGONISTS MODULATES CYTOKINE PRODUCTION IN DC

Data obtained from the MTS assay and dose response experiments allowed us to determine the optimum dose of RXR agonist to use for future experiments. A 1μM dose was selected and used for all future RXR work. BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control), PA024 or HX630 for 7 days (long term exposure) or treated for 1hr (short term exposure) prior to LPS. Supernatants were subsequently analysed for IL-12p70, IL-12p40, IL-23 and TNFα. Long term exposure to both HX630 and PA024 resulted in a significant decrease in both IL-12p40 and IL-12p70 production (p<0.001) **[Figure 3.18]** Short term exposure to these RXR agonists also resulted in a significant decrease in IL-12p40 (p<0.001) and IL-12p70 production (HX630 p<0.01; PA024 p<0.001) **[Figure 3.19].** Long term but not short term exposure to PA024 and HX630 also resulted in a significant increase in IL-23 (p<0.001 and p<0.01 respectively) **[Figure 3.18]**. PA024 also caused a significant increase in the proinflammatory cytokine TNFα when treated over a short term period (p<0.001) **[Figure 3.19]**. For these reasons we chose to use these RXR agonists over a long term

treatment period as this resulted in a decrease in IL-12p40 and IL-12p70 as well as having no effect on TNFα.

3.2.13 THE RXR AGONIST PA024 MODULATES LPS-INDUCED CYTOKINE PRODUCTION IN BMDC

Following optimisation of the RXR dose and time of exposure, we then examined its effects on a wide range of cytokines. BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or PA024 (1μM) for 7 days. DC were then stimulated with LPS (100ng/ml) for 24hr, supernatants were removed and levels of IL-12p40, IL-12p70, IL-27 and IL-23 **[Figure 3.20]**, and TNFα, IL-10, IL-6 and IL-1β **[Figure 3.21]** were assessed using ELISA. RXR activation in these cells significantly decreases the production of IL-12p40 ($p<0.001$), IL-12p70 ($p<0.01$) and IL-27 ($p<0.001$) in response to LPS while also increasing the production of IL-23 ($p<0.05$) **[Figure 3.20]**. PA024 treatment also resulted in a significant increase in the anti-inflammatory cytokine IL-10 ($p<0.01$) whilst having no affect on other DC related cytokines such as IL-1β, TNFα and IL-6 **[Figure 3.21]**.

3.2.14 THE RXR AGONIST PA024 MODULATES CELL SURFACE MARKER EXPRESSION IN LPS MATURED BMDC.

BMDC were harvested from Balb/c mice, differentiated in the presence of GMCSF (40ng/ml) and concurrently cultured with DMSO (vehicle control) or PA024 (1μM) for 7 days. Cells were then stimulated with 100ng/ml LPS for 24hours before being washed and stained with fluorochrome labelled monoclonal antibodies for CD11c, CD40, CD80, CD86, and MHCII. Cells were then gated on the CD11c positive population.

As expected LPS upregulated the expression of CD86, CD80, CD40 and MHCII **[Figure 3.22].** PA024 had the most potent effect on MHCII as seen by a significant downregulation in its expression following LPS stimulation compared to DMSO control. PA024 treatment also significantly decreased CD86, CD80 and CD40 expression **[Figure 3.23]**.

3.2.15 THE EXPRESSION OF THE NUCLEAR RECEPTORS, LXR AND RXR IN THE COLON ARE SIGNIFICANTLY DECREASED IN DSS INDUCED COLITIS.

Total RNA was isolated from colonic tissue of mice at various stages after induction of colitis with DSS (early acute, late acute and chronic disease) along with controls. 1µg of RNA was converted to cDNA and used to examine the expression of LXRα, RXRα and PPARγ. The relative levels of these transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control tissue was adjusted to 1 and served as the basal reference value throughout experiments. Results are representative of fold change within the sample.

The expression of LXRα is significantly increased during the initial onset of disease i.e. the early acute stage ($p<0.001$), however its expression is then at normal levels by the late acute phase and then significantly decreased compared to healthy controls in mice that have chronic colitis ($p<0.01$) **[Figure 3.24].** There is no increase in RXRα, however its expression is also decreased in mice with chronic colitis ($p<0.01$) and to a lesser extent in mice that are in the late acute stage of disease ($p<0.001$). The expression of the nuclear receptor PPARγ does not change in the late phase or chronic stages of

disease however there is a significant increase in the expression of this receptor in the early acute stage of colitis (p<0.001) **[Figure 3.24].**

3.2.16 THE EXPRESSION OF THE IL-12, IL-23 AND IL-27 CYTOKINE SUBUNITS ARE UPREGULATED IN DSS INDUCED COLITIS

Total RNA from healthy and diseased mice was converted to cDNA as previously described and used to examine the expression of all the subunits of the IL-12 family; IL-12p35, IL-12p40, IL-27p28, EBI3 and IL-23p19 by RT-PCR. The relative levels of these transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control tissue was adjusted to 1 and served as the basal reference value throughout experiments. Results are representative of fold change within the sample.

The expression of IL-12p35 is significantly increased in the early acute (p<0.001), late acute (p<0.05) and chronic stages (p<0.05) of DSS induced colitis **[Figure 3.25]**. The expression of IL-12p40 was significantly increased only in the early acute stage of disease (p<0.001) whereas IL-23p19 expression was not significantly increased until the chronic stage of colitis (p<0.001) **[Figure 3.25].** The expression of the IL-27 cytokine subunits i.e. IL-27p28 and EBI3 were both significantly increased only in the early acute stage of DSS induced colitis (p<0.001) **[Figure 3.26]**.

3.2.17 THE EXPRESSION OF LXR IS SIGNIFICANTLY INCREASED FOLLOWING *Citrobacter rodentium* INFECTION.

We also examined the expression of the nuclear receptors in another model of intestinal inflammation, which is induced with *Citrobacter rodentium* infection. Total RNA from

healthy controls and mice infected with *Citrobacter rodentium* over a period of 28 days was converted to cDNA as previously described. The expression of LXRα, RXRα and PPARγ was then assessed using RT-PCR. The relative levels of these transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control tissue was adjusted to 1 and served as the basal reference value throughout experiments. Results are representative of fold change within the sample.

The expression of RXR and PPARγ does not significantly change over the course of infection however LXRα expression is significantly increased after 21 days exposure to the *Citrobacter rodentium* bacteria ($p<0.01$) **[Figure 3.27]**.

3.2.18 THE EXPRESSION OF THE IL-12 FAMILY OF CYTOKINES ARE SIGNIFICANTLY INCREASED DURING Citrobacter *rodentium* INFECTION.

Total RNA from healthy controls and mice infected with *Citrobacter rodentium* over a period of 28 days was converted to cDNA as previously described. The expression of IL-12p35, IL-12p40, IL-23p19, IL-27p28 and EBI3 was then assessed using RT-PCR. The relative levels of these transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control tissue was adjusted to 1 and served as the basal reference value throughout experiments. Results are representative of fold change within the sample.

The expression of IL-12p35 was significantly increased after 14 and 21 days infection ($p<0.05$). However by day 28, the expression of this cytokine subunit has returned to normal **[Figure 3.28]**. IL-12p40 expression increases early in infection with a significant increase by day 14 ($p<0.05$) whereas the expression of IL-23p19 was not significantly increased until towards the end of the infection period at day 28 ($p<0.01$)

[Figure 3.28]. The IL-27 subunit p28 is significantly increased after 9, 14 and 21 days of infection ($p<0.05$) whereas EBI3, the remaining IL-27 subunit is only increased after 9 days of infection ($p<0.05$) **[Figure 3.29]**.

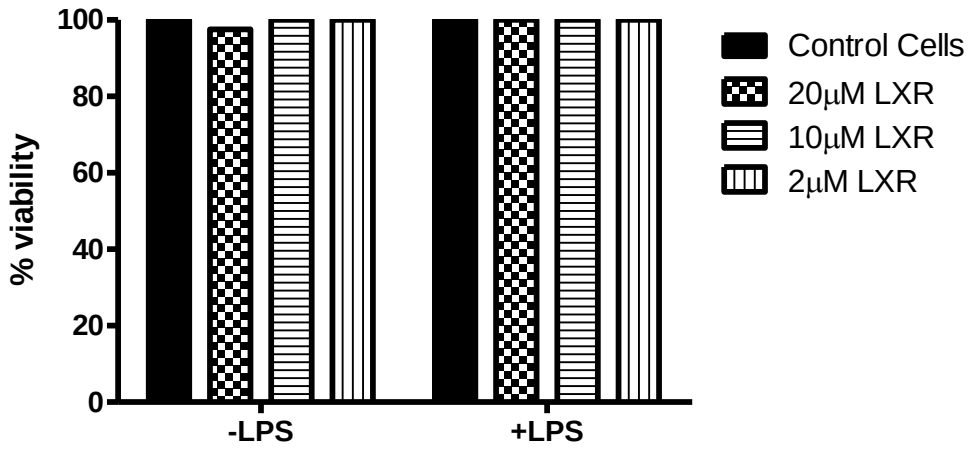


FIGURE: 3.1

Increasing concentrations of a specific LXR agonist (T0901317) does not alter the viability of bone marrow derived dendritic cells (BMDC)

BMDC where differentiated in GMCSF for 7 days in the presence of varying concentrations of T0901317 (20μM, 10μM or 2μM) or DMSO (vehicle control). After this time cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution). Results are expressed as a percentage of untreated cells.

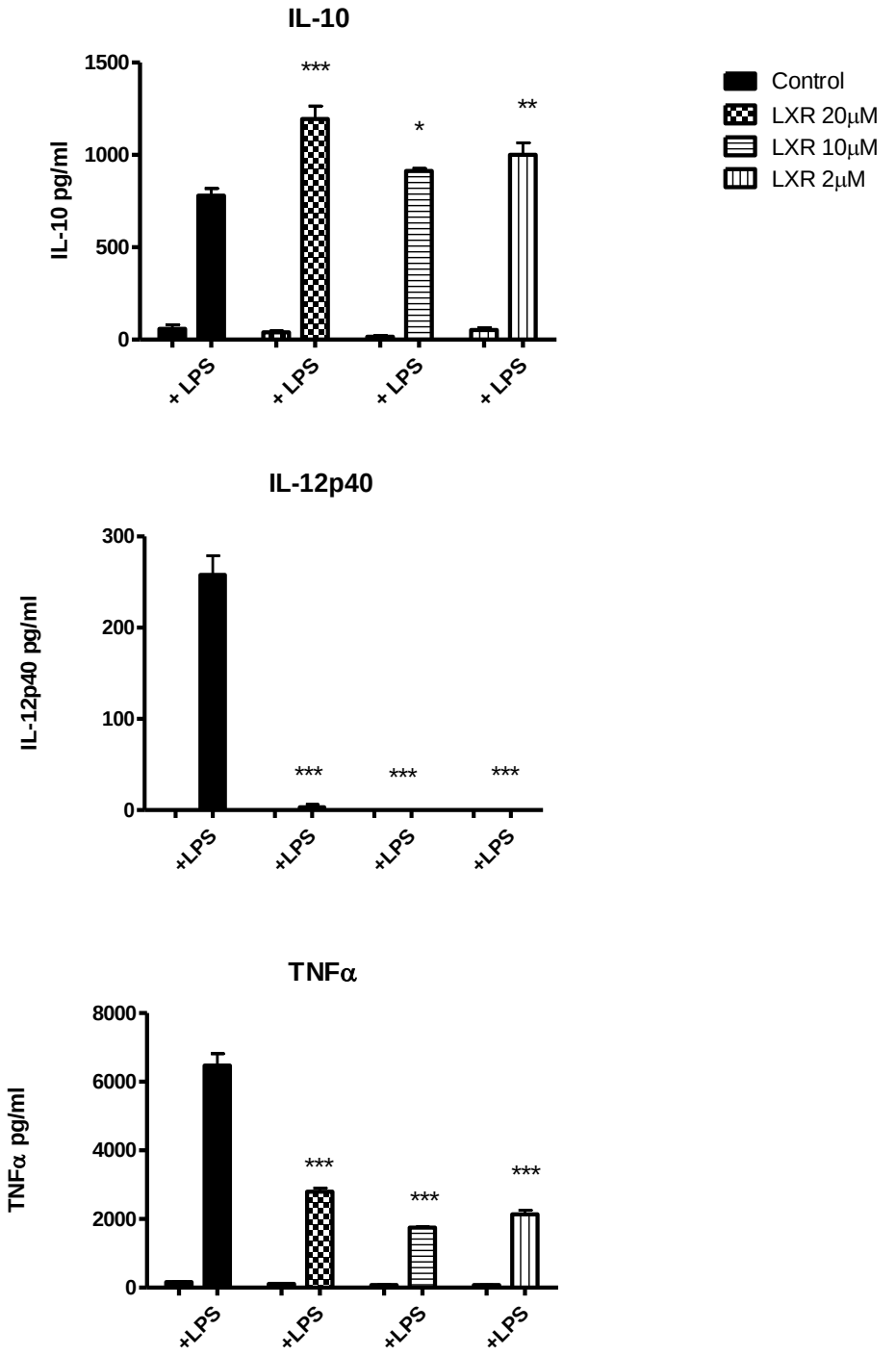


FIGURE: 3.2

Activation of LXR with T0901317 modulates the production of cytokine from BMDC:
BMDC were differentiated in GMCSF for 7 days in the presence of a specific LXR agonist (T0901317) at increasing concentrations (2μM, 10μM and 20μM). After 7 days cells were stimulated for 24hr with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-10, IL-12p40 and TNFα using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

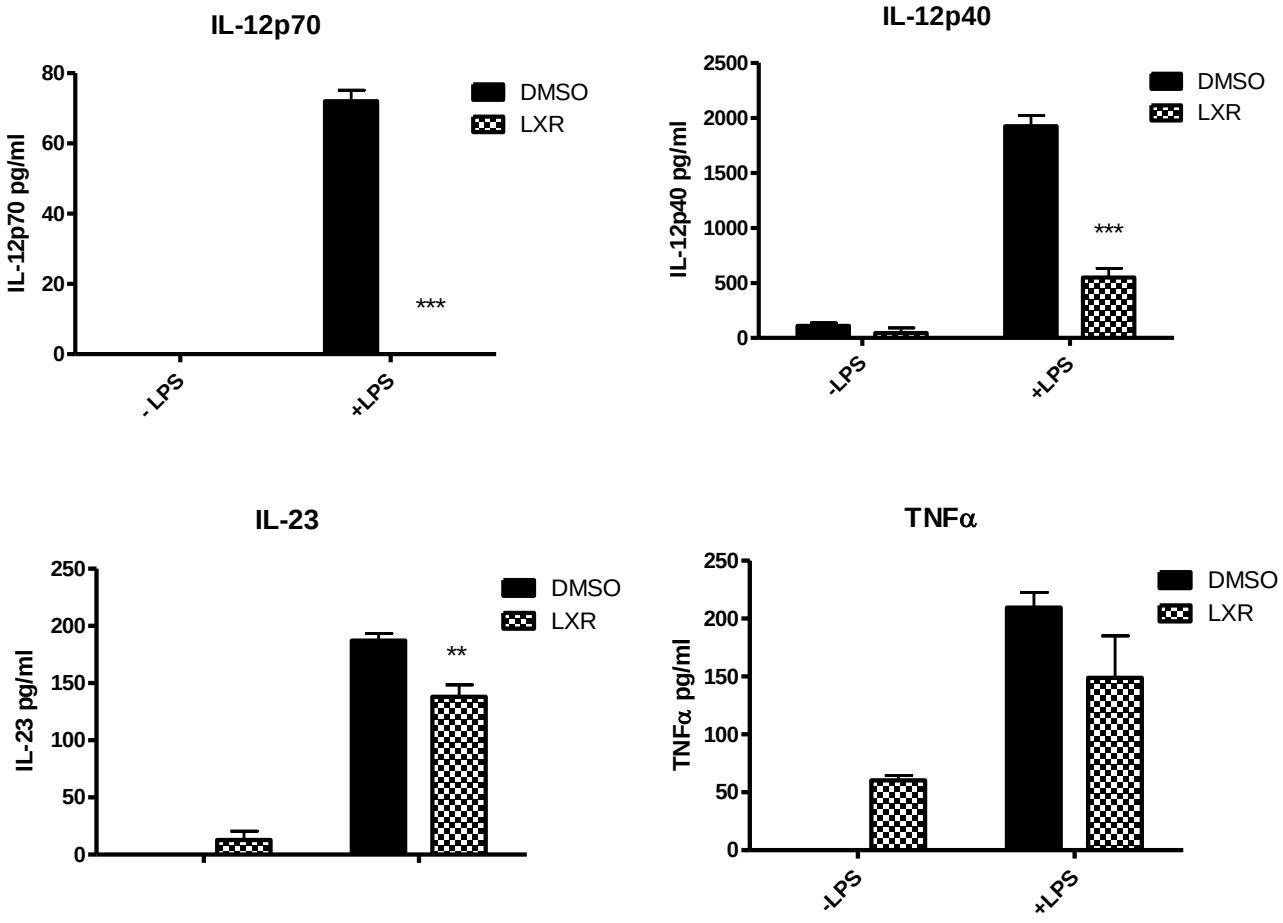


FIGURE 3.3

Long term exposure of DC to the LXR agonist T0901317 is needed to suppress the IL-12 family of cytokines:

BMDC were differentiated in GMCSF in the presence of 2μM T0901317 for 7 days and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

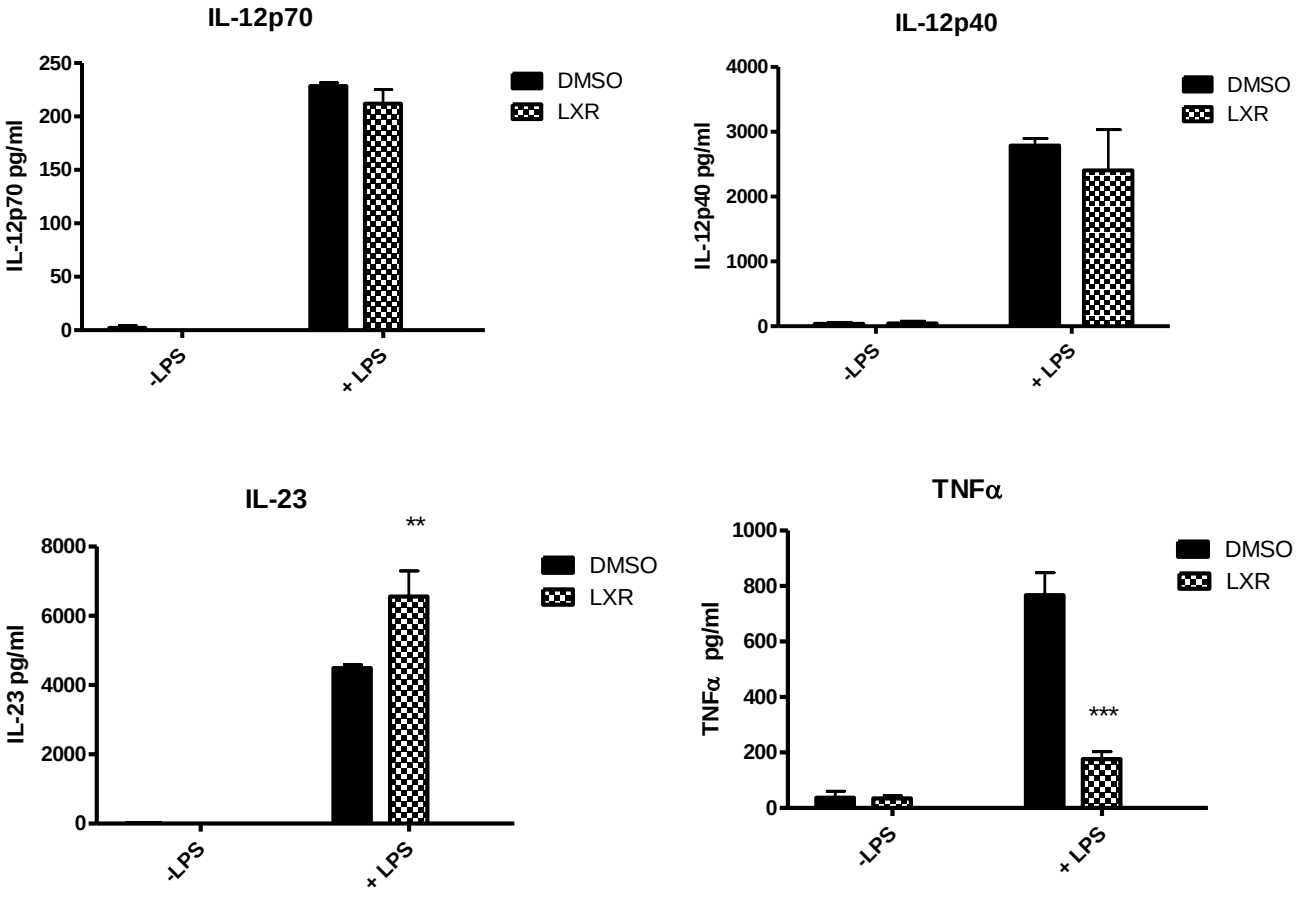


FIGURE 3.4

Short term exposure of DC to the LXR agonist T0901317 does not suppress the IL-12 family of cytokines: BMDC were differentiated in GMCSF for 7 days and stimulated for 24hrs with 100ng/ml LPS. 1hr prior to LPS stimulation 2μM T0901317 was added to cells. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

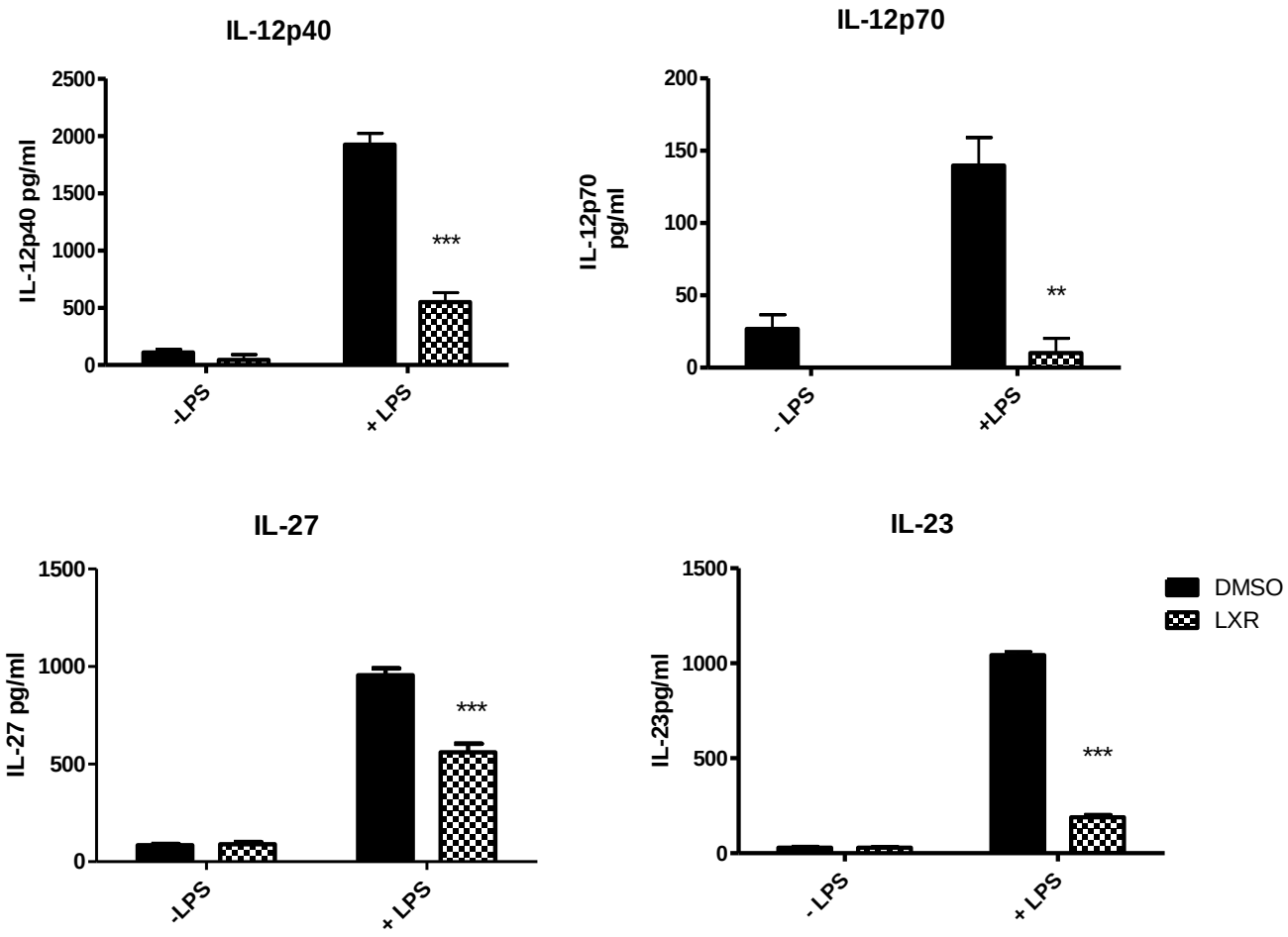


FIGURE 3.5

Activation of LXR with T0901317 suppresses the production of the IL-12 family of cytokines: BMDC were differentiated in GMCSF in the presence of 2μM T0901317 for 7 days and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and IL-27 using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

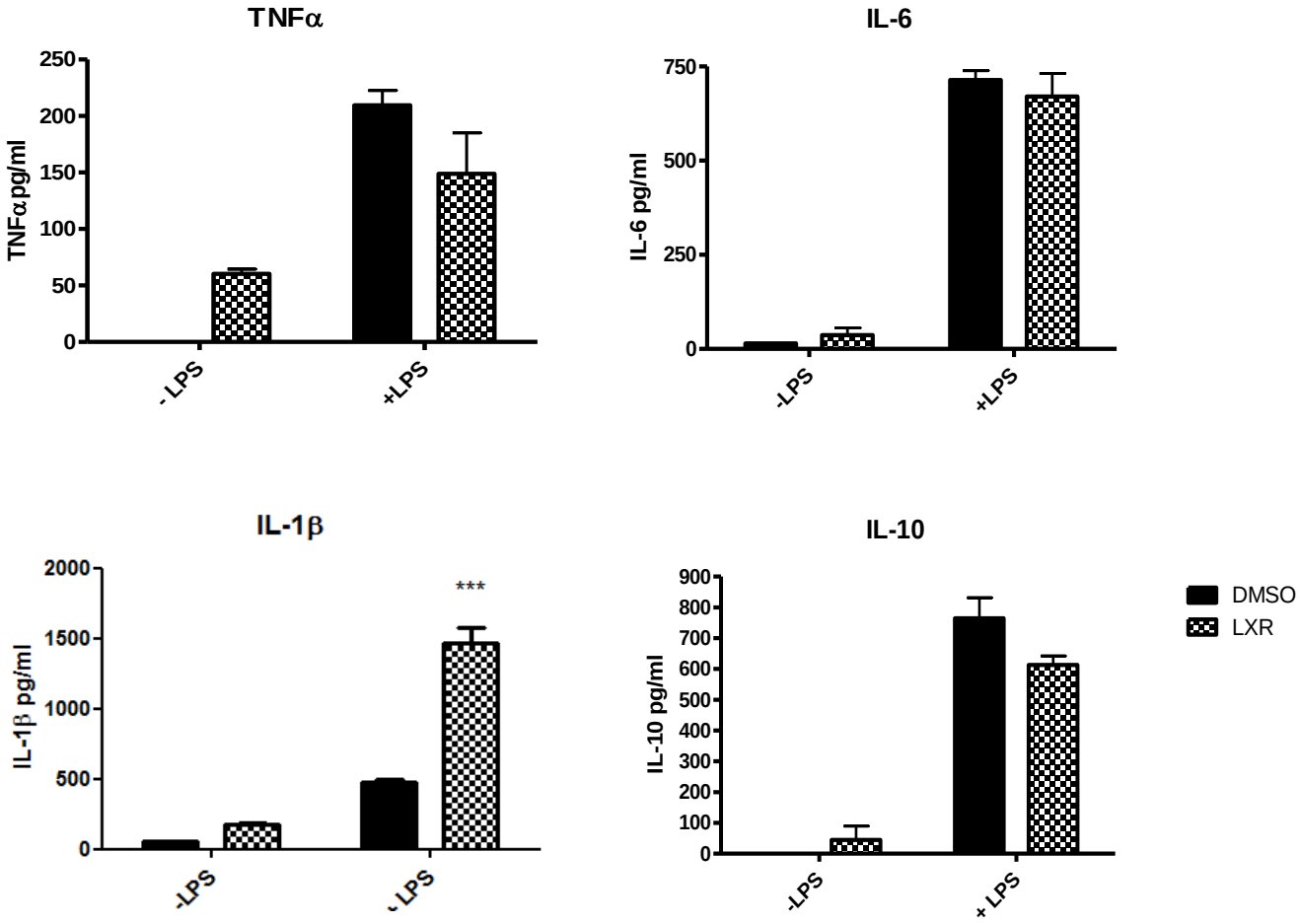


FIGURE 3.6

Activation of LXR with T0901317 increased the production of IL-1β. BMDC were differentiated in GMCSF in the presence of 2μM T0901317 for 7 days and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of TNFα, IL-6, IL-1β and IL-10 using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

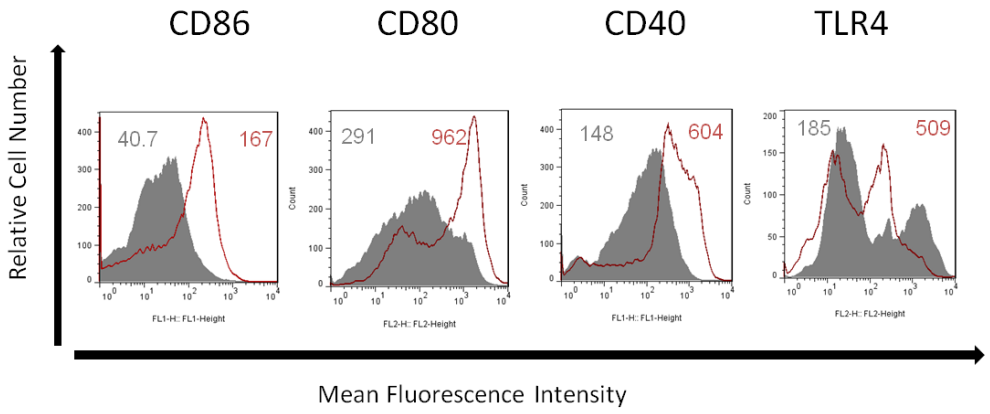


FIGURE 3.7

LPS stimulation enhances the expression of the surface markers CD86, CD80, CD40 and TLR4: BMDC were differentiated over 7 days in the presence of GMCSF and DMSO and subsequently stimulated for 24hr with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80, CD40 and TLR4. Cells were gated on the CD11c positive population. Grey shaded peaks represent DMSO control cells and the red overlaid line represents LPS stimulated DMSO cells.

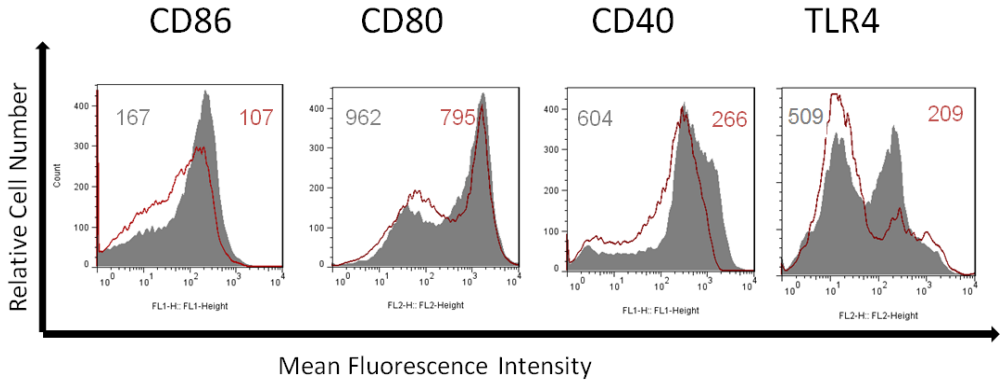


FIGURE 3.8

Activation of LXR decreases surface marker expression in LPS matured BMDC: BMDC were differentiated over 7 days in GMCSF in the presence of DMSO or LXR (T0901317 2µM), and subsequently stimulated for 24hr with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80, CD40 and TLR4. Cells were gated on the CD11c positive population. Grey shaded peaks represent LPS treated cells and the red overlaid line represents T0901317 treated LPS stimulated cells.

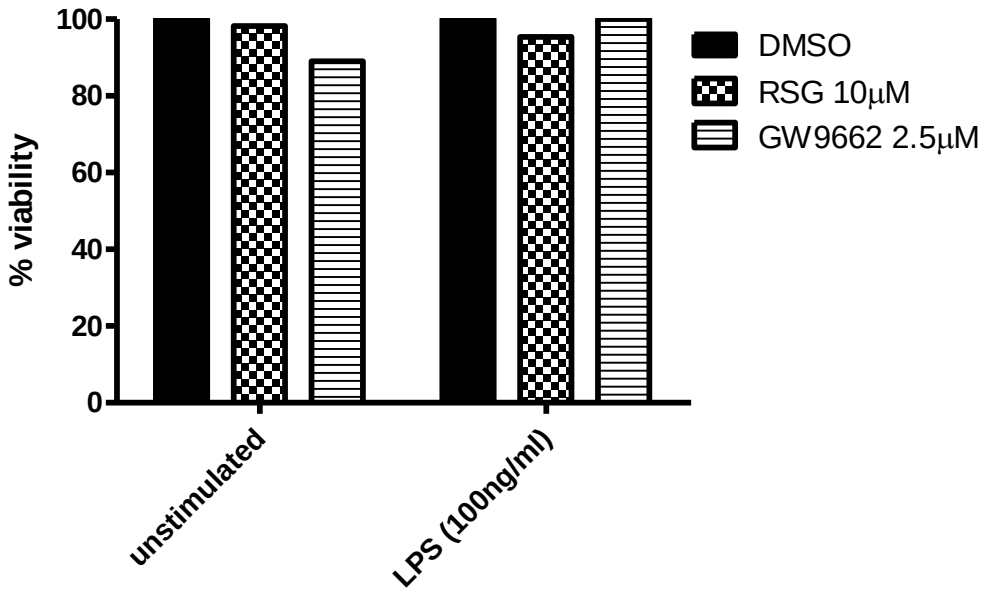


FIGURE: 3.9

The PPARγ specific agonist Rosiglitazone (RSG) and specific antagonist GW9662 do not alter the viability of (BMDC)

BMDC where differentiated for 7 days in GMSCF in the presence of RSG 10μM, GW9662 2.5μM or DMSO (vehicle control). After this time cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution). Results are expressed as a percentage of untreated cells.

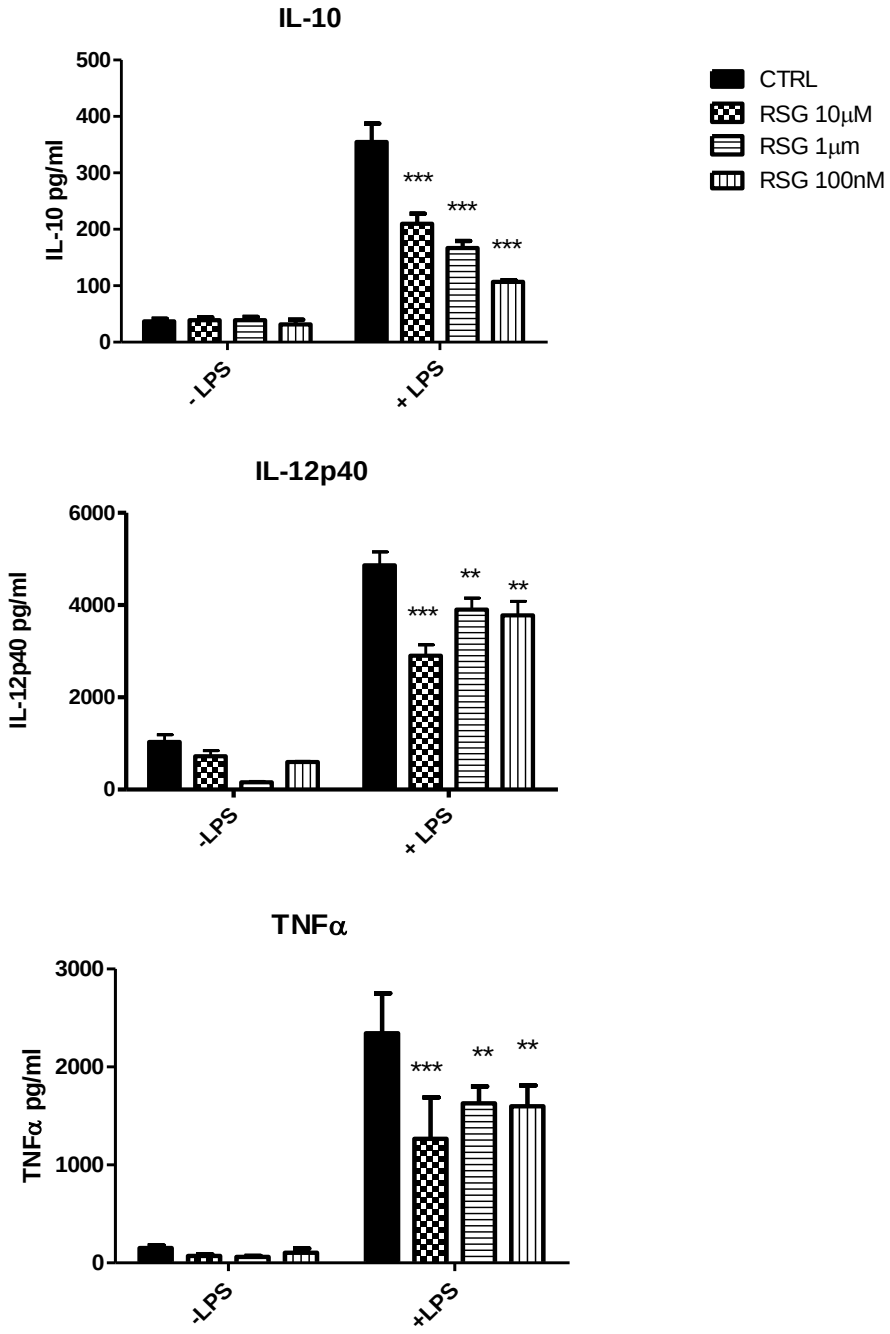


FIGURE 3.10

Activation of PPARγ modulates cytokine production by BMDC: BMDC were differentiated GMCSF for 7 days in the presence of increasing concentrations of RSG (10μM, 1μM and 100nM and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. RSG/LPS groups as determined by one-way ANOVA test.

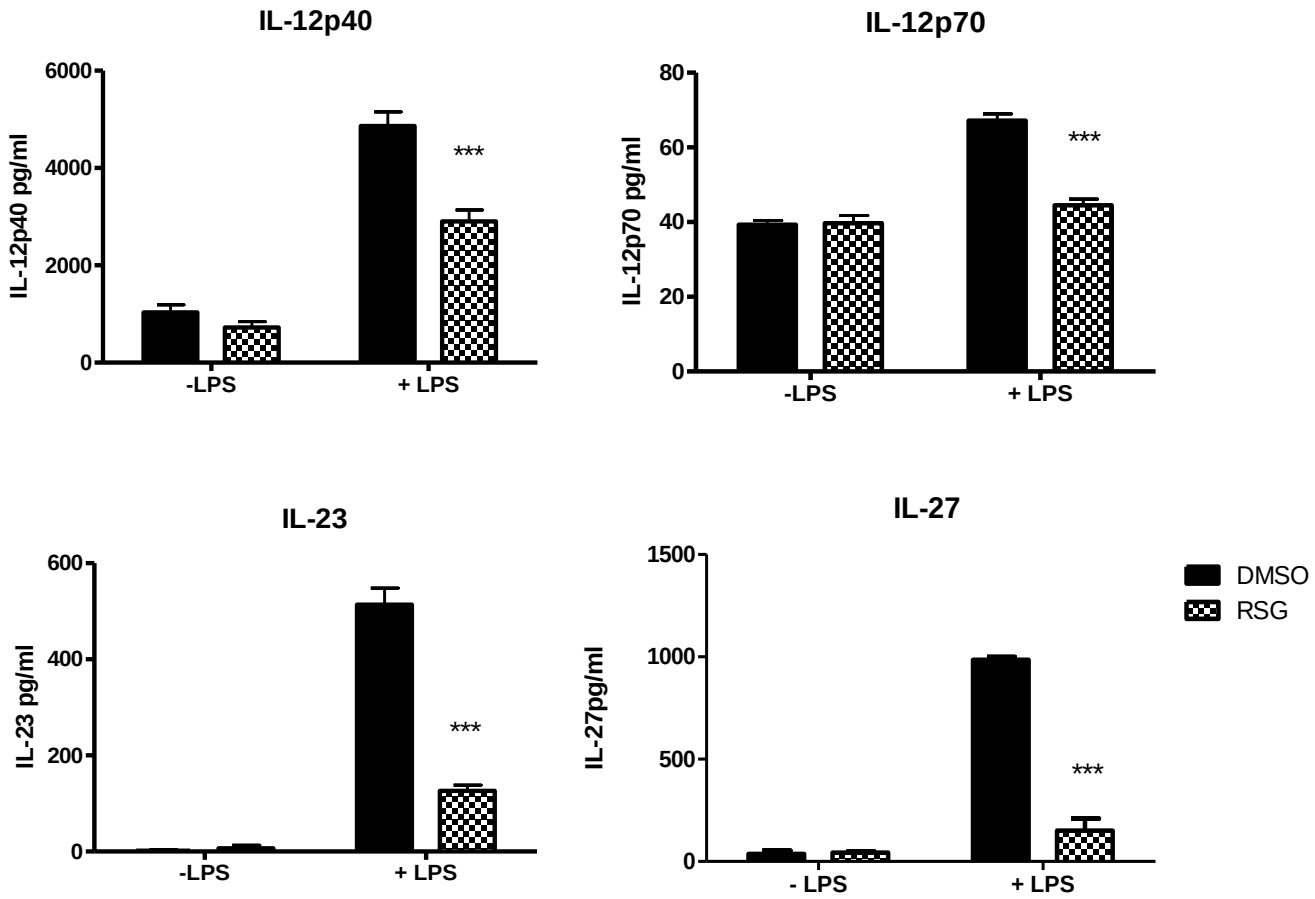


FIGURE 3.11

Activation of PPARγ suppresses the production of the IL-12 family of cytokines by BMDC: BMDC were differentiated in GMCSF in the presence of 10μM RSG for 7 days and stimulated for 24hr with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and IL-27 using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001 comparing DMSO/LPS vs. RSG/LPS groups as determined by one-way ANOVA test.

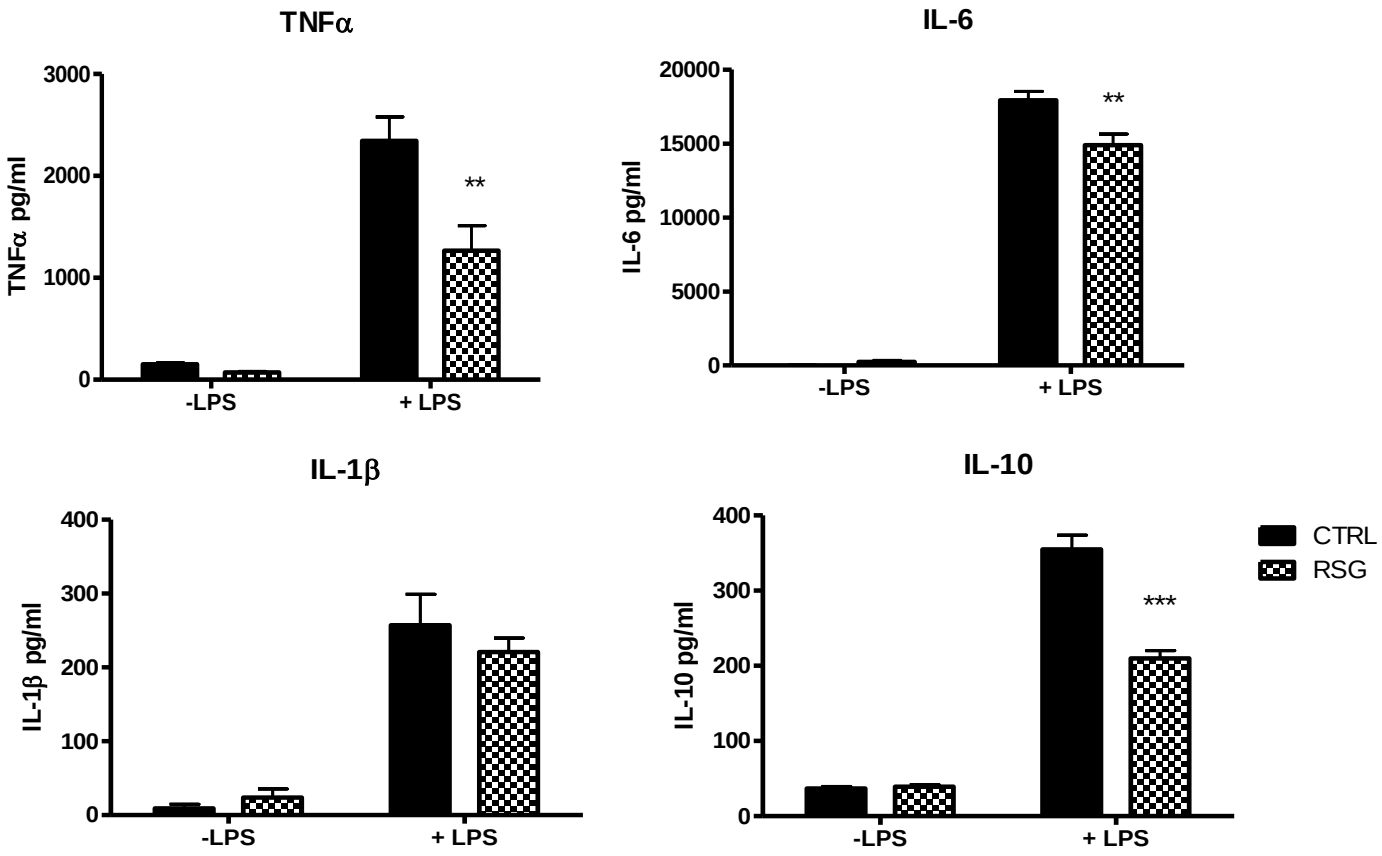


FIGURE 3.12

Activation of PPARγ decreases the production of TNFα, IL-6 and IL-10 from BMDC:
BMDC were differentiated in GMCSF in the presence of 10μM RSG for 7 days and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of TNFα, IL-6, IL-1β and IL-10 using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. RSG/LPS groups as determined by one-way ANOVA test.

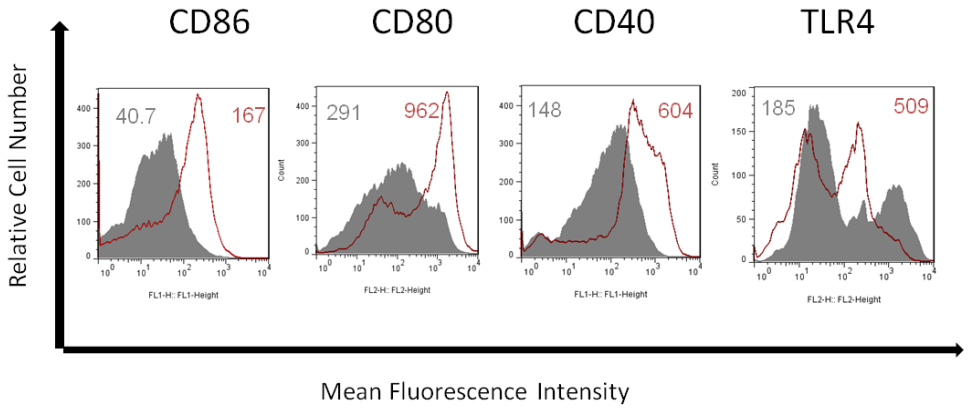


FIGURE: 3.13

LPS stimulation enhances the expression of the surface markers CD86, CD80, CD40 and TLR4: BMDC were differentiated over 7 days in GMCSF in the presence of DMSO and subsequently stimulated for 24hr with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80, CD40 and TLR4. Cells were then gated on the CD11c positive population. Grey shaded peaks represent DMSO control cells and the red overlaid line represents LPS stimulated DMSO treated cells.

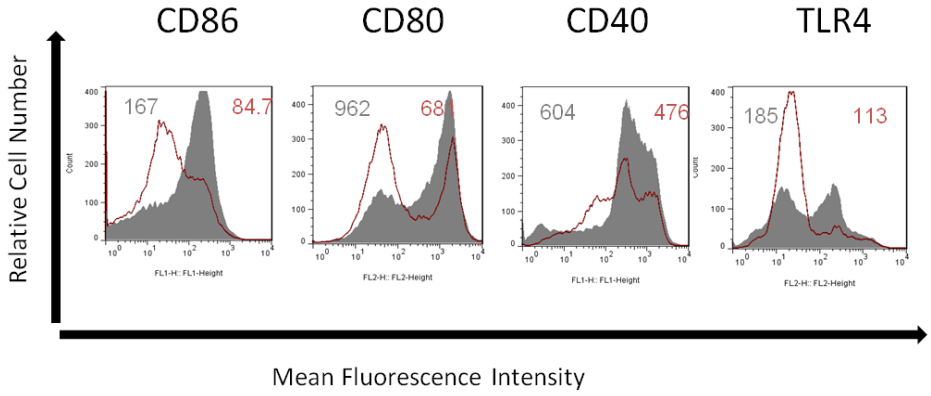


FIGURE 3.14

Activation of PPARγ decreases surface marker expression in LPS matured BMDC: BMDC were differentiated over 7 days in the presence of DMSO or RSG 10µM and subsequently stimulated for 24hr with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80, CD40 and TLR4. Grey shaded peaks represents LPS stimulated cells and the red overlaid line represents LPS stimulated RSG treated cells.

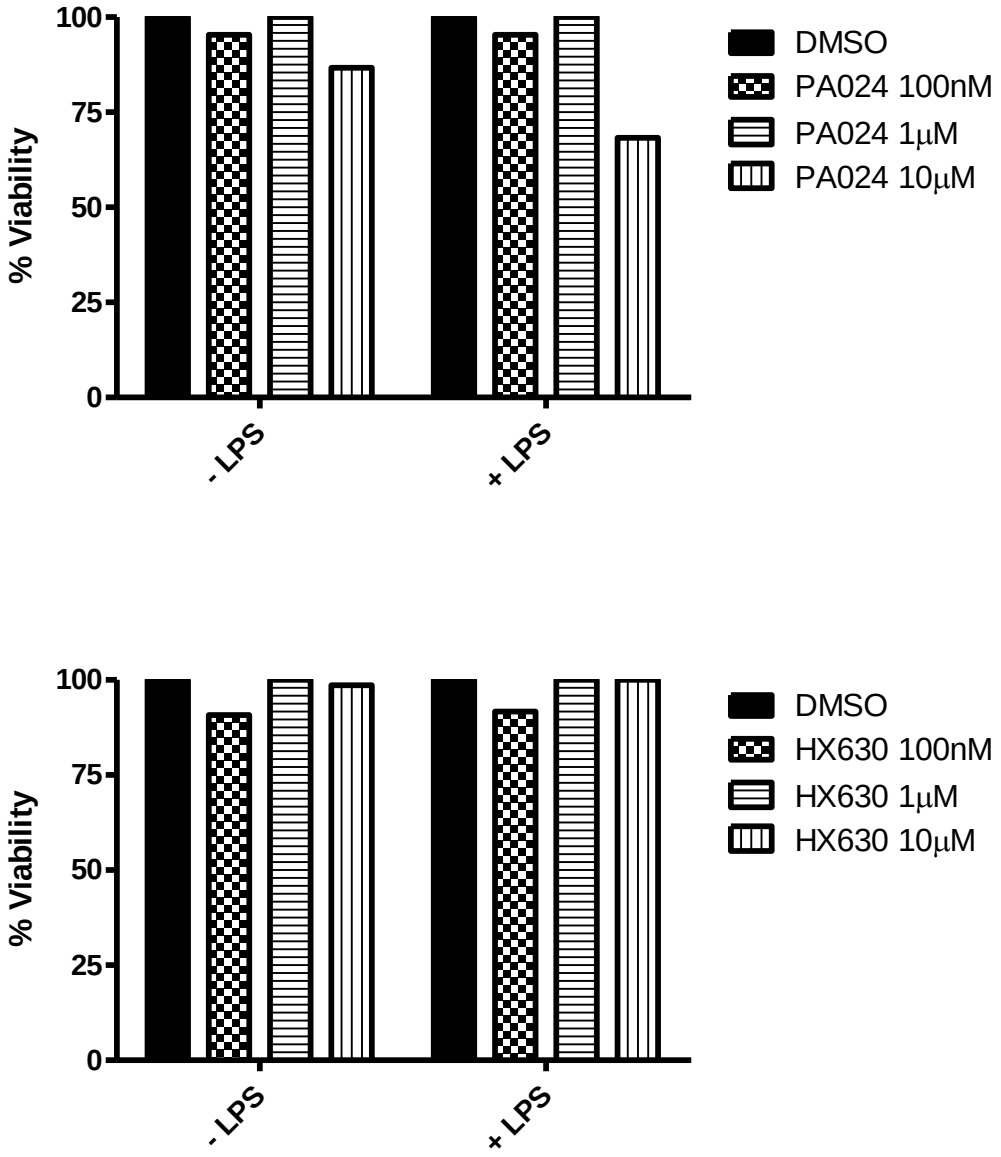


FIGURE: 3.15

Effect of the RXR agonists PA024 and HX630 on the viability of BMDC: BMDC where differentiated for 7 days in GMCSF in the presence of varying concentrations of PA024 and HX630 (10μM, 1μM or 100nM) or DMSO (vehicle control). After this time cellular viability was assessed using an MTS assay (Cell Titer 96 Aqueous One Solution). Results are expressed as a percentage of untreated cells.

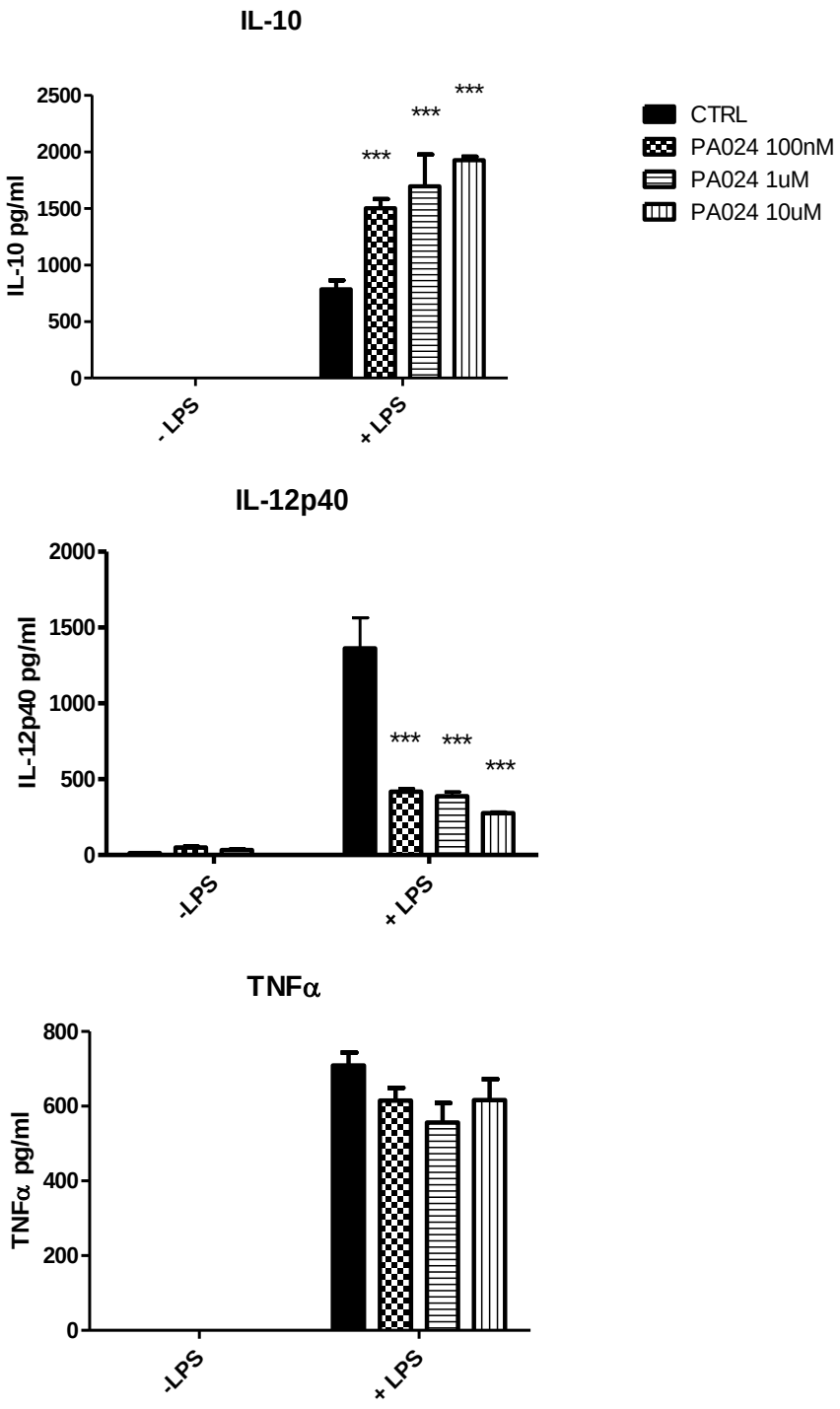


FIGURE 3.16

Activation of RXR with PA024 dose dependently inhibits the IL-12 family of cytokines: BMDC were differentiated in GMCSF for 7 days in the presence of increasing concentrations of PA024 (10µM, 1µM and 100nM) and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-10, IL-12p40 or TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001 comparing DMSO/LPS vs. PA024/LPS groups as determined by one-way ANOVA test.

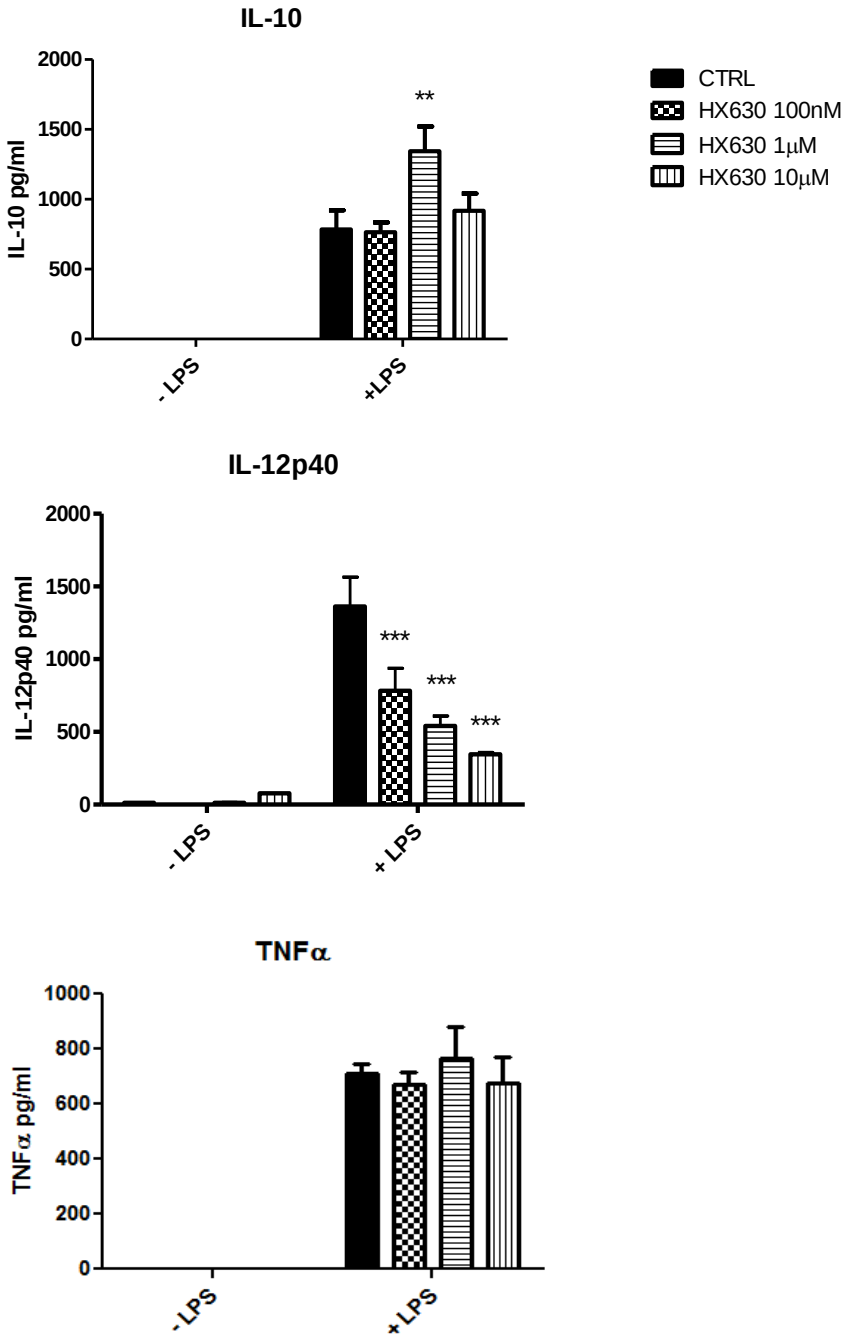


FIGURE 3.17

Activation of RXR with HX630 dose dependently modulates IL-10 production from BMDC:

BMDC were differentiated in GMCSF for 7 days in the presence of increasing concentrations of HX630 (10μM, 1μM and 100nM) and stimulated for 24hr with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-10, IL-12p40 and TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. HX630/LPS groups as determined by one-way ANOVA test.

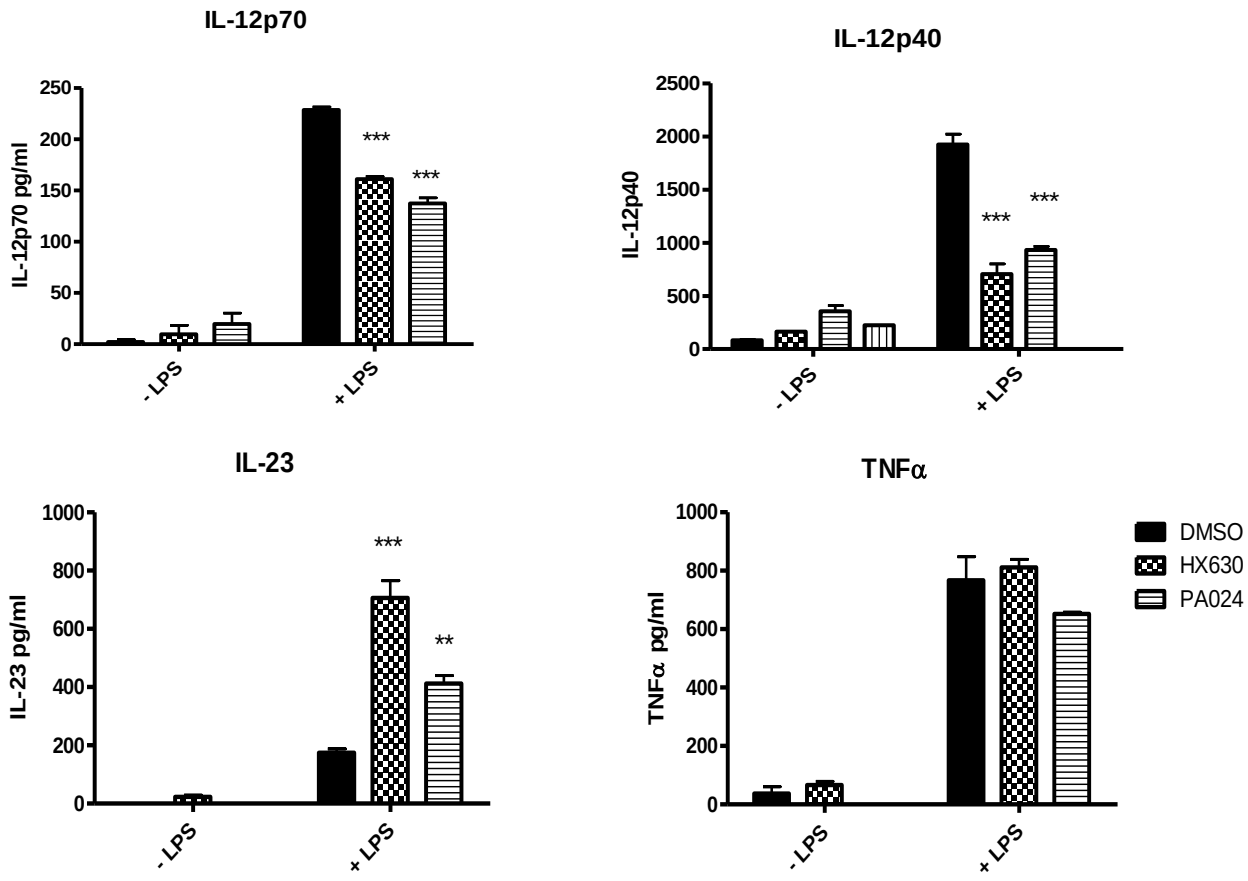


FIGURE 3.18

Long term exposure to RXR agonists modulates the production of the IL-12 family of cytokines by BMDC:

BMDC were differentiated in GMCSF in the presence of 1μM PA024 or HX630 for 7 days and stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. PA024/LPS groups or HX630/LPS groups as determined by one-way ANOVA test.

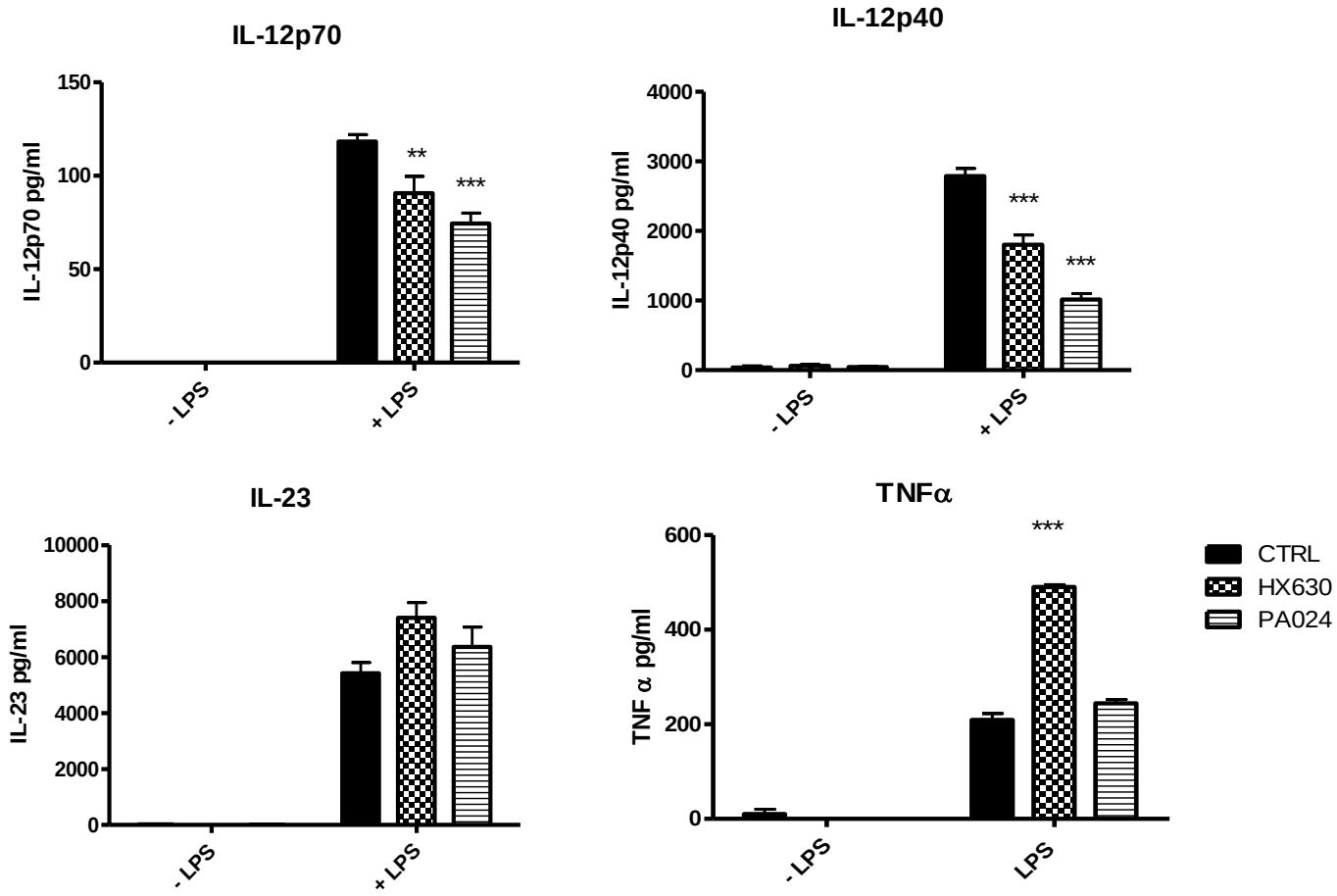


FIGURE 3.19

Short term exposure to RXR agonists is less potent in modulating the production of the IL-12 family of cytokines by BMDC:

BMDC were differentiated in GMCSF for 7 days and then exposed to 1μM PA024 or HX630 1hr prior to LPS (100ng/ml) stimulation. After 24hr LPS stimulation, supernatants were harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and TNFα using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. PA024/LPS groups or HX630/LPS groups as determined by one-way ANOVA test.

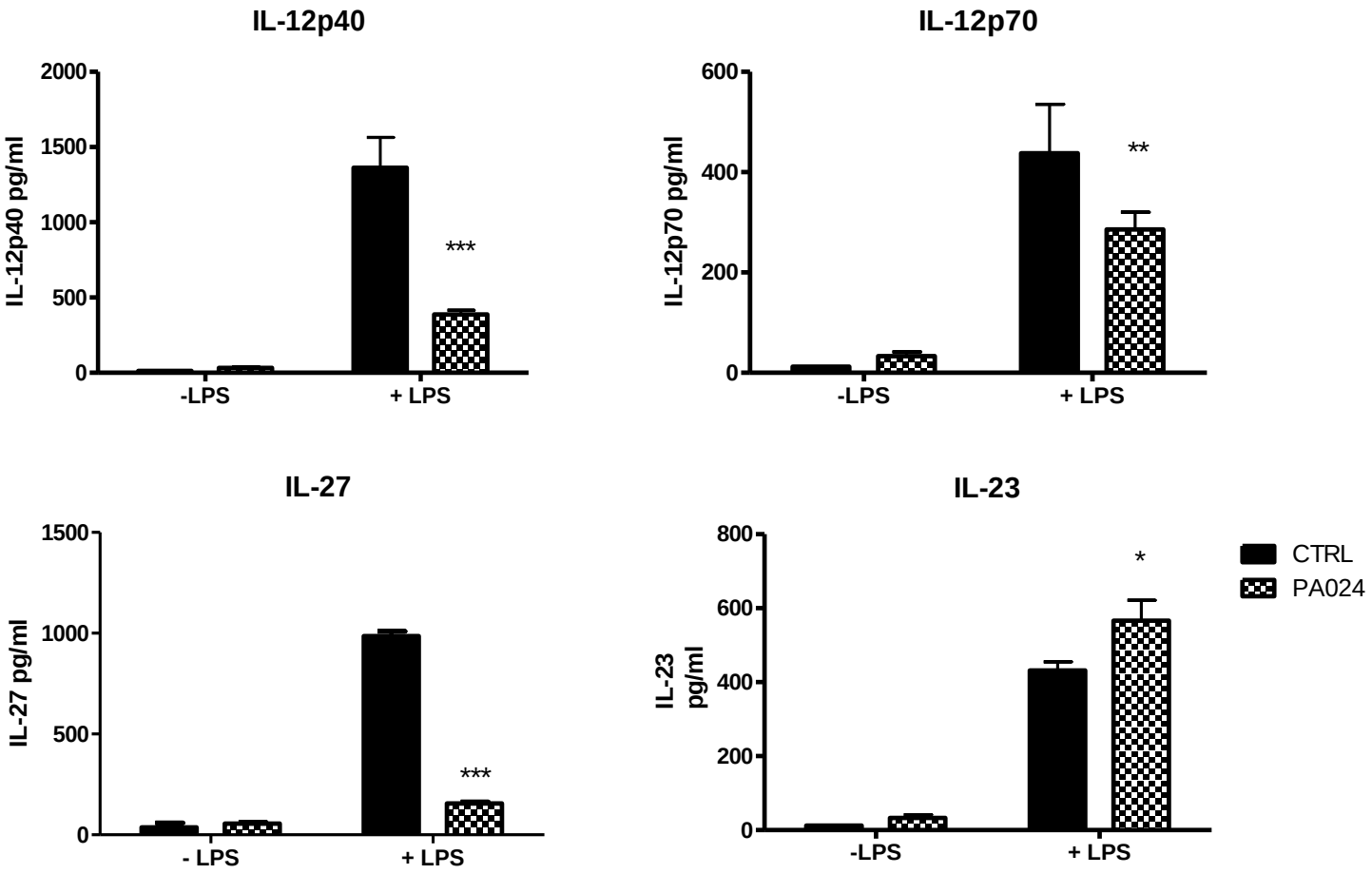


FIGURE 3.20

Activation of RXR with PA024 modulates the IL-12 family of cytokines in BMDC: BMDC were differentiated in GMCSF in the presence of 1μM PA024 for 7 days and stimulated for 24hr with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70, IL-23 and IL-27 using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. PA024/LPS groups as determined by one-way ANOVA test.

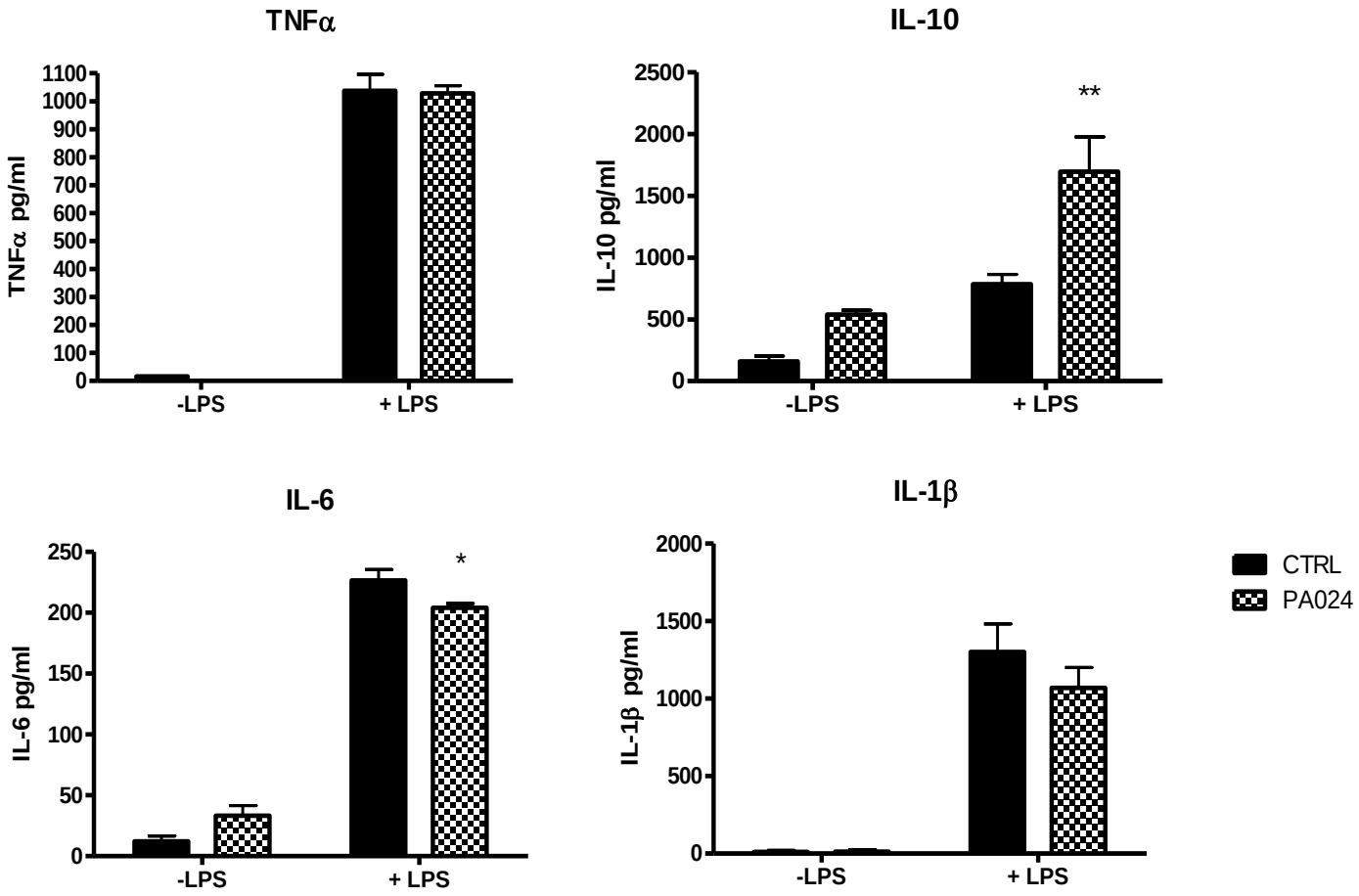


FIGURE 3.21

Activation of RXR with PA024 enhances the production of the anti-inflammatory cytokine IL-10 while decreasing the production of IL-6 from LPS matured BMDC:

BMDC were differentiated in GMCSF on the presence of 1μM PA024 for 7 days and stimulated for 24hr with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of TNFα, IL-6, IL-1β and IL-10 using specific immunoassays. Results are ± SEM of triplicate assays and represent three independent experiments. ** $p<0.01$, * $p<0.05$ comparing DMSO/LPS vs. PA024/LPS groups as determined by one-way ANOVA test.

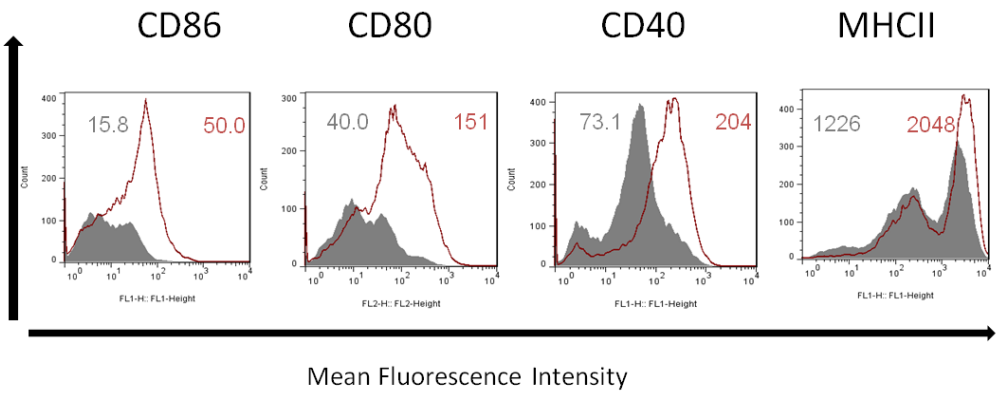


FIGURE: 3.22

LPS stimulation enhances the expression of the surface markers CD86, CD80, CD40 and MHCII: BMDC were differentiated over 7 days in GMCSF in the presence of DMSO and subsequently stimulated for 24hr with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80, CD40 and MHCII. Cells were then gated on the CD11c positive population. Grey shaded peaks represent DMSO control cells and the red overlaid line represents LPS stimulated DMSO treated cells.

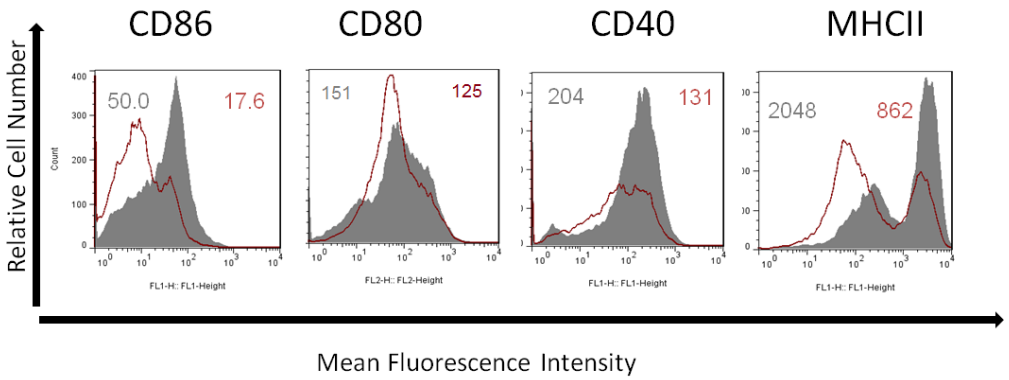


FIGURE: 3.23

Activation of RXR decreases surface marker expression in LPS matured BMDC: BMDC were differentiated over 7 days in the presence of DMSO or PA024 (1µM) and subsequently stimulated for 24hr with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80, CD40 and MHCII.Cells were gated on the CD11c population. Grey shaded peaks represents LPS stimulated cells and the red overlaid line represents LPS stimulated PA024 treated cells.

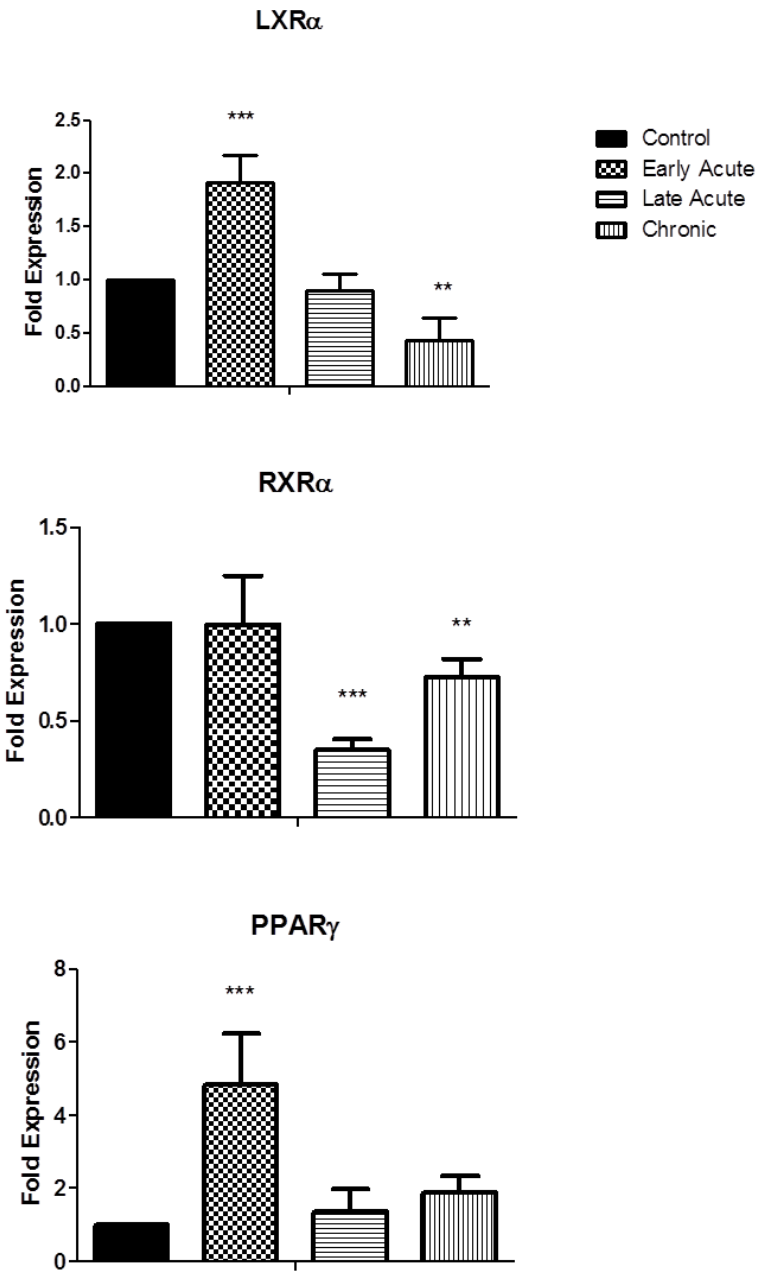


FIGURE: 3.24

The expression of LXR, RXR and PPARγ in DSS induced colitis. Total RNA was isolated from colonic tissue of mice at various stages after induction of colitis with DSS (early acute, late acute and chronic disease) along with controls. 1µg of RNA was converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represents 5 seperate mice per group ***p<0.001, **p<0.01, * comparing control vs. disease groups as determined by one-way ANOVA test.

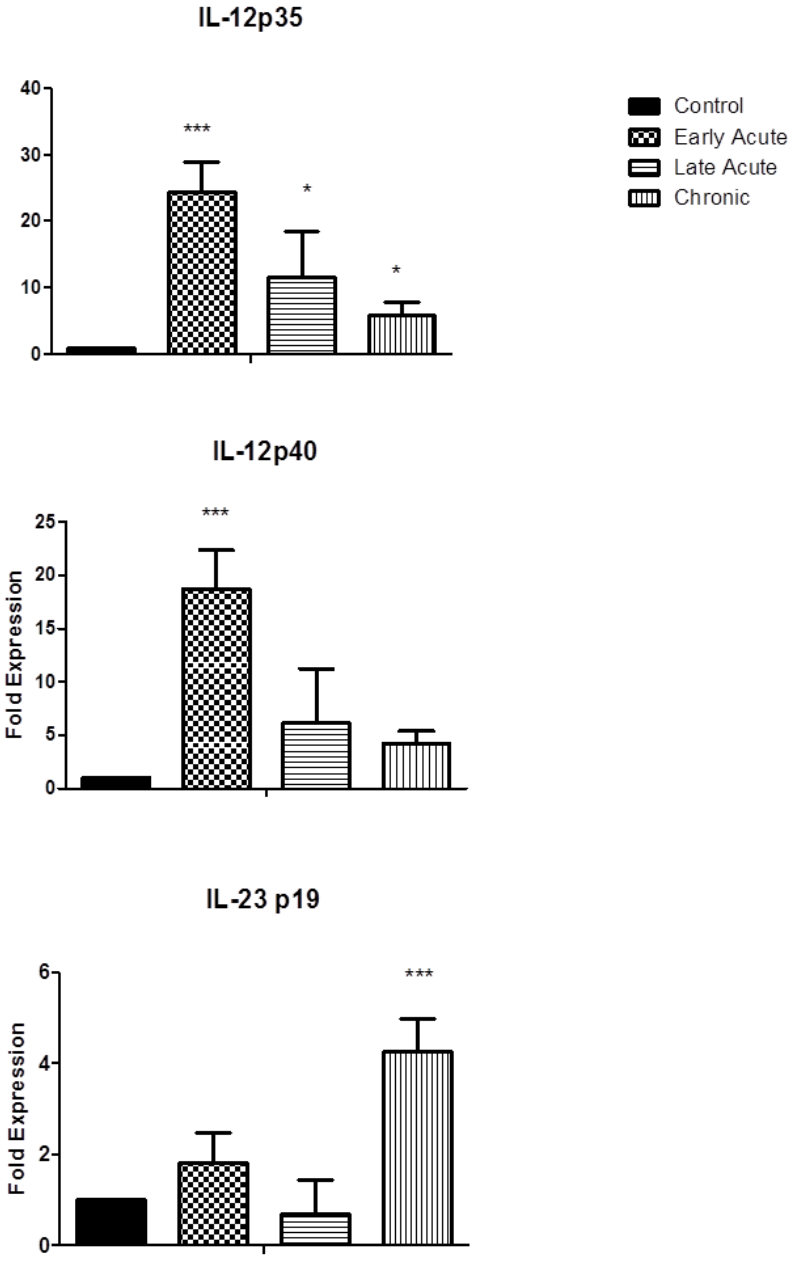


FIGURE: 3.25

The expression of the IL-12 and IL-23 cytokine subunits is upregulated in DSS induced colitis. Total RNA was isolated from colonic tissue of mice at various stages after induction of colitis with DSS (early acute, late acute and chronic disease) along with controls. 1µg of RNA was converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represents 5 seperate mice per group ***p<0.001, *p<0.05 comparing control vs. disease groups as determined by one-way ANOVA test.

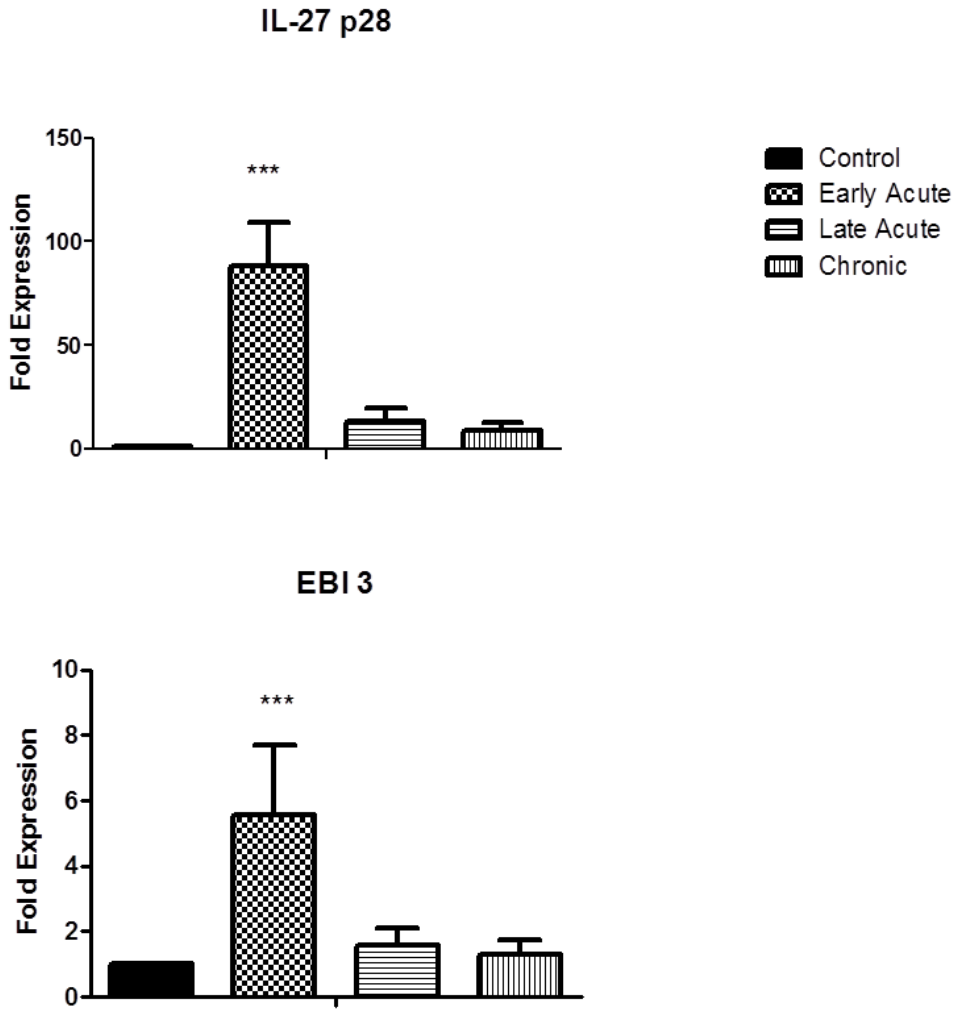


FIGURE: 3.26

The expression of IL-27 is upregulated in early acute colitis in DSS induced mice. Total RNA was isolated from colonic tissue of mice at various stages after induction of colitis with DSS (early acute, late acute and chronic disease) along with controls. 1µg of RNA was converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represents 5 seperate mice per group ***p<0.001 comparing control vs. disease groups as determined by one-way ANOVA test

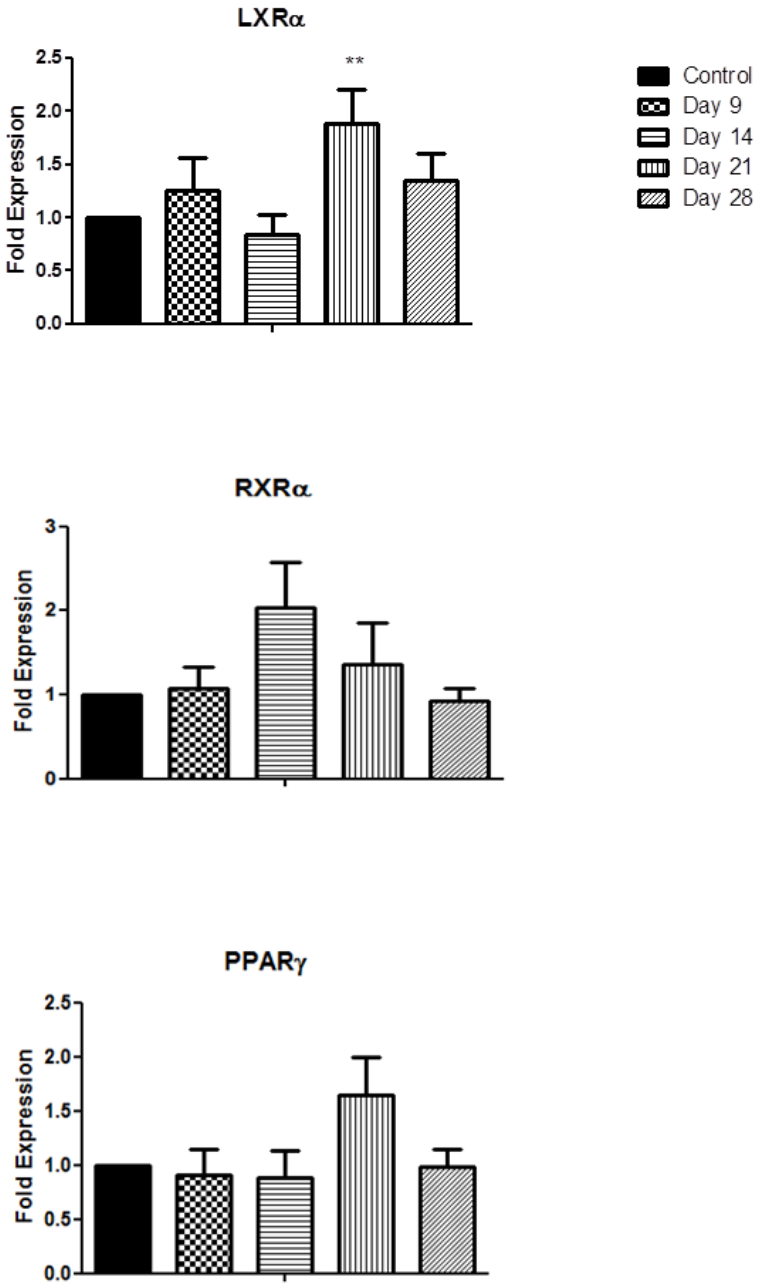


FIGURE: 3.27

The expression of LXRα but not RXRα or PPARγ is significantly altered following Citrobacter rodentium infection. Total RNA was isolated from colonic tissue of healthy mice and mice infected with *Citrobacter rodentium* over the course of 28 days. 1µg of RNA was converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represents 4 seperate mice per group $^{**}p<0.01$ comparing control vs. infected groups as determined by one-way ANOVA test.

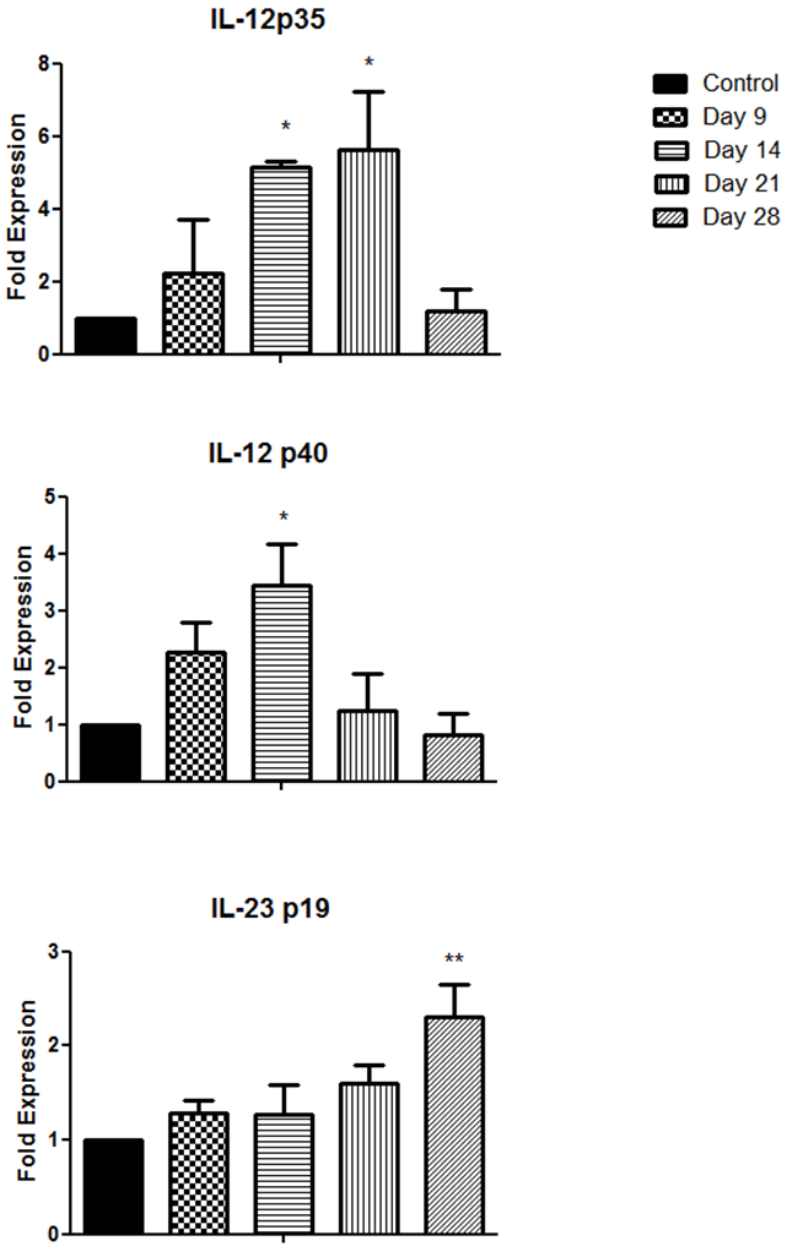


FIGURE: 3.28

The expression of the IL-12 and IL-23 cytokine subunits is significantly increased during Citrobacter rodentium infection. Total RNA was received from colonic tissue of healthy mice and mice infected with *Citrobacter rodentium* over the course of 28 days. 1µg of RNA was converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represent 4 seperate mice per group $^{**}p<0.01$, $^{*}p<0.05$ comparing control vs. infected groups as determined by one-way ANOVA test.

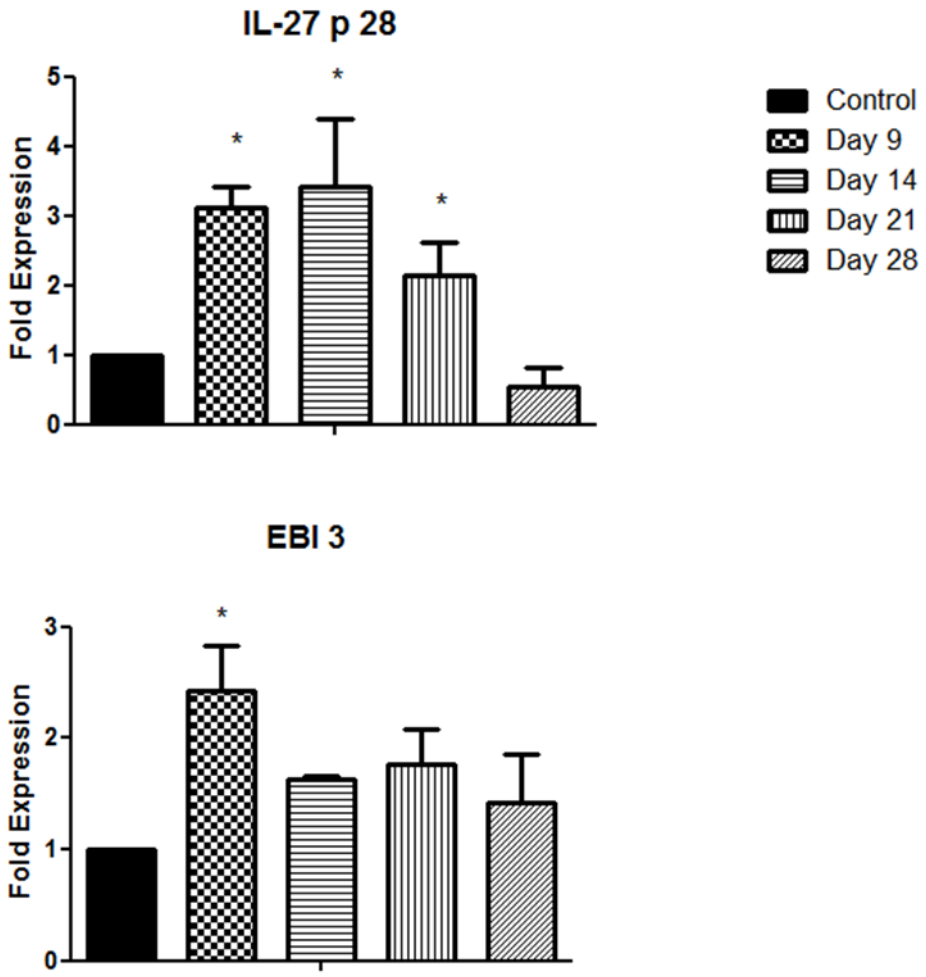


FIGURE: 3.29

The expression of the IL-27 cytokine subunits is significantly increased during Citrobacter rodentium infection. Total RNA was received from colonic tissue of healthy mice and mice infected with *Citrobacter rodentium* over the course of 28 days. 1µg of RNA was converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represents 4 seperate mice per group *p<0.05 comparing control vs. infected groups as determined by one-way ANOVA test.

TARGET	EFFECT OF LXR	EFFECT OF PPARγ	EFFECT OF RXR
↓ IL-12p40	✔	✔	✔
↓ IL-12p70	✔	✔	✔
↓ IL-23	✔	✔	x
↓ IL-27	✔	✔	✔
↓ CD80	✔	✔	✔
↓CD86	✔	✔	✔
↓CD40	✔	✔	✔
↓TLR4	✔	✔	n/a
↓MHCII	n/a	n/a	✔

Table 3.1: The effect of nuclear receptor activation on cytokine production and cell surface marker expression on BMDC.

TARGET	EARLY ACUTE DSS INFECTION	LATE ACUTE DSS INFECTION	CHRONIC DSS INFECTION
↑ IL-12p40	✔	x	x
↑ IL-12p35	✔	✔	✔
↑ IL-23p19	x	x	✔
↑ IL-27p28	✔	x	x
↑ EBI3	✔	x	x
LXR	↑	No Change	↓
PPARγ	↑	No Change	No Change
RXR	No Change	↓	↓

Table 3.2: The expression of nuclear receptors and the IL-12 family of cyokines in early acute, late acute and chronic DSS induced colitis.

TARGET	DAY 9 *Citrobacter rodentium*	DAY 14 *Citrobacter rodentium*	DAY 21 *Citrobacter rodentium*	DAY 28 *Citrobacter rodentium*
↑ IL-12p40	x	✔	x	x
↑ IL-12p35	x	✔	✔	x
↑ IL-23p19	x	x	x	✔
↑ IL-27p28	✔	✔	✔	x
↑ EBI3	✔	x	x	x
↑ LXR	x	x	✔	x
↑ PPARγ	x	x	x	x
↑ RXR	x	x	x	x

Table 3.3: The expression of nuclear receptors and the IL-12 family of cytokines in Citrobacter rodentium induced colitis.

3.3 DISCUSSION

The results presented in this chapter provide evidence that nuclear receptors, specifically PPARγ, LXR and RXR can affect DC maturation *in vitro.* DC are essential APCs that are capable of efficiently responding to danger signals and initiating a specific immune response. However a number of studies in human autoimmune disorders have indicated that DC are found in autoimmune lesions. Similarly, in animal models of autoimmune disease, DC are amongst the first cells to infiltrate target tissues (Bayry et al. 2004). Given that these cells are unique in their ability to drive T helper cell responses and these responses are also implicated in autoimmune diseases, they are ideal targets for the treatment of these conditions

Interestingly, our results show that activation of LXR, RXR or PPARγ in DC can specifically and significantly inhibit the production of the IL-12 family of cytokines. Although these cytokines are structurally related to one another and have unique functions within the immune system, they have also been implicated in inflammatory and autoimmune disorders.

IL-12p70 for example which consists of IL-12p40 and IL-12p35 is a potent inducer of IFNγ from T cells and NK cells and is also essential for the differentiation of naive T cells to Th1 cells *in vitro* and *in vivo*. However mRNA expression of IL-12p40 has been detected in acute MS lesions – specifically it has been found in early disease cases suggesting that this cytokine may be important in initiating disease (Duvallet et al. 2011). Similarly IL-12p35-/- mice have been shown to develop exacerbated CIA compared to WT mice (Vasconcellos et al. 2011). A role for IL-12p40 in CD has also been highlighted in murine models of the disease where anti- IL-12p40 antibodies lessened disease severity (Benson et al. 2011). Our data shows that PPARγ, LXR and

RXR agonist treatment significantly reduces the production of IL-12 (both IL-12p40 and IL-12p70) from LPS matured DC. Given that this cytokine has been widely implicated in autoimmune diseases, this result suggests that there is promising therapeutic potential for targeting these nuclear receptors in such diseases.

Our results also show that both LXR and PPARγ activation result in a significant decrease in the production of the IL-12 related cytokine IL-23. Interestingly, this reduction is not seen following RXR activation, suggesting that despite the structural and mechanistic similarities between these nuclear receptors and their ability to interact with each other they still have their own distinct roles. IL-23 is composed of the IL-12 subunit p40 and the newly identified p35 related subunit, p19. Although it is important for protection against fungal infections it is also essential for maintaining a Th17 phenotype (Wang et al. 2011). This Th17/IL-23 axis has been established as a major player in intestinal inflammation and the development of CD. Indeed, a significant association between the IL-23R and CD was established following a genome wide association study undertaken by Duerr *et al* (Duerr et al. 2006). IL-23 is also increased in DC isolated from MS patients (Vaknin-Dembinsky, Balashov & Weiner 2006). Therefore suppressing the production of this cytokine could be beneficial in ameliorating disease.

We also see a significant decrease in IL-27 following LXR, PPARγ and RXR activation in DC. As well as inhibiting the differentiation of Th17 cells, IL-27 also synergises with IL-12 to promote Th1 differentiation and expansion. However as with other IL-12 related cytokines, IL-27 has also been linked to disease. A study undertaken by Goldberg *et al* showed that in a rat model of arthritis and a murine model of MS,

disease severity was reduced upon administration of IL-27p28 neutralizing antibodies (Goldberg et al. 2004).

The remaining DC secreted cytokines i.e. TNFα, IL-6, IL-1β and IL-10 are all affected differently depending on which nuclear receptor is activated. TNFα for example is a proinflammatory cytokine which activates other immune cells such as macrophage and NK cells and enhances further proinflammatory cytokine and chemokine production. PPARγ activation significantly suppresses the production of this proinflammatory cytokine in LPS matured DC. TNFα production can also synergize the effect of IL-23 on IL-17 production from Th17 cells (Notley et al. 2008). Given that PPARγ activation also significantly decreases IL-23, this result further emphasises the effect PPARγ may have downstream on Th17 polarisation. Since the production of TNFα has long been associated with increased disease severity in RA, MS and IBD, any changes in its cytokine levels could prove beneficial in the treatment of such autoimmune diseases (Kollias et al. 1999).

The production of the proinflammatory cytokine IL-6 can also be regulated by both RXR and PPARγ as its production is significantly decreased following activation of these nuclear receptors in DC. IL-6 is a powerful proinflammatory cytokine that not only stimulates T and B cells but also leads to the infiltration of further immune cells, thus heightening the immune response. The expression of this cytokine is also increased in many inflammatory diseases and treatments blocking IL-6 have been proven successful in treating models of colitis and rheumatoid arthritis (Gabay 2006). Given that RXR and PPARγ have the ability to decrease this cytokine; this again highlights their potential as novel therapeutic targets in the treatment of inflammatory diseases.

IL-10 is a potent anti-inflammatory cytokine which is necessary for downregulating pro-inflammatory cytokines after bacterial clearance and is therefore essential in maintaining immunological homeostasis. Its production is increased following RXR activation, again emphasising the receptors anti-inflammatory capabilities. PPARγ activation however decreases the production of IL-10. Although high levels of this cytokine are ideal in the treatment and prevention of dysregulated inflammatory diseases, targeting one or several of the proinflammatory cytokines that contribute to the disease has also been used as a successful approach. Therefore PPARγ can still be considered as having potent therapeutic potential in the treatment of these diseases.

It is important to note that although the results represented in this chapter represent a consistent trend in the effect of these nuclear receptors on proinflammatory cytokine production i.e. decrease in the IL-12 cytokines, there are however batch to batch variations in the levels produced in each experiment. While some experiments resulted in large amounts of proinflammatory cytokine production, others produced only modest levels of the cytokines examined. Throughout these studies BMDC were harvested from Balb/c mice between the ages of 6-12 weeks. It is therefore possible that the age of each mouse prior to BMDC harvest had an effect on its ability to respond efficiently to LPS or other TLR maturation signals. Indeed, work by Paula *et al* (2008) reported that aged mice are less responsive to LPS than younger mice and show diminished DC maturation (Paula et al. 2009).While the ages of mice used in this study ranged from 2 months (young) to 18 months (aged), the conclusion of this report still highlights that age can affect DC function. The immune function of Balb/c mice can also be affected by the housing densities in which they are caged. Laber *et al* reported that mice caged at a high density (10 per cage) have lower levels of $CD4^+$ T helper cells compared to those housed at lower housing densities (Laber et al. 2008). Throughout these experiments

mice where initially housed at 10 mice per cage, however this number decreased as mice were culled for BMDC experiments. We aimed to minimise the variability in our experiments were possible in relation to these factors, however slight changes in housing numbers in combination with slight changes in age could account for batch to batch variability in cytokine production observed throughout this study and those following in the remaining chapters.

The cytokines examined in this chapter have important and nonredundant roles in T helper cell differentiation. However in the absence of signal 2 i.e. costimulation between the T cell and DC, naive T cells fail to differentiate and polarize into their specific subsets. Therefore we also examined the effect of nuclear receptor activation on costimulatory marker expression in LPS matured DC. Treatment with agonists specific for LXR, PPARγ and RXR resulted in a significant decrease in the costimulatory marker CD40 whereas LXR has the most potent effect on this surface marker. CD40 can interact with CD40L on T cells to further differentiate the subset while simultaneously enhancing T cell activation and expansion through upregulation of the B7 costimulatory markers CD80 and CD86(Sharpe, Freeman 2002). CD40 has also been implicated in a number of autoimmune diseases such as MS and IBD. A study undertaken by Polese *et al* showed a significant increase in CD40 expression in lamina propria leukocytes in both Crohns and Ulcerative colitis patients compared to healthy controls elegantly highlighting a link between CD40 and disease (Polese et al. 2003). Therefore inhibiting this CD40-CD40L interaction should induce T cell tolerance and subsequently reduce inflammatory cytokine expression. Indeed Neurath *et al* effectively used a CD40L antibody (anti-gp39) in colitis induced in mice to significantly prevent disease activity (Neurath et al. 1995). Further studies demonstrated that the loss of

disease activity following anti-gp39 treatment was due to an inhibition in IL-12 signalling (Stuber, Strober & Neurath 1996).

CD80 and CD86 are also upregulated in DC maturation and again these receptors contribute to T cell differentiation via interaction with CD28 on naive T cells. Taken together these costimulatory markers are referred to as the B7 markers and both have been detected in MS lesions (Boylan et al. 1999). In support of the hypothesis that these markers contribute to MS disease progression Perrin *et al* both showed that administering anti-CD80 injections to mice during the initiation of the murine model of MS –EAE significantly reduced disease severity (Perrin et al. 1996). RXR, LXR and PPARγ activation results in a downregulation in both CD86 and CD80. Although there have been reports on the implications of targeting one of the B7 molecules as potential therapeutic targets, one of the most successful costimulatory blockers on the market to date, Abatacept (Orencia) targets both. Abatacept is a fusion protein that binds with very high affinity to CD28 on T cells thus preventing B7 ligation. This ultimately prevents signal 2 from being effectively delivered, thus turning down the T cell response (Chen 2010). Similarly our results show that through nuclear receptor activation, B7 expression is inhibited with possible downstream effects on T helper cell polarization.

PPARγ activation decreases the expression of the LPS signalling receptor, TLR4. Since LPS is solely recognized by TLR4, a reduction in this pattern recognition receptor could reduce DC responsiveness to LPS thus inhibiting downstream cytokine and surface marker expression. RXR activation has the most potent effect on MHCII as demonstrated by a significant downregulation in its expression suggesting that the antigen presentation capabilities in these cells would be diminished. Presentation of antigen on MHC and subsequent interactions with T cells is considered signal one and

so changes in this marker could have effects for downstream T cell differentiation and expansion (Kunz, Ibrahim 2009)

Activation of these nuclear receptors in immune cells has also been previously documented and results generated from these studies show similarities with results presented in this chapter. A study by Gosset *et al* showed the effect of PPARγ activation on LPS matured human monocyte derived dendritic cells (MDDC). These cells showed a significant decrease in IL-12p70 production as well as a downregulation in CD80 expression. Similarly these cells show no change in IL-1β production which correlates with the data presented here (Gosset et al. 2001). PPARγ activation in BMDC was also shown to downregulate CD86, CD80 and CD40 as well as suppressing IL-12 production by Klotz *et al* (Klotz et al. 2007). Another study undertaken by Xu *et al* showed that RSG treated – LPS stimulated microglia cells also showed an inhibition in IL-12p40, Il-12p70, IL-23 and IL-27p28 secretion – again correlating with the data we have presented (Xu, Drew 2007).

Work undertaken by Zapata-Gonzalez *et al* on the role of RXR in human immature and mature DC also complements our work on RXR activation in DC (Zapata-Gonzalez et al. 2007). In this study a natural ligand for RXR (9-cis-Retinoic Acid -9cRA) was used to determine the effects of RXR activation on LPS-matured DC. This group reported a significant downregulation in the co-stimulatory marker CD86, CD80 as well as a decrease in MHCII expression.

Given the anti-inflammatory properties these nuclear receptors displayed *in vitro*, we next examined if the expression of these receptors were altered over the course of an inflammatory disease – in this case experimentally induced colitis. The DSS model of colitis is a chemically induced model of disease which involves the administration of

DSS to mice via their drinking water. Mice exposed to DSS for 5 -7 days develop acute inflammation whereas those exposed to repeated cycles of DSS for 5 days followed by administration of normal drinking water for 3 weeks, develop chronic inflammation (Kawada, Arihiro & Mizoguchi 2007). Since IBD is classed as a condition with acute, recovery and chronic phases of inflammation, the DSS model represents an excellent colitis model for examining proteins or therapies of interest. Our results show that the expression of LXR is significantly increased in early acute inflammation but becomes significantly decreased in chronic inflammation compared to healthy controls. Given that we previously reported an antiinflammatory role for LXR in response to an acute inflammatory signal (LPS), it is possible that during the early onset of colitis this receptor is increased in order to regulate and suppress the production of proinflammatory cytokines that contribute to disease. During this early onset of inflammation there is also increased infiltration of immune cells such as dendritic cells, macrophages and T cells to the colon (Kawada, Arihiro & Mizoguchi 2007). Given the receptors expression in these cells, this could also account for increased LXR expression in early acute colitis. However a loss in the expression of this receptor – as seen during chronic inflammation could then subsequently account for increased disease severity as inflammation progresses. Interestingly the expression of RXR is also significantly decreased during chronic inflammation and also in late acute inflammation. Since these receptors function by heterodimerising with each other, if one receptor is significantly decreased i.e. LXR the expression of its associated signalling partner could also be affected. PPARγ, undoubtedly the most widely studied nuclear receptor in inflammatory disease, is significantly increased in early acute inflammation. This observation could also be a result of increased infiltration of immune cells expressing PPARγ to the inflamed colon. This receptor may also be upregulated as a

protective mechanism within the gut to negatively regulate acute inflammation. Indeed it already widely accepted that activation of this nuclear receptor in models of colitis greatly attenuates disease severity (Desreumaux et al. 2001, Shah, Morimura & Gonzalez 2007).

We also examined expression of the IL-12 family of cytokines in this model of colitis to emphasise the role that these cytokines play in disease. The expression of IL-12p35, IL-12p40, IL-27p28 and EBI3 are all significantly upregulated in early acute inflammation with their expression decreasing over the course of disease. The expression of IL-23p19 however is not significantly increased until chronic disease, suggesting that the production of IL-12 and IL-27 is involved in the acute phase of intestinal inflammation whereas IL-23 is increased later in disease to mediate chronic inflammation in the colon. Since both IL-12 and IL-27 are involved in the differentiation of Th1 cells and IL-23 drives the differentiation of Th17, our results suggest acute intestinal inflammation is associated with a Th1 response were as the maintenance of chronic inflammation is regulated predominantly by a Th17 response. This observation has been seen by other groups who have also come to this conclusion (Alex et al. 2009, Fichtner-Feigl et al. 2007).

Given the multifactorial mechanisms underlying IBD, we also examined the expression of nuclear receptors and the IL-12 family of cytokines in another model of experimental colitis. *Citrobacter rodentium* is a gram negative bacterium which, upon infection, colonises the distal colon and results in IBD-like symptoms and responses within the mouse (Mundy et al. 2005). Colonisation of the intestinal mucosa following infection of *C.rodentium*, peaks after 1 -2 weeks and is then cleared over the following 3-4 weeks (Eckmann 2006). Our results show that there is a significant increase in LXR expression after 3 weeks (Day 21) of infection whereas there are no significant changes in any of

the other nuclear receptors examined. This suggests that the expression of LXR is upregulated as the infection begins to clear. Since this model results in a bias towards a Th1 response to effectively clear the *C.rodentium* infection, it is fitting that the expression of LXR would not be upregulated early in infection - we have previously shown that LXR can inhibit the production of IL-12 and IL-27 both of which are essential for Th1 differentiation. Increased expression of LXR during the initial onset of infection could result in an inhibited Th1 response and thus affect the clearance of the pathogen from the mouse. However once the infection begins to clear, it is possible that LXR expression is increased to inhibit the production of these cytokines and subsequently reduce the Th1 response. As expected, the expression of the IL-12 and IL-27 cytokine subunits are significantly increased during the first 2 weeks of infection, again emphasising the need for a Th1 response for bacterial clearance. Interestingly the expression of the IL-23 subunit p19 does not become significantly increased until day 28 – when the infection has been cleared. Although it has previously been reported that IL-23 is essential and nonredundant for the full clearance of *C. rodentium* infection (Mangan et al. 2006) our results would suggest that an increase in the IL-12p40 subunit of IL-23 is sufficient in increasing the production of IL-23 early in infection.

To the best of our knowledge, this is the first time the expression of these nuclear receptors has been characterised following *C. Rodentium* infection and (with the exception of PPARγ) following DSS induced colitis. Although an increase in IL-12 and IL-23 expression has previously been reported following *C. rodentium* infection (Higgins et al. 1999, Zheng et al. 2008), this is the first time the expression of IL-27 has been characterised over the course of infection. Similarly this is the first time a complete profile of the entire proinflammatory IL-12 family over both acute and chronic DSS induced colitis has been described. The results presented in this study highlight the

importance of targeting these IL-12 cytokines and also how the presence of these nuclear receptors could be a promising method to do so.

The role of LXR in immunobiology still remains somewhat elusive. Although numerous studies have determined the receptors role in lipid metabolism and cholesterol efflux, its role in dendritic cell function still remains relatively unexplored. However two conflicting reports have been described recently. Two separate studies undertaken by Geyeregger *et al* and Hanley *et al* identified an anti-inflammatory role for LXR in human MDDC. Geyeregger et al reported a downregulation in CD86 and a significant decrease in both IL-12p40 and Il-12p70 cytokine production in LXR activated LPS matured DC whereas Hanley *et al* reported similar findings as seen by a decrease in CD86 and CD80 expression (Hanley et al. 2010, Geyeregger et al. 2007) These reports correlate with the results presented above and would suggest an anti-inflammatory role for LXR activation in DC. However a recent study by Torocsik *et al* reported a pro-inflammatory role for this receptor in LPS matured human MDDC as seen by an increase in CD80 and CD86 expression as well as an increase in IL-12p70 production (Torocsik et al. 2010). This study involved the treatment of MDDC with a synthetic partial agonist known as GW3965 where as we and other studies carried out by Hanley *et al* and Geyeregger *et al* used the full agonist T0901317. It has previously been reported that these two ligands can differentially regulate LXR target genes. Although both ligands, upon LXR occupancy can induce conformational changes at helix 12, there are distinct differences in the ligand-binding pocket of those treated with GW3965 compared with those treated with T0901317 (Farnegardh et al. 2003). This suggests that the induction of LXR dependent genes is ligand-specific and would account for the variability seen amongst these studies. These studies also highlight the importance of

synthetic ligand design and suggest that it may be possible to design LXR ligands in the future which have more specific targets and less unwanted side effects.

Although certain aspects of this work have been shown previously to some extent amongst other groups, this is the first time that a comprehensive examination of DC secreted cytokines and surfaces markers has been reported following nuclear receptor activation. Indeed, we show for the first time the effect of LXR, RXR and PPARγ on many of the IL-12 family of cytokines i.e. IL-12 p70, IL-12p40, IL-23 and IL-27p28 in LPS matured DC. Although previous work has shown the effect of RXR activation on DC surface marker expression, this is the first time that selective RXR agonists have been used i.e. compounds which activate RXR depending on whether it is associated with LXR or PPARγ. This result therefore gives us greater understanding into the mechanism of action of RXR and which nuclear receptor it heterodimerises with in order to function. The results reported for the role of LXR in DC are extremely novel. We highlight the receptors ability to not only downregulate CD80 and CD86, but also the costimulatory marker CD40 and TLR4 as well as showing for the first time this receptors overall effect on a panel of DC secreted cytokines.

The results presented here provide evidence that nuclear receptors have anti-inflammatory properties and can modulate DC maturation *in vitro*. In the next chapter we will explore the mechanism in which these receptors can carry out these effects.

CHAPTER 4

EXAMINATION OF THE TLR PATHWAY HIGHLIGHTS THE MECHANISM OF ACTION OF NUCLEAR RECEPTORS

4.1 INTRODUCTION

It is widely accepted that in order to function, nuclear receptors must form homodimers with each other or heterodimers with other members of the nuclear receptor family. These heterodimers can be activated by agonists to either or both receptors and can subsequently control numerous biological processes by regulating gene expression (Huang, Glass 2010). Indeed, these nuclear receptors can act in very different ways depending on their heterodimerisation partner. RXR for example can positively regulate the expression the ATP- binding cassette transporters A1 and G1 (ABCA1 and ABCG1) when it associates with LXR (Uehara et al. 2007). Rosemary *et al* also confirmed this observation, however they also expanded this study and showed that while RXR:LXR can significantly induce ABCA1 when RXR heterodimerises with PPARγ the ability to regulate these proteins is lost and it becomes a weak activator of ABCA1 (Cesario et al. 2001). We therefore aimed in this study to elucidate the dimer partners for each nuclear receptor of interest. Through the use of selective nuclear receptor antagonists we investigated the effect of inhibiting one nuclear receptor while simultaneously activating another. We used the synthetic compound GW9662 which has been described as a potent PPARγ full antagonist that covalently modifies a cysteine residue in the PPARγ LBD thus inhibiting its activation (Leesnitzer et al. 2002). Similarly HX531 and PA452 have been described as two synthetic pan antagonists that inhibit RXR transactivation. Interestingly HX531 acts as an antagonist toward the PPARγ-RXR heterodimer but does not affect other RXR heterodimers. Whereas PA452 has been described as a more potent antagonist which can inhibit PPARγ- RXR activation as well as LXR-RXR activation (Kagechika, Shudo 2005). Thus this antagonist can selectively inhibit certain nuclear receptor interactions that are of interest to us. As of yet there is no commercially available LXR antagonists and so our study is limited to inhibiting these two nuclear

receptors. By using these antagonists in this way, we aimed to identify they heterodimer partner for each of the nuclear receptors we examined in Chapter 3.

As mentioned earlier, DC are positioned at sites of potential pathogen entry and thus are one of the first cells to come in contact with invading organisms. For this reason it is essential that DC can respond to a wide variety of pathogens to prevent an infection from occurring. In order to do this, DC and other APC are equipped with a set of germline encoded receptors known as pattern recognition receptors (PRR) which recognise conserved pathogen associated molecular patterns (PAMPs) on eukaryotic cells (Janeway, Medzhitov 2002). The most common PRRs are the TLRs- an evolutionary ancient set of conserved proteins that have been found in plants, insects and mammals (Basset et al. 2003). As shown in Chapter 3, TLR4 signalling via LPS initiates a program of DC maturation and nuclear receptor activation in these cells can interfere with this maturation process. However, TLR4 is just one member of the TLR family which consists of 10 other members. These TLRs use different intracellular signalling molecules which ultimately lead to different transcriptional responses. We therefore aimed to examine the effects of nuclear receptor activation on DC matured with a panel of TLR ligands to examine if these nuclear receptors have a specific target within the TLR pathway.

Although TLR4 is undoubtedly the most extensively studied TLR to date, our understanding of the remaining TLR members has improved dramatically in recent years. Similar to TLR4, TLR2 is also expressed on the cell wall and responds to bacterial antigens. However unlike other TLRs, TLR2 can only respond to PAMPs by dimerising with either TLR1 or TLR6. The ligands for these are PGN or Pam3CSK4 for TLR2/1 or Zymosan for TLR2/6 (Kawai, Akira 2007). TLR5 is also expressed on the cell surface and recognizes flagellin – the protein that makes up bacterial flagella. TLR

9 which is expressed in endosomes recognizes unmethylated CpG motifs present in the bacterial genome. TLR7 recognizes guanine analogs such as loxoribine as well as recognizing ssRNA viruses such as HIV. Finally TLR3 specifically recognizes dsRNA from dsRNA viruses such as neovirus as well as the synthetic analogue PolyIC (used in this study)(Kawai, Akira 2010).

After recognizing PAMPs TLRs activate intracellular signalling pathways that lead to the activation of the transcription factor NFκB or IRF. TLRs can then activate either the MyD88 dependent or MyD88 independent pathway/TRIF dependent pathway. MyD88 is a universal adaptor that is shared by all TLRs with the important exception of TLR3. Recruitment of MyD88 leads to the activation of MAP kinases and NFκB which subsequently controls the expression of inflammatory genes. Although TLR2 and TLR4 signal through the MyD88 dependent pathway, unlike other TLRs they also use the adaptor molecule Mal to mediate downstream signalling (Kawai, Akira 2008). TLR3 and TLR4 use the adaptor molecule TRIF to signal and thus utilize the MyD88 independent pathway leading to the induction of IRF3 and IFNβ production and late NFκB activation. TLR4 is unique in its ability to signal through both the MyD88 dependent and independent pathway (Moynagh 2005). We therefore used this information on the TLR pathway to decipher if nuclear receptors target a specific signalling element within this pathway.

4.2 RESULTS

4.2.1 THE EFFECT OF THE LXR AGONIST T0901317 ON THE IL-12 FAMILY OF CYTOKINES IS COMPLETELY REVERSED IN THE PRESENCE OF THE RXR INHIBITOR PA452.

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) for 7 days in the presence or absence of the RXR inhibitors PA452 and HX531 (1μM). RXR inhibitors were added 2hr prior to LXR agonist treatment. DC were then stimulated with LPS (100ng/ml) for 24hr, supernatants were removed and levels of IL-12p40, IL-23, IL-12p70 and IL-27 **[Figure 4.1]** were assessed using ELISA.

As shown in Chapter 3, T0901317 has a profound and significant effect on the IL-12 family of cytokines as seen by a significant decrease in IL-12p40, IL-23 ($p<0.001$), IL-12p70 ($p<0.05$) and IL-27($p<0.01$) **[Figure 4.1]**. Addition of PA452 but not HX531 reversed the effect of T0901317 on these cells with the suppression of IL-12p40, IL-23, IL-12p70, and IL-27 no longer present.

4.2.2 THE MODULATION OF CELL SURFACE MARKER EXPRESSION IN T0901317 TREATED BMDC IS NOT ALTERED IN THE PRESENCE OF THE RXR INHIBITOR PA452

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) for 7 days in the presence or absence of the RXR inhibitor PA452 (1μM). The RXR inhibitor was added 2hr prior to LXR agonist treatment. Cells were then stimulated with 100ng/ml LPS for 24hr before being washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface

markers (CD11c, CD40, CD80,and CD86). Cells were then gated on the CD11c positive population.

As expected LPS upregulated the expression of CD86,CD80 and CD40 **[Figure 4.2; Row 1]** where DMSO unstimulated cells are represented by the shaded grey peak and LPS stimulated DMSO control cells are overlaid with a red line. As seen previously in Chapter 3, expression of the costimulatory marker CD40 was significantly downregulated in LPS stimulated T0901317 treated cells compared to LPS stimulated DMSO control cells **[Figure 4.2 Row 2].** T0901317 treatment also decreases the expression of the costimulatory markers CD86 and CD80 **[Figure 4.2 Row 2]** compared to DMSO vehicle control. With the presence of the RXR inhibitor PA452 in T0901317 treated cells the decrease in CD40, CD80 and CD86 expression was still observed and thus the effect of LXR activation in these cells was not reversed when RXR was inhibited **[Figure 4.2;Row 3]**

4.2.3 THE EFFECT OF THE LXR AGONIST T0901317 ON THE IL-12 FAMILY OF CYTOKINES IS PARTIALLY REVERSED IN THE PRESENCE OF THE PPARγ INHIBITOR GW9662.

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) for 7 days in the presence or absence of the PPARγ inhibitor GW9662 (1μM). GW9662 was added 2hr prior to LXR agonist treatment. DC were then stimulated with LPS (100ng/ml) for 24hr, supernatants were removed and levels of IL-12p40, IL-12p70 and IL-23 **[Figure 4.3]** were assessed using ELISA.

As expected, T0901317 significantly decreased the production of IL-12p40, IL-23 ($p<0.001$) and IL-12p70 ($p<0.01$) **[Figure 4.3]**. The suppressive effect of T0901317 on IL-23 was reversed in the presence of the PPARγ antagonist GW9662 **[Figure 4.3]**.

However, the effect of LXR activation on IL-12p70 and IL-12p40 was not reversed in the presence of the PPARγ antagonist GW9662 since a significant decrease in both cytokines was still observed ($p<0.01$ and $p<0.001$ respectively).

4.2.4 THE MODULATION OF CELL SURFACE MARKER EXPRESSION IN T0901317 TREATED BMDC IS NOT ALTERED IN THE PRESENCE OF THE PPARγ INHIBITOR GW9662.

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) for 7 days in the presence or absence of the PPARγ inhibitor GW9662 (1μM). The inhibitor was added 2hr prior to LXR agonist treatment. Cells were then stimulated with 100ng/ml LPS for 24hr before being washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80 and CD86). Cells were then gated on the CD11c positive population.

As expected LPS upregulated the expression of CD86, CD80 and CD40 in DMSO control cells **[Figure 4.4 Row 1]**. T0901317 treatment decreased the expression of the cell surface markers CD80, CD86 and CD40 **[Figure 4.4; Row 2].** In T0901317 cells that were pretreated with the PPARγ antagonist GW9662, the effect of LXR activation on CD40 and CD86 was not reversed as a decrease in the expression of these surface markers was still observed. In the presence of GW9662 however, the effect of LXR activation on CD80 appears to be partially reversed **[Figure 4.4;Row 3]**.

4.2.5 THE EFFECT OF PPARγ ACTIVATION ON THE IL-12 FAMILY OF CYTOKINES IS NOT REVERSED IN THE PRESENCE OF SPECIFIC RXR INHIBITORS

BMDC were harvested and differentiated as previously described and cultured with RSG (10μM) for 7 days in the presence or absence of the RXR inhibitors PA452 and HX531 (1μM). RXR inhibitors were added 2hr prior to RSG treatment. DC were then stimulated with LPS (100ng/ml) for 24hr, supernatants were removed and levels of IL-12p70, IL-12p40 and IL-23 **[Figure 4.5]** were assessed using ELISA.

As seen in Chapter 3, PPARγ activation in BMDC significantly decreased the production of IL-23, IL-12p70 ($p<0.001$) and IL-12p40 ($p<0.05$). The effect of PPARγ activation was not reversed in cells that were pretreated with the RXR inhibitors PA452 and HX531 as IL-23, IL-12p70 and IL-12p40 remain decreased **[Figure 4.5]**.

4.2.6 THE MODULATION OF CELL SURFACE MARKER EXPRESSION IN PPARγ ACTIVATED BMDC IS NOT ALTERED IN THE PRESENCE OF THE RXR INHIBITOR PA452.

BMDC were harvested and differentiated as previously described and treated with RSG (10μM) for 7 days in the presence or absence of the RXR inhibitor PA452 (1μM). The inhibitor was added 2hr prior to RSG treatment. Cells were then stimulated with 100ng/ml LPS for 24hr before being washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80,CD86 and TLR4). Cells were then gated on the CD11c positive population.

As expected LPS upregulated the expression of CD86, CD80, CD40 and TLR4 in DMSO control cells **[Figure 4.6 Row 1]**. RSG has a significant effect on the costimulatory markers CD80, CD86, CD40 and TLR4 as seen by a downregulation in their expression following LPS stimulation compared to DMSO control **[Figure 4.6 Row 2].** In RSG treated cells that have been pretreated with the RXR antagonist PA452, the decrease in these costimulatory markers CD40, CD80 and CD86 aswell as TLR4 is not reversed and instead a more enhanced affect is observed compared to RSG treated cells **[Figure 4.6;Row 3]**.

4.2.7 THE EFFECT OF RXR ACTIVATION ON THE IL-12 FAMILY OF CYTOKINES IS NOT REVERSED IN THE PRESENCE OF THE SPECIFIC PPARγ INHIBITOR GW9662

BMDC were harvested and differentiated as previously described and cultured with PA024 (1μM) for 7 days in the presence or absence of the PPARγ inhibitor GW9662 (1μM). Inhibitors were added 2hr prior to PA024 treatment. DC were then stimulated with LPS (100ng/ml) for 24hr, supernatants were removed and levels of IL-12p70, IL-12p40 and IL-23 **[Figure 4.7]** were assessed using ELISA.

RXR activation in BMDC significantly decreased the production of IL-12p40 ($p<0.001$) and IL-12p70 ($p<0.05$) as well as potently increasing the production of IL-23 ($p<0.001$). However the effect of RXR activation on these cytokines was not reversed in the presence of the PPARγ antagonist GW9662 as IL-12p40 and IL-12p70 were still decreased while IL-23 was also still increased compared to DMSO control **[Figure 4.7]**

4.2.8 THE MODULATION OF CELL SURFACE MARKER EXPRESSION ON BMDC BY RXR IS NOT ALTERED IN THE PRESENCE OF THE PPARγ INHIBITOR GW9662.

BMDC were harvested and differentiated as previously described and treated with PA024 (1μM) or DMSO for 7 days in the presence or absence of the PPARγ inhibitor GW9662 (1μM). The inhibitor was added 2hr prior to PA024 treatment. Cells were then stimulated with 100ng/ml LPS for 24hr before being washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80,CD86, and MHCII). Cells were then gated on the CD11c positive population.

As expected LPS upregulated the expression of CD86, CD80 and CD40 in DMSO control cells **[Figure 4.8 Row 1]**. As shown in Chapter 3, PA024 has the most potent effect on MHCII as seen by a significant decrease in its expression compared to DMSO control. RXR activation in LPS matured cells also decreased CD86, CD40 and to a lesser extent CD80 expression [**Figure 4.8 Row 2]**. In PA024 cells that were pretreated with the PPARγ antagonist GW9662, the effect of RXR activation on CD80, CD86, CD40 and MHCII was not reversed as a decrease in the expression of these surface markers was still observed.

4.2.9 LXR ACTIVATION IN DENDRITIC CELLS MODULATES THE IL-12 FAMILY OF CYTOKINES FOLLOWING STIMULATION WITH A PANEL OF TLR LIGANDS

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) or DMSO for 7 days. Cells were then stimulated for 24hr with either LPS (100ng/ml), Pam3CSK4 (5μg/ml), CpG (2μM) Zymosan (10μg/ml), Poly:(IC)

(10μg/ml), Flagellin (5μg/ml) or Loxoribine (5μg/ml). Supernatants were removed and levels of IL-12p40, IL-12p7, IL-27 and IL-23 were assessed using ELISA.

T0901317 had a significant effect on the production of IL-12p40 in TLR stimulated cells as seen by a significant decrease in its production following stimulation with LPS (p<0.05), Poly:(IC) (p<0.001), Loxoribine (p<0.01) and Zymosan (p<0.01) [**Figure 4.9].** However there was no change in IL-12p40 production in LXR activated cells following stimulation with the TLR5 ligand Flagellin or the TLR2/6 ligand Pam3CSK4 **[Figure 4.9]**. In LXR activated cells the production of IL-12p70 was also significantly decreased following stimulation with either LPS (p<0.01), CpG or Loxoribine (p<0.001) **[Figure 4.9].**

LPS, Loxoribine, Zymosan and Flagellin stimulated DC to produce IL-27 **[Figure 4.10]**. T0901317 treatment resulted in a significant decrease in IL-27 following LPS (p<0.001) Zymosan (p<0.05) Loxoribine (p<0.001) stimulation but not following stimulation with Flagellin or Pam3CSK4 **[Figure 4.10]**.

IL-23 was also significantly decreased in T0901317 treated cells stimulated with LPS (p<0.05), Pam3CSK4 (p<0.01) and Zymosan (p<0.001) however there was no change in IL-23 in treated cells that were stimulated with Loxoribine. **[Figure 4.10]**.

4.2.10 LXR ACTIVATION IN DENDRITIC CELLS MODULATES CELL SURFACE MARKER EXPRESSION FOLLOWING STIMULATION WITH A PANEL OF TLR LIGANDS.

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) for 7 days. Cells were then stimulated for 24hr with either LPS

(100ng/ml) ,Zymosan (10μg/ml), Pam3CSK4 (5μg/ml), Poly:(IC) (10μg/ml), Flagellin (5μg/ml), Loxoribine (5μg/ml) and CpG (2μM). Cells were washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80,CD86, and MHCII). Cells were then gated on the CD11c positive population.

Stimulation with a panel of TLR ligands caused an upregulation in the expression of the surface markers CD86, CD80, CD40 and MHCII **[Figure 4.11 – 4.14 A & B Row 1]**. As seen previously T0901317 treatment in LPS stimulated cells caused a decrease in the expression of the costimulatory markers CD80, CD86 and CD40 as well as a significant decrease in the antigen presentation receptor MHCII. T0901317 treated cells stimulated with Pam3CSK4 **[Figure 4.11 B Row 2]**, Zymosan **[Figure 4.12 A Row 2]**, PolyIC **[Figure 4.12 B Row 2]**, CpG **[Figure 4.13 B Row 2]** or Flagellin **[Figure 4.12 Row 2]** all show a decrease in expression of CD86, CD80, CD40 and MHCII compared to DMSO stimulated cells. Cells that were stimulated with the TLR7 ligand Loxoribine also showed a decrease in CD86, CD80 and MHCII expression however there was no change in CD40 expression in these cells **[Figure 4.13 A Row 2]**.

4.2.11 PPARγ ACTIVATION IN DENDRITIC CELLS MODULATES THE IL-12 FAMILY OF CYTOKINES FOLLOWING STIMULATION WITH A PANEL OF TLR LIGANDS

BMDC were harvested and differentiated over 7 days in the presence or absence of the PPARγ agonist RSG (10μM) as previously described. Cells were then stimulated for 24hr with either LPS (100ng/ml), Pam3CSK4 (5μg/ml), CpG (2μM) Zymosan (10μg/ml), Poly:(IC) (10μg/ml), Flagellin (5μg/ml) and Loxoribine (5μg/ml).

Supernatants were removed and levels of IL-12p40, IL-12p70 IL-23 and IL-27 [**Figure 4.15; Figure 4.16**] were assessed using ELISA.

PPARγ activation had a significant effect on the production of IL-12p40 in all TLR stimulated cells (except those stimulated through TLR9) as seen by a significant decrease in its production following stimulation with LPS ($p<0.05$), Pam3CSK4 ($p<0.01$), Poly:(IC) ($p<0.001$), Loxoribine ($p<0.05$), Zymosan ($p<0.001$) and Flagellin ($p>0.01$) [**Figure 4.15]**. Following stimulation with either LPS, CpG or Loxoribine in RSG treated cells there was a significant decrease in the production of IL-12p70 ($p<0.01$; $p<0.001$ respectively) [**Figure 4.15]**.

Similarly PPARγ activation can inhibit IL-23 production from DC matured with a panel of TLR ligands as seen by a significant decrease in the cytokine following stimulation with LPS ($p<0.01$), Zymosan ($p<0.001$) and CpG ($p<0.05$) **[Figure 4.16]**. As highlighted in the previous chapter, RSG treatment can also inhibit the production of IL-27 from LPS stimulated cells. Again we see a significant decrease in IL-27 following this stimulation ($p<0.001$) and also in those cells stimulated with the CpG ($p<0.01$) and Zymosan ($p<0.001$) **[Figure 4.16]**

4.2.12 PPARγ ACTIVATION IN DENDRITIC CELLS MODULATES CELL SURFACE MARKER EXPRESSION FOLLOWING STIMULATION WITH A PANEL OF TLR LIGANDS.

BMDC were harvested and differentiated over 7 days in the presence or absence of the PPARγ agonist RSG (10μM) as previously described. Cells were then stimulated for 24hr with either LPS (100ng/ml), Zymosan (10μg/ml), Pam3CSK4 (5μg/ml), Poly:(IC) (10μg/ml), Flagellin (5μg/ml), Loxoribine (5μg/ml) and CpG (2μM). Cells were then washed and stained with fluorochrome labelled monoclonal antibodies for specific

costimulatory and cell surface markers (CD11c, CD40, CD80,CD86, and MHCII). Cells were then gated on the CD11c positive population.

Stimulation with a panel of TLR ligands caused an upregulation in the expression of the surface markers CD86, CD80, CD40 and MHCII **[Figure 4.17 – 4.20 A & B Row 1]**. The expression of the costimulatory marker CD80 was decreased in RSG treated cells stimulated with LPS, Pam3CSK4 **[Figure 4.17 A and B Row 2]** Zymosan, Poly:(IC) **[Figure 4.18 A and B Row 2]** and Flagellin **[Figure 4.20]**. However the expression of this surface marker remained unchanged in RSG treated cells that were stimulated with Loxoribine and CpG **[Figure 4.19].** CD86 was also decreased in RSG treated cells stimulated with LPS, **[Figure 4.17 A Row 2]** PolyIC, Zymosan **[Figure 4.18 A and B Row 2]** and Flagellin **[Figure 4.20].** CD86 expression however remained unchanged in RSG treated cells that were stimulated with Pam3CSK4 **[Figure 4.17 B]**, Loxoribine and CpG **[Figure 4.19].**The expression of the surface marker CD40 fluctuated the most depending on which TLR stimulation was used. In LPS, Zymosan and Poly:(IC) stimulated RSG treated cells there was a decrease in CD40 expression. However following Pam3CSK4, Loxoribine, CpG and Flagellin stimulation there was a small upregulation in its expression in RSG treated cells **[Figure 4.17 B, Figure 4.19 & 4.20].** Finally, the expression of the antigen presentation receptor MHCII was increased in RSG treated cells that were activated with any of the TLR ligands used within this study.

4.2.13 RXR ACTIVATION IN DENDRITIC CELLS MODULATES THE IL-12 FAMILY OF CYTOKINES FOLLOWING STIMULATION WITH A PANEL OF TLR LIGANDS

BMDC were harvested and differentiated over 7 days in the presence or absence of PA024 or HX630 (1μM) as previously described. Cells were then stimulated for 24hr with either LPS (100ng/ml), Pam3CSK4 (5μg/ml), CpG (2μM) Zymosan (10μg/ml), Poly:(IC) (10μg/ml), Flagellin (5μg/ml) or Loxoribine (5μg/ml). Supernatants were removed and levels of IL-12p40, IL-12p70 IL-23 and IL-27 **[Figure 4.21; Figure 4.22]** were assessed using ELISA.

The RXR agonist PA024 significantly suppressed the production of IL-12p40 in LPS ($p<0.001$), Zymosan ($p<0.001$), Poly:(IC) ($p<0.001$) and Flagellin ($p<0.001$) stimulated cells. However PA024 did not suppress the production of IL-12p40 when stimulated with the TLR7 ligand Loxoribine, the TLR2/1 ligand Pam3CSK4 and the TLR9 ligand CpG **[Figure 4.21]**. PA024 also significantly inhibited IL-12p70 production following stimulation with LPS ($p<0.05$) and Loxoribine ($p<0.001$). The production of IL-27 was also significantly decreased in PA024 treated cells that were stimulated with either LPS ($p<0.001$) or Zymosan ($p<0.01$) however there was no inhibition in the production of this cytokine in CpG stimulated cells **[Figure 4.22].** The production of IL-23 was increased significantly in PA024 treated LPS ($p<0.001$), Pam3CSK4 ($p<0.001$), and Loxoribine ($p<0.001$) stimulated cells. However PA024 decreases the production of IL-23 in Zymosan stimulated cells ($p<0.01$) **[Figure 4.22]**

HX630 was less potent at inhibiting the IL-12 family of cytokines than PA024 which can activate a larger variety of nuclear receptor heterodimerisation partners. There was no change in IL-12p40 production in HX630 treated cells following stimulation with

LPS, Pam3CSK4, Loxoribine, Zymosan CpG or Flagellin however there was a significant decrease in its production following stimulation with the TLR3 ligand Poly:(IC) (p>0.001). The production of IL-12p70 was not affected following HX630 treatment and different TLR stimulations however IL-27 was significantly decreased in HX630 treated LPS stimulated (p<0.001) or Zymosan stimulated cells (p<0.01). Finally HX630 significantly increased the production of IL-23 from LPS (p<0.001), Pam3CSK4 (p<0.001) and Loxoribine (p<0.001) stimulated cells. Stimulation of these cells through TLR9 caused a significant decrease in the production of IL-23 (p<0.01). However there was no significant change in the production of IL-23 in Zymosan stimulated cells **[Figure 4.22].**

4.2.14 RXR ACTIVATION IN DENDRITIC CELLS MODULATES CELL SURFACE MARKER EXPRESSION FOLLOWING STIMULATION WITH A PANEL OF TLR LIGANDS.

BMDC were harvested and differentiated over 7 days in the presence or absence of the RXR agonist PA024 (1μM) as previously described. Cells were then stimulated for 24hr with either LPS (100ng/ml) ,Zymosan (10μg/ml), Pam3CSK4 (5μg/ml), (10μg/ml), Poly:(IC) (10μg/ml), Flagellin (5μg/ml), Loxoribine (5μg/ml) or CpG (2μM). Cells were then washed and stained with fluorochrome labelled monoclonal antibodies for specific costimulatory and cell surface markers (CD11c, CD40, CD80,CD86, and MHCII). Cells were gated on the CD11c positive population.

Stimulation with a panel of TLR ligands caused an upregulation in the expression of the surface markers CD86, CD80, CD40 and MHCII **[Figure 4.23 – 4.29 Row 1]**. PA024 treatment caused a decrease in the expression of CD86 in cells that were stimulated with either LPS, Pam3CSK4, Flagellin, Poly:(IC), Loxoribine, CpG or Zymosan. Similarly

CD80 expression was also decreased following PA024 treatment with LPS, Pam3CSK4, Flagellin, Poly:(IC), CpG or Zymosan however there was a small increase in the expression of this surface markers following Loxoribine stimulation **[Figure 4.27 Row 2]**.There was also a small increase in CD40 expression following CpG and Loxoribine stimulation **[Figure 4.27 & 4.28 Row 2]** however a decrease in the expression of this costimulatory maker was seen following stimulation with all other TLR ligands. PA024 treatment also decreased the expression of MHCII following stimulation with this panel of TLR ligands with the exception of Flagellin-stimulated cells which showed an increase in MHCII expression **[Figure 4.29 Row 2]**.

The HX630 treatments also had potent effects on the expression of these surface markers on TLR stimulated cells. CD86 expression was decreased on HX630 treated cells that were stimulated with all the TLR ligands examined in this study. There was also a decrease in the expression of CD80 on HX630 treated cells following stimulation with LPS, Pam3CSK4, Flagellin, CpG or Zymosan however the expression of this receptor remained unchanged following Poly:(IC) and Loxoribine stimulation **[Figure 4.26 & Figure 4.27 Row 3]**. CD40 expression was also decreased following LPS, Pam3CSK4, Flagellin and Zymosan stimulation **[Figure 4.23, 4.24, 4.25 & 4.29 Row 3]** however there was no change in the expression of this costimulatory marker following Poly:(IC), Loxoribine or CpG stimulation **[Figure 4.26, 4.27 &4.28]**. Finally, HX630 also decreased the expression of MHCII following stimulation with all of the TLR ligands used within this study.

4.2.15 NUCLEAR RECEPTOR ACTIVATION SELECTIVELY INHIBITS THE ACTIVATION OF NFκB AND IRF3 DOWNSTREAM OF TLR4 ACTIVATION

Results thus far have led us to believe that nuclear receptors, once activated must target an element of the TLR pathway which is common to all receptors. Consequently we next examined the effect of nuclear receptor activation on two important downstream transcription factors – NFκB and IRF3. HEK293 cells stably expressing TLR4-CD14-MD2 (HEK-MTC) were cultured for 7 days in the presence of DMSO, T0901317 (2μM), RSG (10μM), PA024 (1μM) or HX630 (1μM). These cells were then plated and transiently transfected with either NFκB or ISRE luciferase reporter plasmid.

As expected, NFκB and ISRE were both activated following exposure to LPS **[Figure 4.30].** LXR (T0901317) and PA024 treatment significantly suppressed NFκB and ISRE compared to DMSO vehicle control ($p<0.001$) whereas PPARγ activation specifically and significantly suppressed ISRE and not NFκB compared to DMSO control ($p<0.001$)

4.2.16 THE ADDITION OF THE RXR INHIBITOR PA452 REVERSES THE SUPPRESSIVE EFFECT OF LXR ON NFκB AND NOT IRF3

Results thus far have highlighted an association of LXR with RXR and not PPARγ in order to exert its effects. Consequently we examined the effect of LXR activation in the presence of RXR inhibitors on NFκB and IRF3. HEK293 cells stably expressing TLR4-CD14-MD2 (HEK-MTC) were treated for 7 days with T0901317 (2μM) in the presence or absence of the PPARγ inhibitor GW9662 (1μM) or RXR inhibitors PA452 or HX531 (1μM). These cells were then plated and transiently transfected with either NFκB or ISRE luciferase reporter plasmid. As seen here and previously in **Figure 4.30** LXR activation significantly inhibits both NFκB and IRF3 ($p<0.001$). In the presence of the RXR inhibitor PA452 (1μM) the suppressive effect of LXR on NFκB but not IRF3 is

reversed. The addition of the PPARγ inhibitor does not change the effect of LXR on NFκB or IRF3 **[Figure 4.31]**.

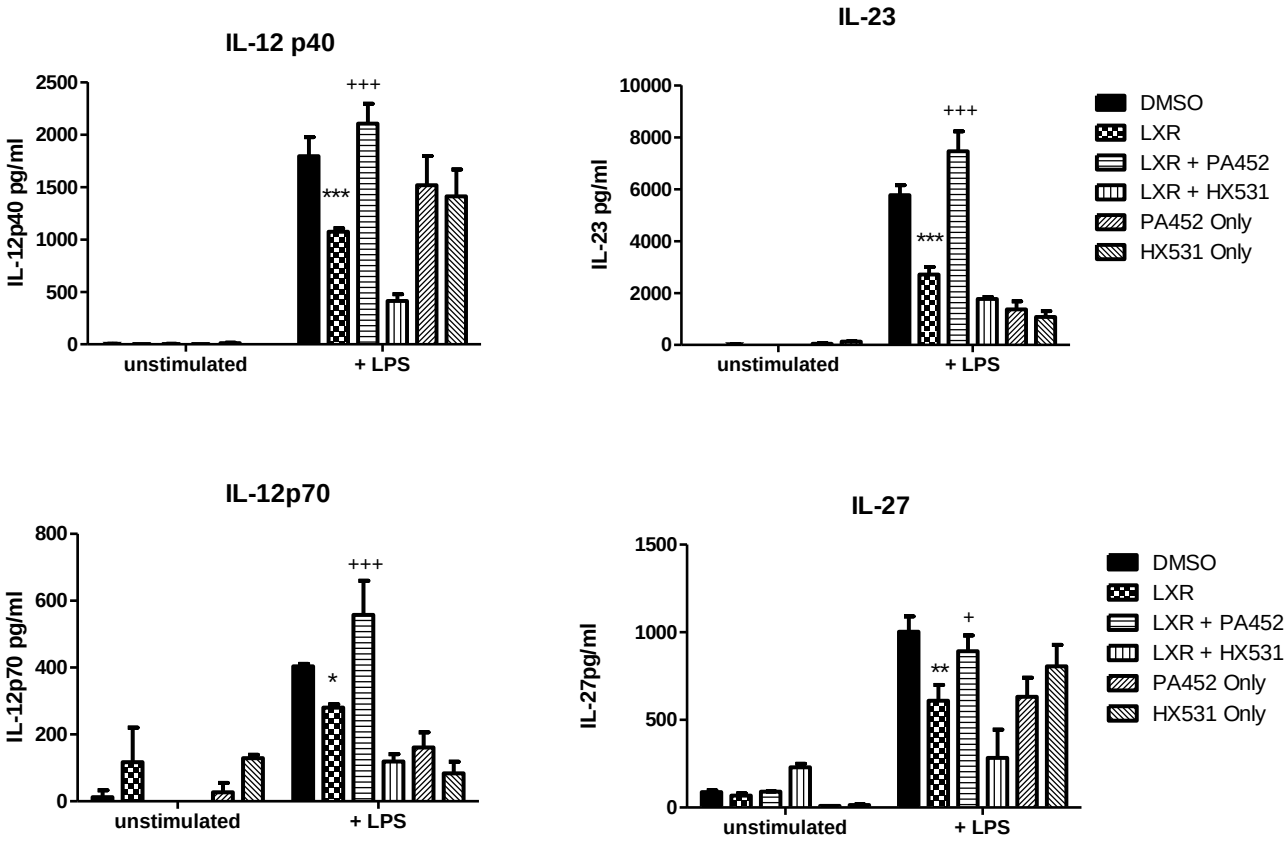


FIGURE: 4.1

The effect of LXR activation on the IL-12 family is completely reversed in the presence of a specific RXR inhibitor:

BMDC were differentiated in GMCSF for 7 days in the presence of the LXR agonist T0901317 (2μM) or DMSO. Specific RXR antagonists HX531 and PA452 (1μM) were added 2 hours prior to the LXR agonist. Cells were stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p70, IL-12p40, IL-27 and Il-23 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test. +++ p<0.001, + p<0.05 comparing T0901317 v's T0901317 + RXR inhibitor as determined by one-way ANOVA test.

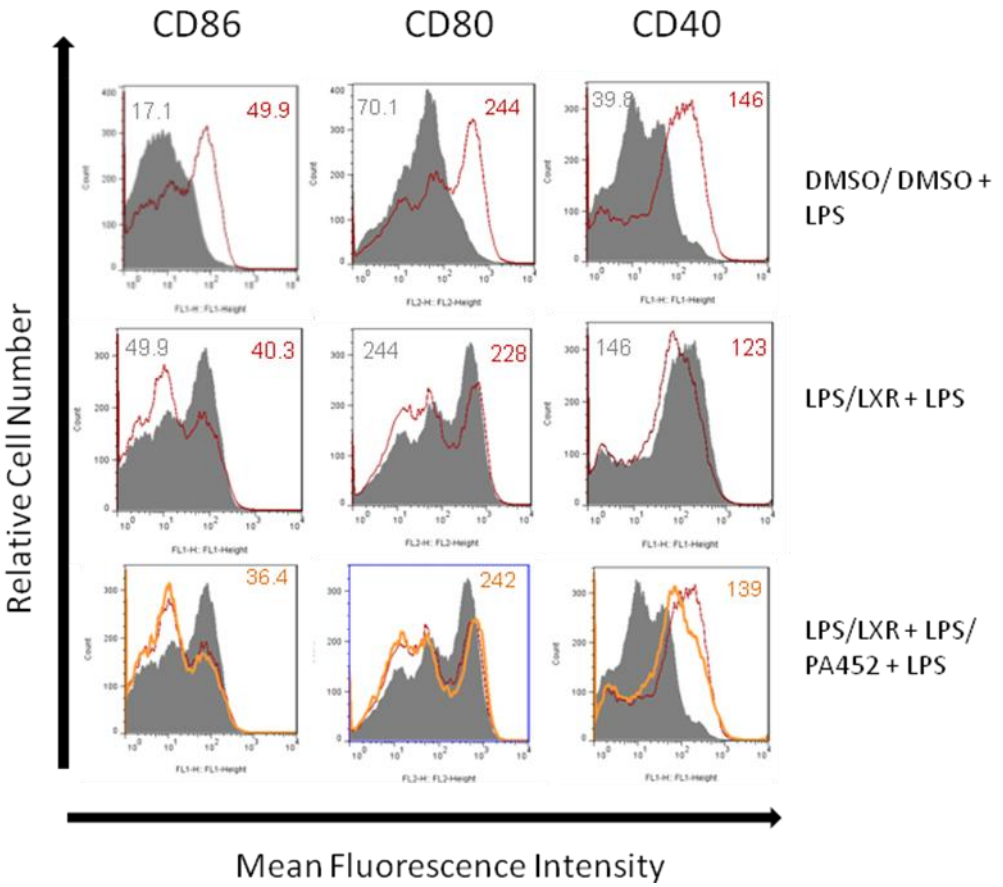


FIGURE 4.2

The effect of LXR activation on mature BMDC surface marker expression is not reversed in the presence of specific RXR inhibitors.

BMDC were differentiated in GMCSF over 7 days in the presence of either DMSO or LXR (T0901317 2µM) and the specific RXR inhibitor PA452 (1µM) and then stimulated for 24hrs with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80 and CD40. Mean Fluorescent Intensity values for each group are represented in the associated group colour and are displayed on the top corners of each histogram. Row 1 represents DMSO control (shaded grey) overlaid with LPS stimulated cells. Row 2 represents LPS stimulated DMSO cells (shaded grey) overlaid with LXR activated LPS stimulated cells.Finally Row 3 represents samples shown in Row 2 overlaid with the RXR inhibitor (orange thick line).

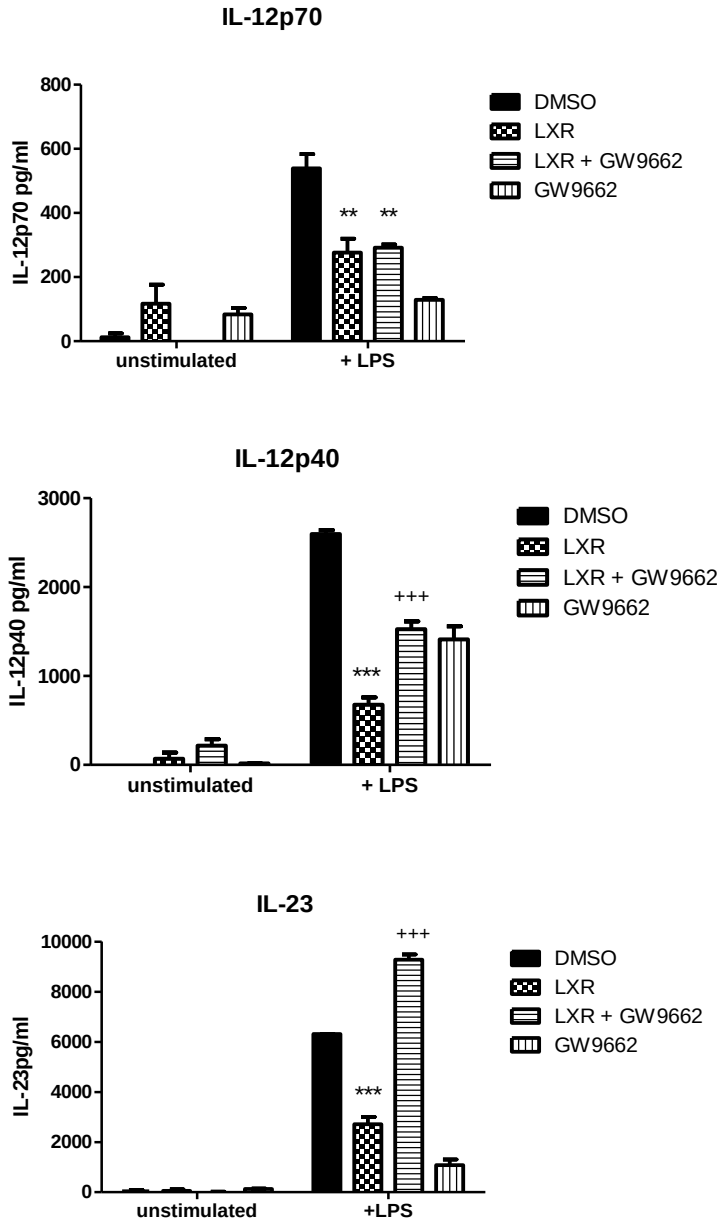


FIGURE: 4.3

The effect of LXR activation on the IL-12 family is only partially reversed in the presence of a specific PPARγ inhibitor:

BMDC were differentiated in GMCSF for 7 days in the presence of the LXR agonist T0901317 (2μM) or DMSO. The specific PPARγ antagonist GW9662 (1μM) was added 2 hours prior to the LXR agonist. Cells were then stimulated for 24hrs with 100ng/ml LPS. Supernatants were harvested and assessed for levels of IL-12p70, IL-12p40 and IL-23 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. T0901317/LPS vs. T0901317/GW9662/LPS groups as determined by one-way ANOVA test. . +++ p<0.001 comparing T0901317 v's T0901317 + GW9662 as determined by one-way ANOVA test.

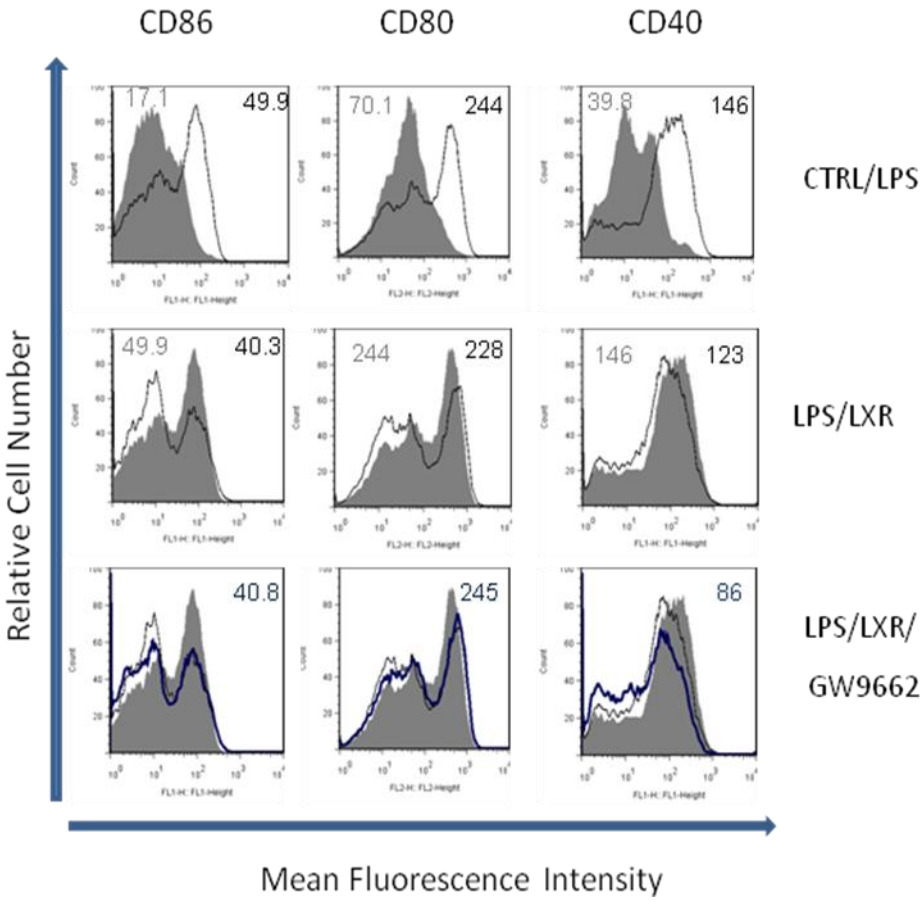


FIGURE 4.4

The effect of LXR activation on mature BMDC surface marker expression is not reversed in the presence of a specific PPARγ inhibitor.

BMDC were differentiated in GMCSF over 7 days in the presence of either DMSO or LXR (T0901317 2µM) and the specific PPARγ inhibitor GW9662 (1µM) and stimulated for 24hrs with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80 and CD40. Mean Fluorescent Intensity values for each group are represented in the associated group colour and are displayed on the top corners of each histogram. Row 1 represents DMSO control (shaded grey) overlaid with LPS stimulated cells. Row 2 represents LPS stimulated DMSO cells (shaded grey) overlaid with LXR activated LPS stimulated cells.Finally Row 3 represents samples shown in Row 2 overlaid with the GW9662 inhibitor (thick blue line).

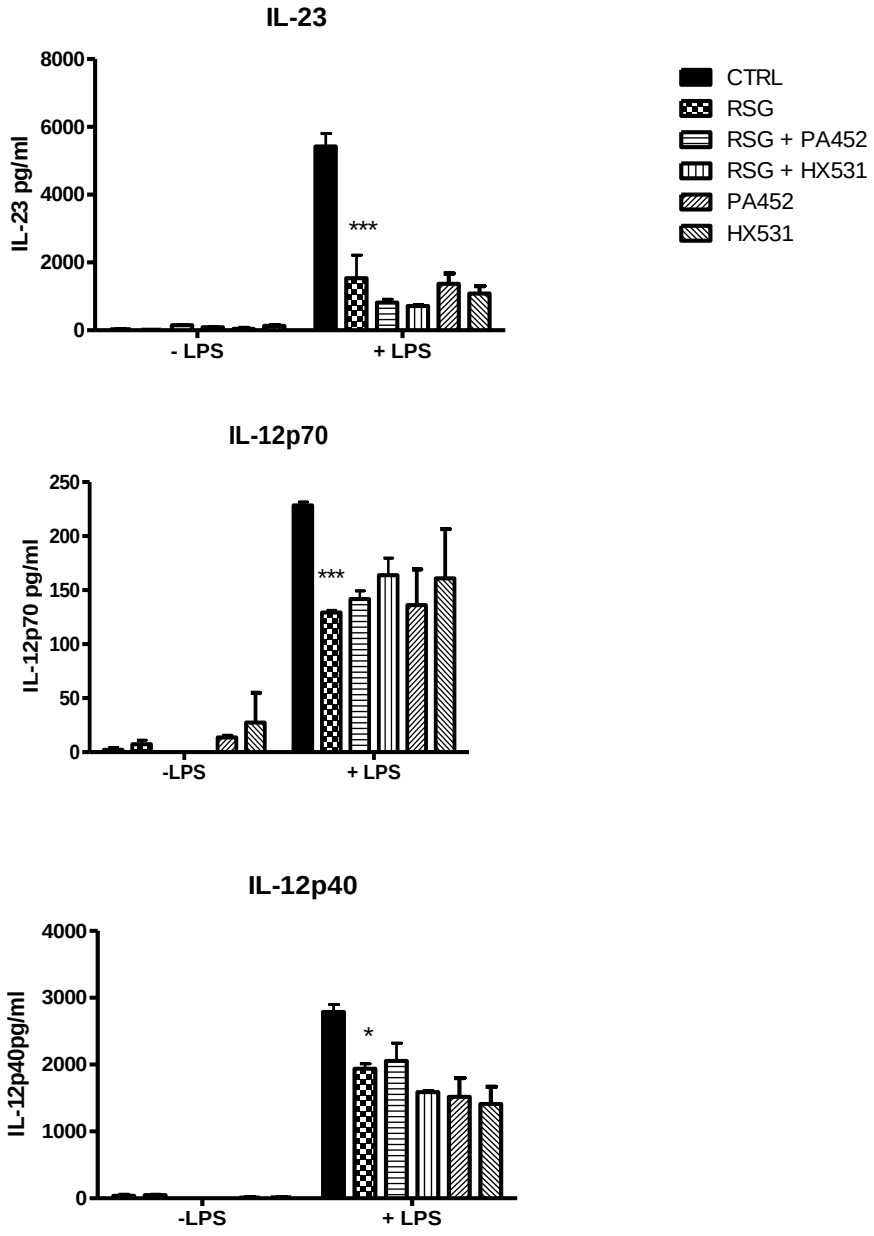


FIGURE 4.5

The effect of PPARγ activation on the IL-12 family is not reversed in the presence of specific RXR inhibitors

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the PPARγ agonist RSG (10µM). The specific RXR antagonists PA452 and HX531 (1µM) were added 2 hours prior to the PPARγ agonist and cells were stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-23, IL-12p70 and IL-12p40 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, *p<0.05 comparing DMSO/LPS vs. RSG /LPS vs. RSG/RXR/LPS groups as determined by one-way ANOVA test.

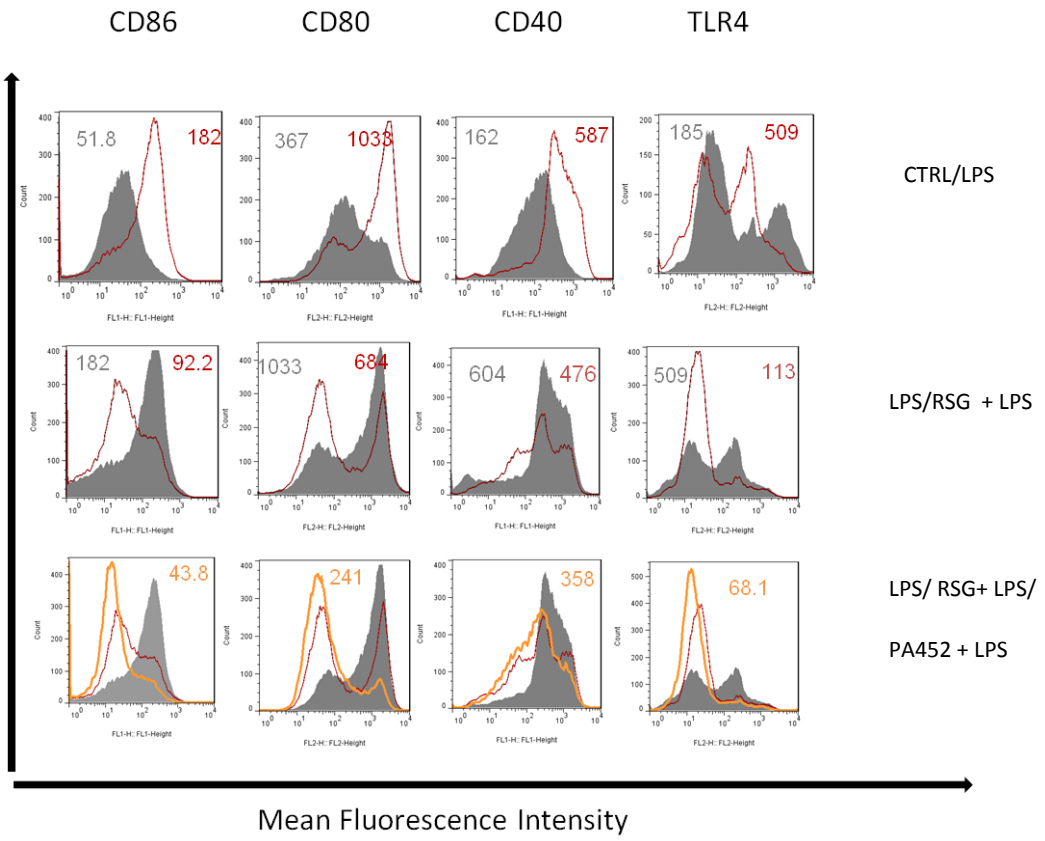


FIGURE 4.6

The effect of PPARγ activation on mature BMDC surface marker expression is not reversed in the presence of a specific RXR inhibitor.

BMDC were differentiated in GMCSF over 7 days in the presence of DMSO or RSG (10µM) and the specific RXR inhibitor PA452 (1µM). Cells were then stimulated for 24hrs with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80 and CD40, and TLR4. Mean Fluorescent Intensity values for each group are represented in the associated group colour and are displayed on the top corners of each histogram. Row 1 represents DMSO control (shaded grey) overlaid with LPS stimulated cells. Row 2 represents LPS stimulated DMSO cells (shaded grey) overlaid with PPARγ activated LPS stimulated cells. Finally Row 3 represents samples shown in Row 2 overlaid with the PA452 inhibitor (thick blue line).

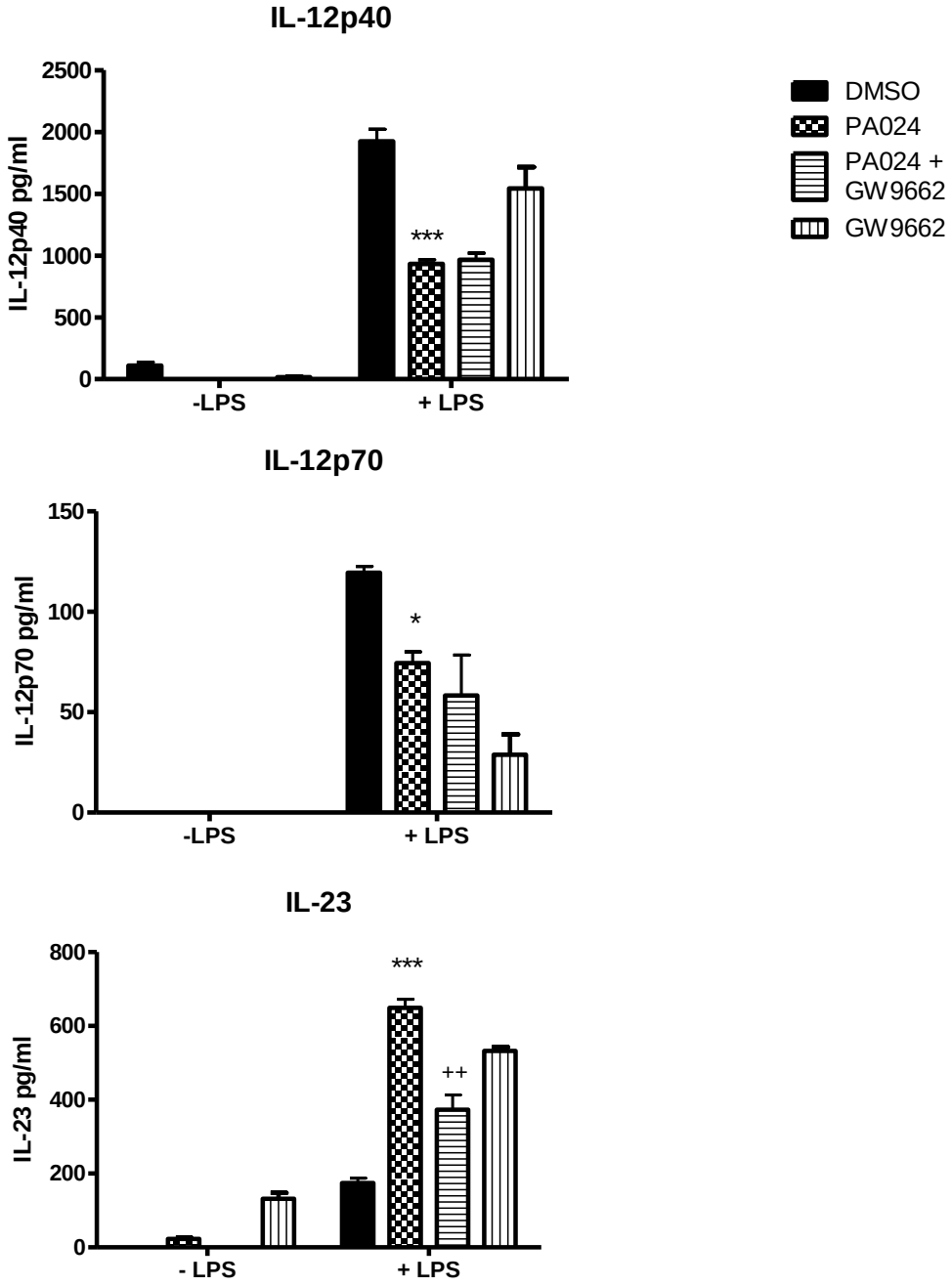


FIGURE 4.7

The effect of RXR activation on the entire IL-12 family is not fully reversed in the presence of a specific PPARγ inhibitor

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonist PA024 (1μM). The specific PPARγ antagonist GW9662 (1μM) was added 2 hours prior to the RXR agonist and cells were then stimulated for 24hrs with 100ng/ml LPS. Supernatants were then harvested and assessed for levels of IL-12p40, IL-12p70 and IL-23 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, *p<0.05 comparing DMSO/LPS vs. PA024/LPS vs. PA024/GW9662/LPS groups as determined by one-way ANOVA test. ++ p<0.01 comparing RXR vs. RXR +GW9662 as determined by one-way ANOVA test.

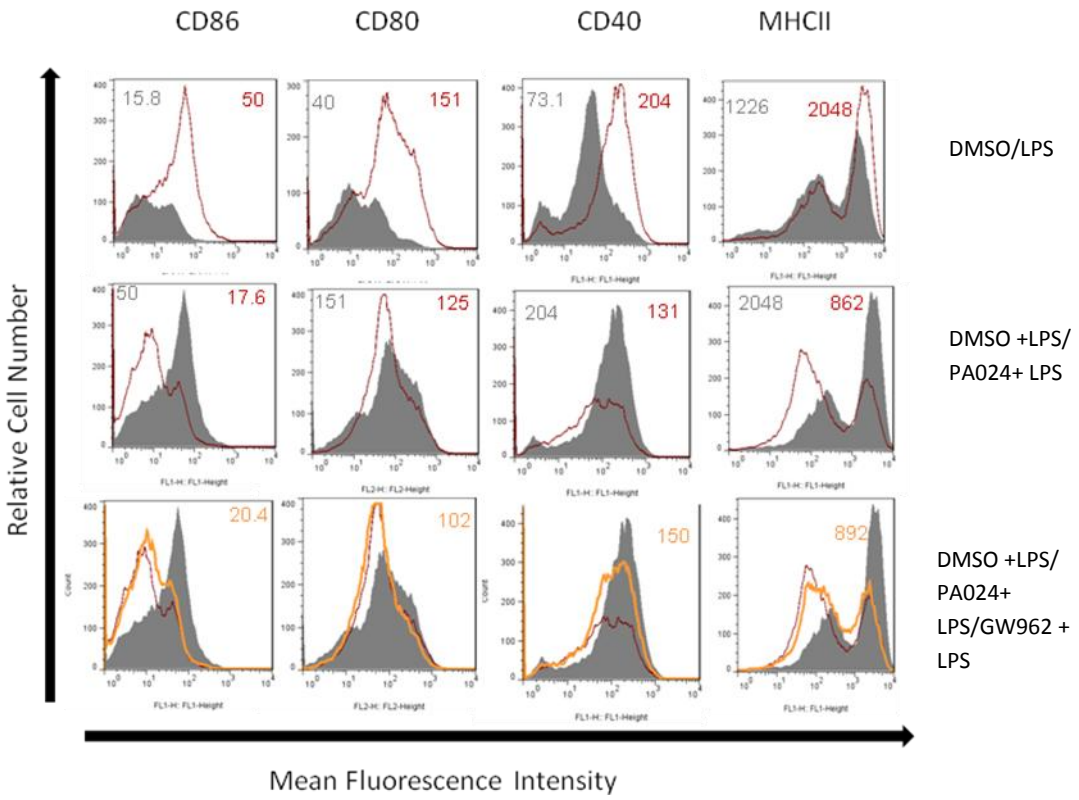


FIGURE 4.8

The effect of RXR activation on mature BMDC surface marker expression is not reversed in the presence of a specific PPARγ inhibitor.

BMDC were differentiated in GMCSF over 7 days in the presence of DMSO or PA024 1µM and the specific PPARγ antagonist GW9662 (1µM). Cells were then stimulated for 24hrs with LPS (100ng/ml). Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII. Mean Fluorescent Intensity values for each group are represented in the associated group colour and are displayed on the top corners of each histogram. Row 1 represents DMSO control (shaded grey) overlaid with LPS stimulated cells. Row 2 represents LPS stimulated DMSO cells (shaded grey) overlaid with RXR activated LPS stimulated cells.Finally Row 3 represents samples shown in Row 2 overlaid with the GW9662 inhibitor (thick orange line).

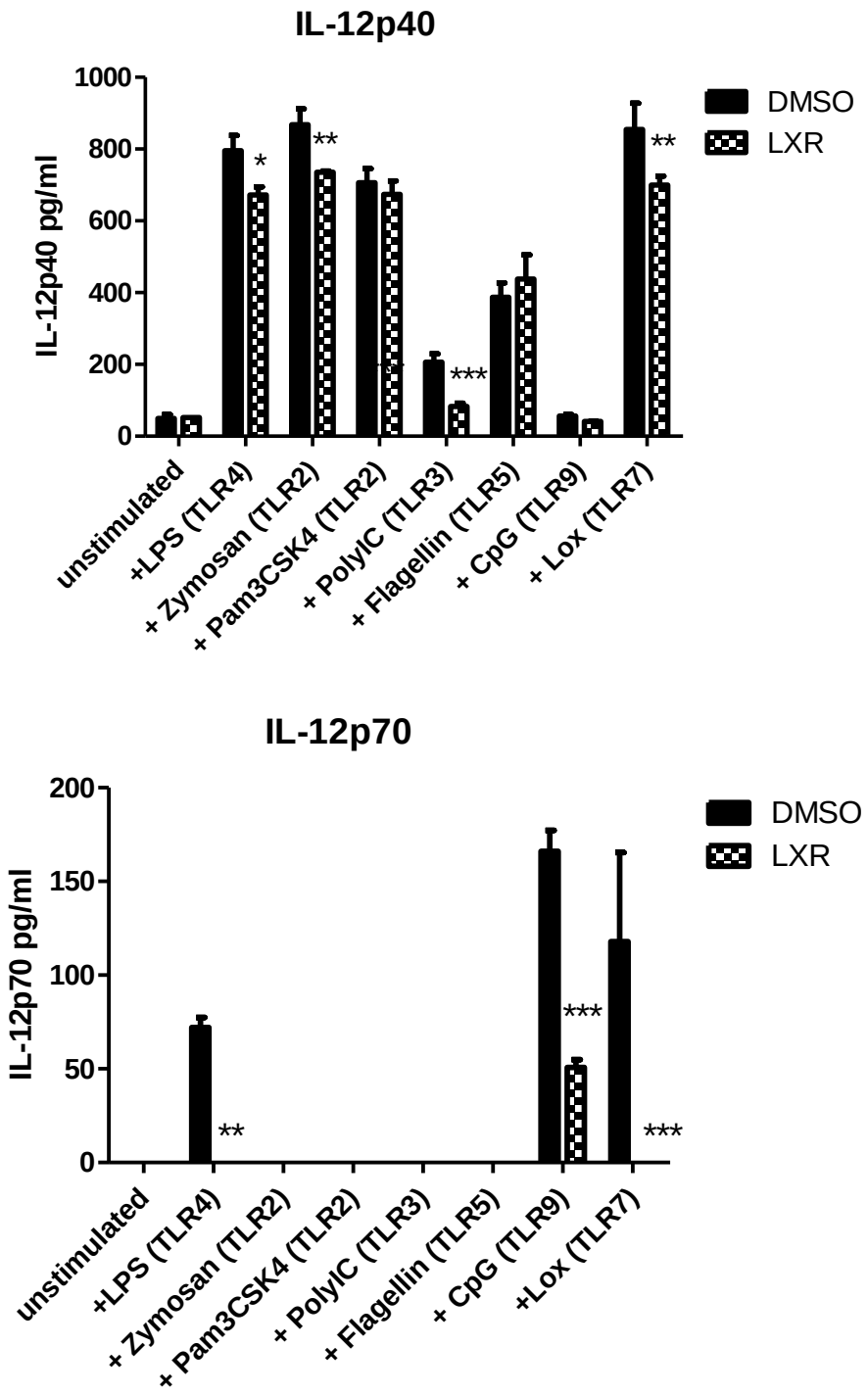


FIGURE 4.9

Activation of LXR modulates the IL-12 family of cytokines in BMDC matured with a panel of TLR ligands

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the LXR agonist T0901317 (2µM) after which cells were plated and stimulated for 24hrs with a panel of TLR ligands. Supernatants were then harvested and assessed for levels of IL-12p40 and IL-12p70 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

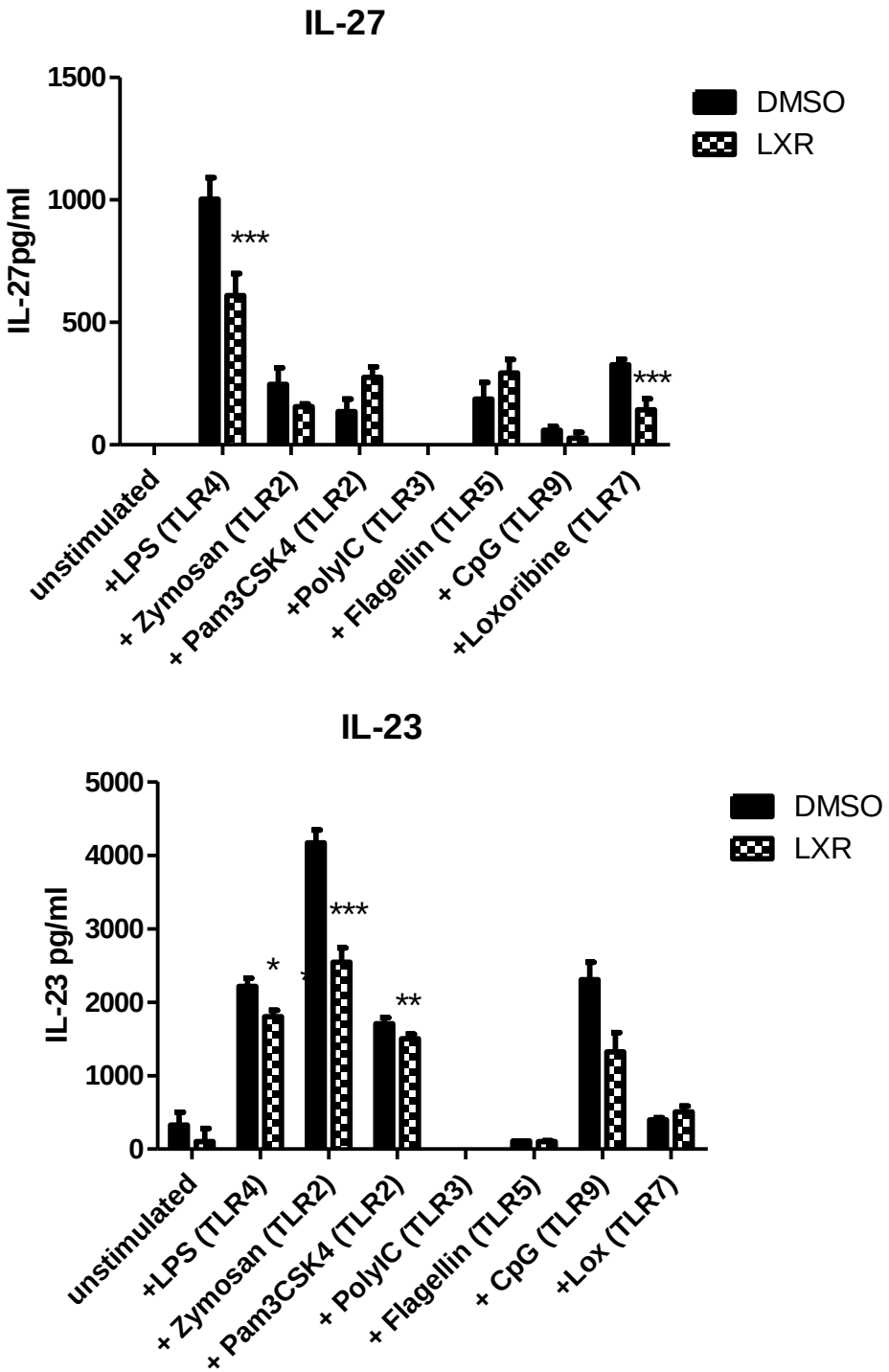


FIGURE 4.10

Activation of LXR modulates the IL-12 family of cytokines in BMDC matured with a panel of TLR ligands

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the LXR agonist T0901317 (2μM) after which cells were plated and stimulated for 24hrs with a panel of TLR ligands. Supernatants were harvested and assessed for levels of IL-27 and IL-23 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. T0901317/LPS groups as determined by one-way ANOVA test.

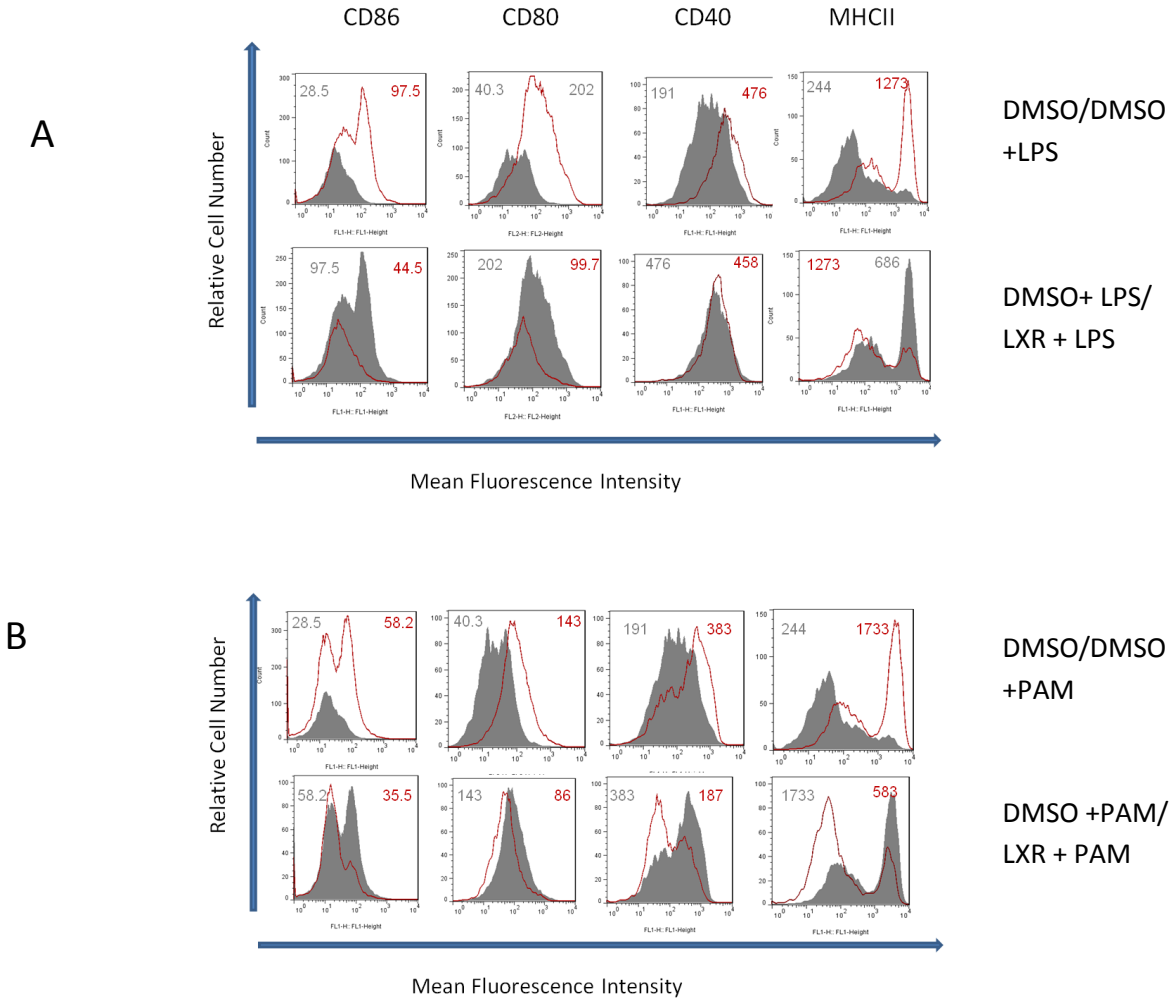


FIGURE 4.11

LXR activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the LXR agonist T0901317 (2µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII. Mean Fluorescent Intensity values for each group are represented in the associated group colour and are displayed on the top corners of each histogram.

Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with LPS 100ng/ml **[A]**or Pam3CSK4 (5µg) **[B]**. Row 2 represents DMSO with the TLR ligand stimulation (shaded grey) overlaid with LXR (2µM T0901317) with the TLR ligand stimulation.

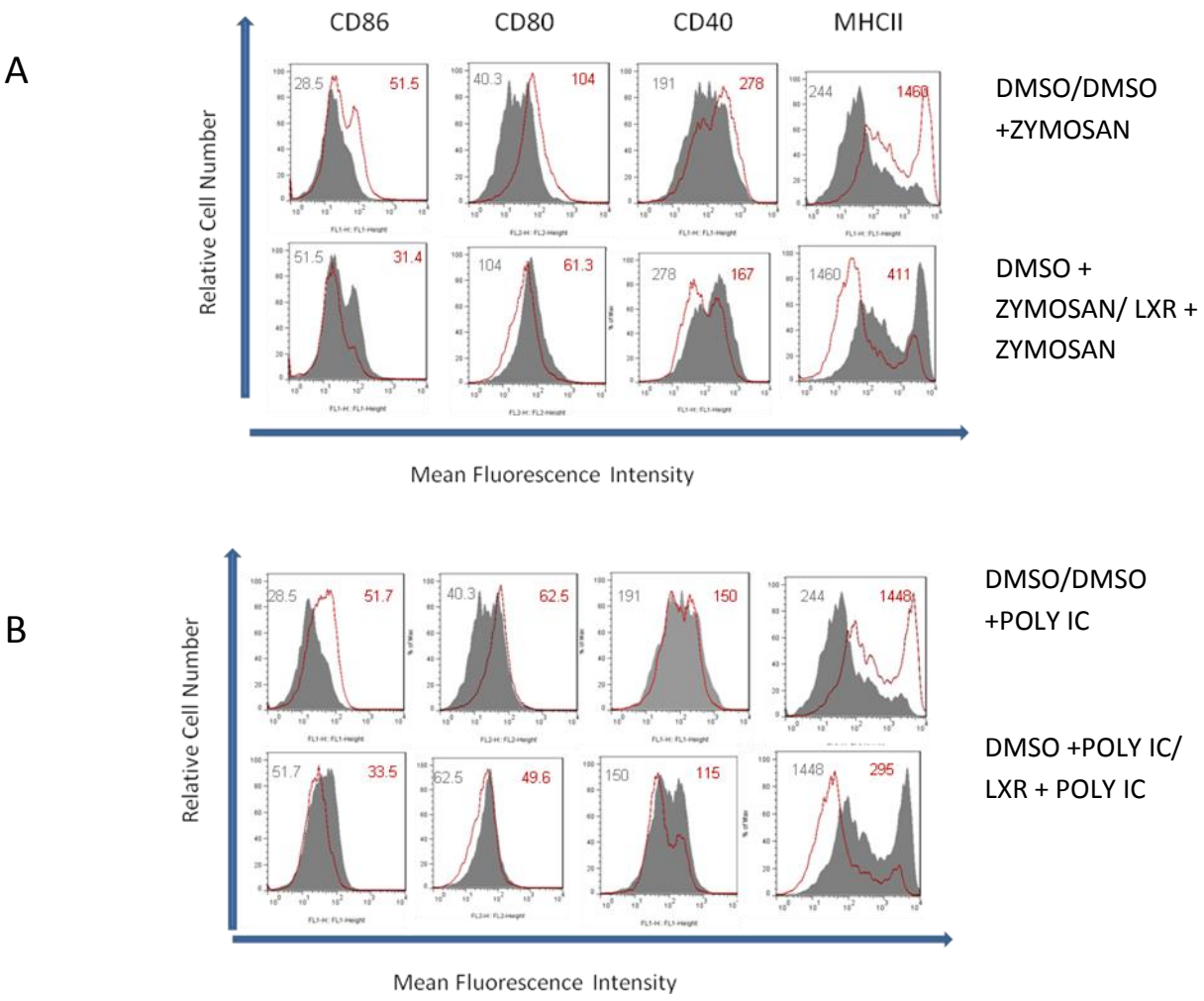


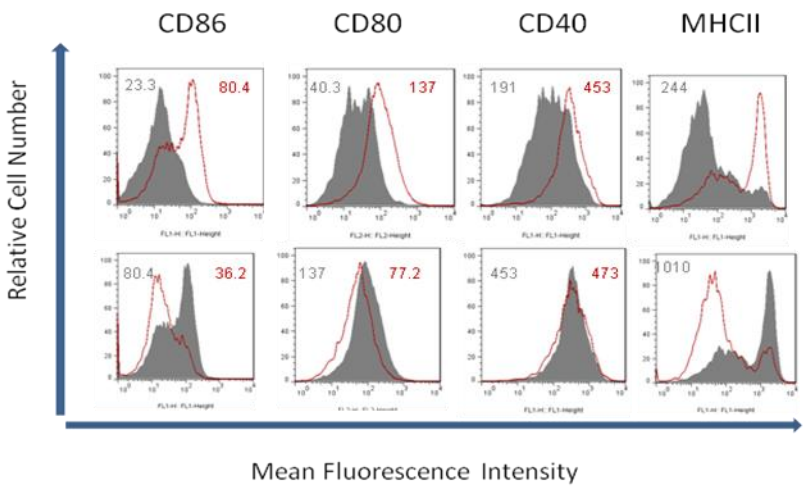
FIGURE 4.12

LXR activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the LXR agonist T0901317 (2µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII.

Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with Zymosan (10µg/ml) **[A]** or Poly: (IC) (10µg) **[B]**. Row 2 represents DMSO with the TLR ligand stimulation (shaded grey) overlaid with LXR (2µM T0901317) with the TLR ligand stimulation.

A



B

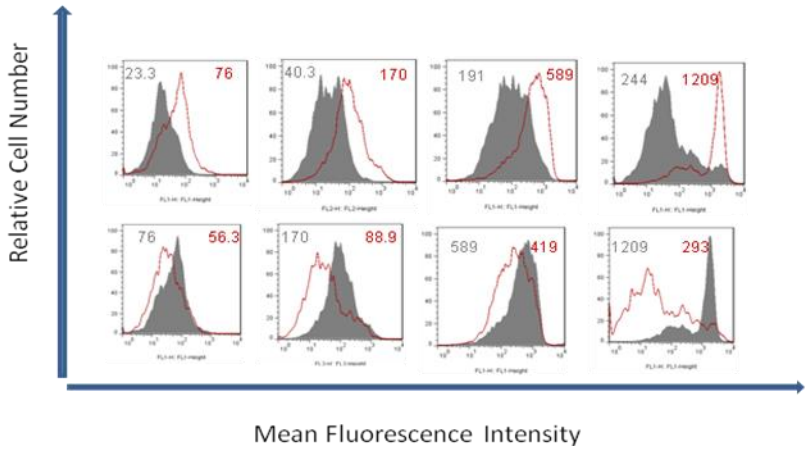


FIGURE 4.13

LXR activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the LXR agonist T0901317 (2µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII.

Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with loxoribine (1µM) **[A]** or CpG (2mM) **[B]**. Row 2 represents DMSO with the TLR ligand stimulation (shaded grey) overlaid with LXR (2µM T0901317) with the TLR ligand stimulation.

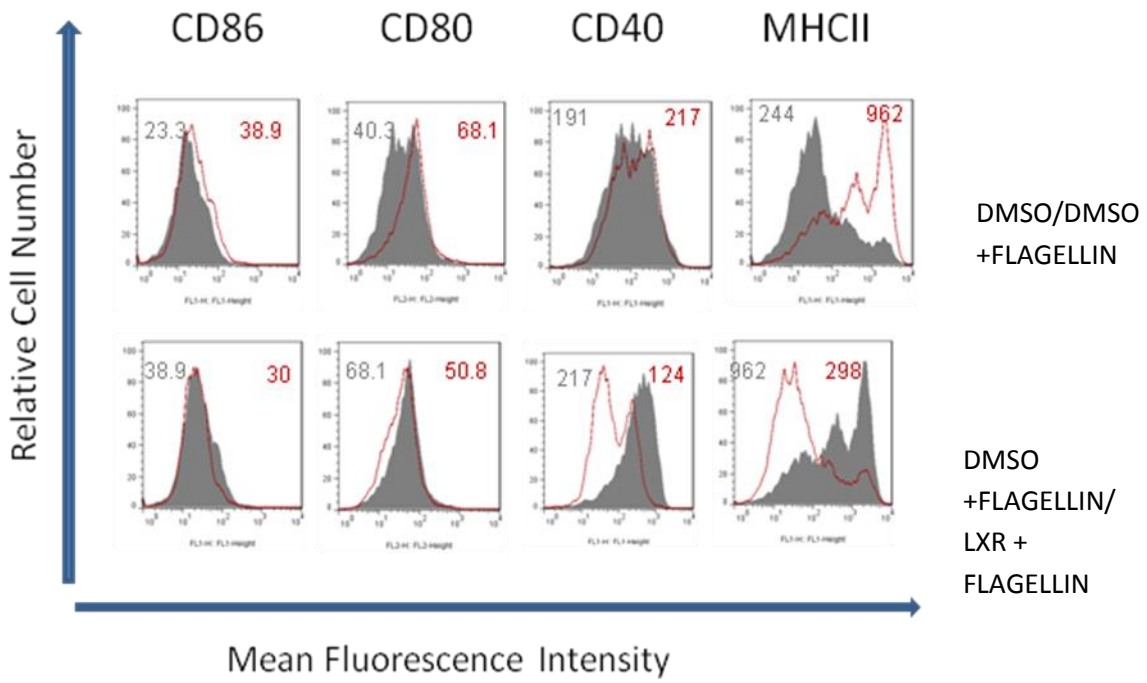


FIGURE 4.14

LXR activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the LXR agonist T0901317 (2µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII.

Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with flagellin (5µg). Row 2 represents DMSO with flagellin (shaded grey) overlaid with LXR (2µM T0901317) with flagellin.

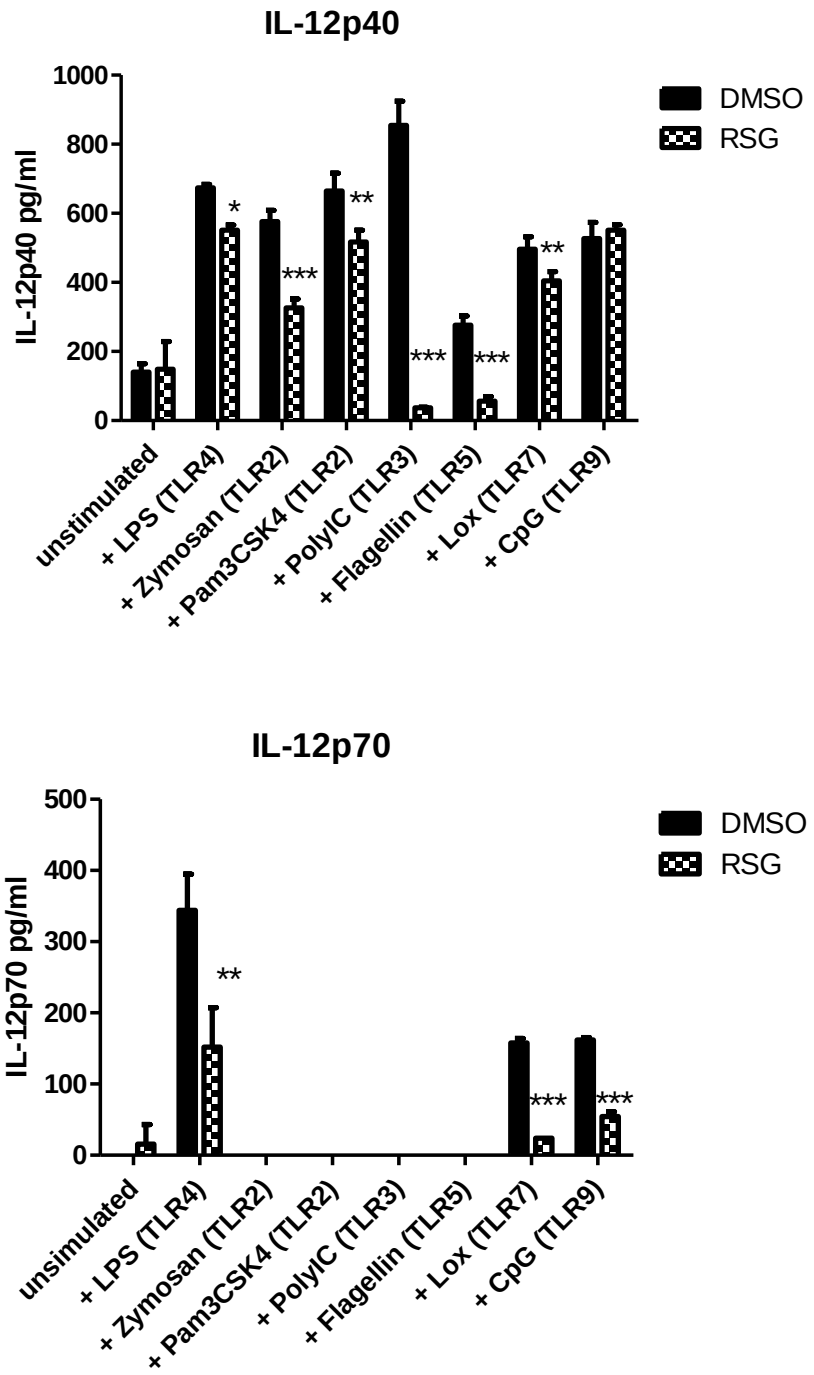


FIGURE 4.15

Activation of PPARγ modulates the IL-12 family of cytokines in BMDC matured with a panel of TLR ligands

BMDC were differentiated in GMCSF for 7 days in the presence of the PPARγ agonist RSG (10μM) after which cells were plated and stimulated for 24hrs with a panel of TLR ligands. Supernatants were then harvested and assessed for levels of IL-12p40 and IL-12p70 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01 comparing DMSO/LPS vs. RSG/LPS groups as determined by one-way ANOVA test.

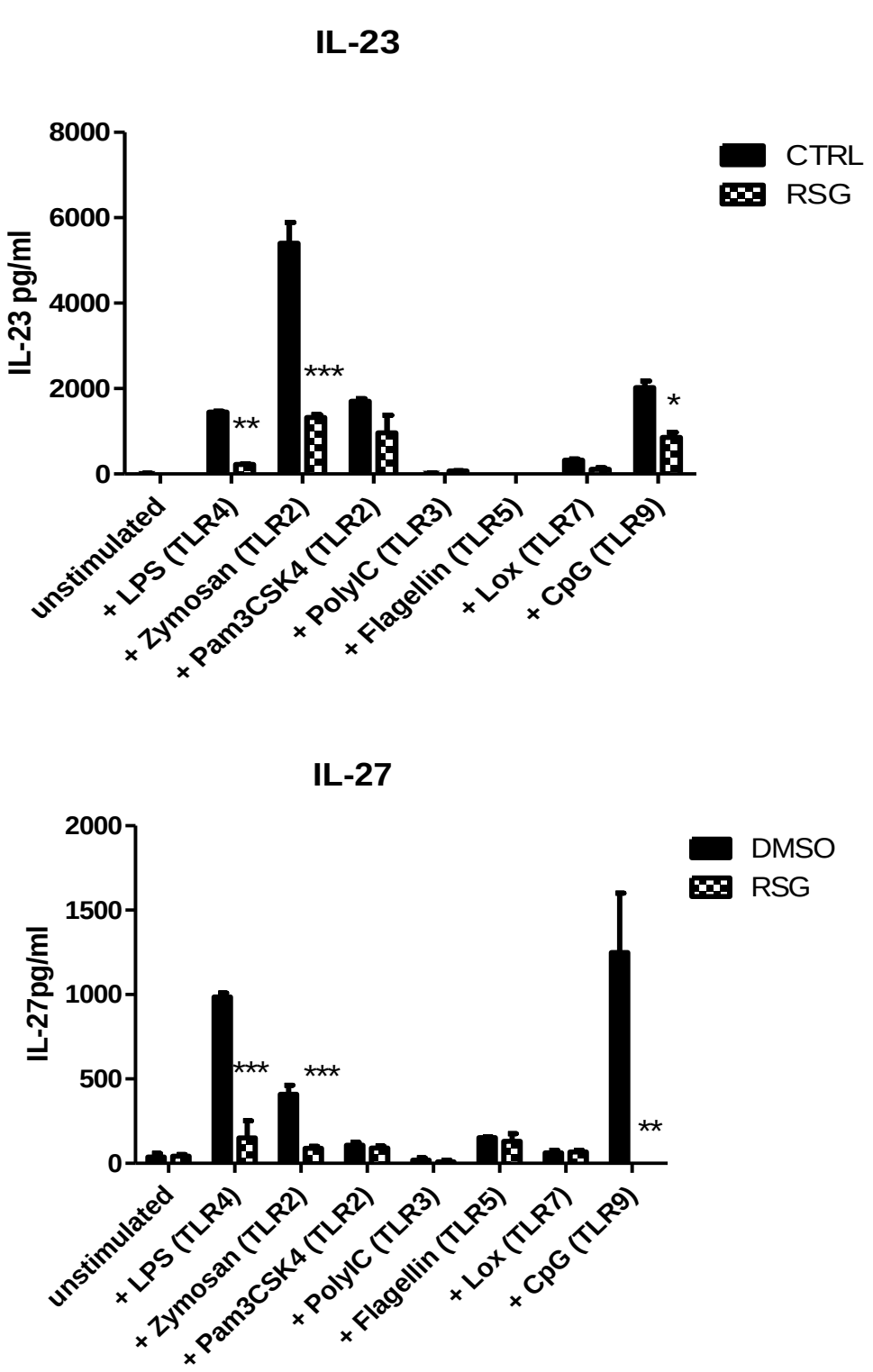


FIGURE 4.16

Activation of PPARγ modulates the IL-12 family of cytokines in BMDC matured with a panel of TLR ligands

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the PPARγ agonist RSG (10μM) after which cells were plated and stimulated for 24hrs with a panel of TLR ligands. Supernatants were then harvested and assessed for levels of IL-23 and IL-27 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. RSG/LPS groups as determined by one-way ANOVA test.

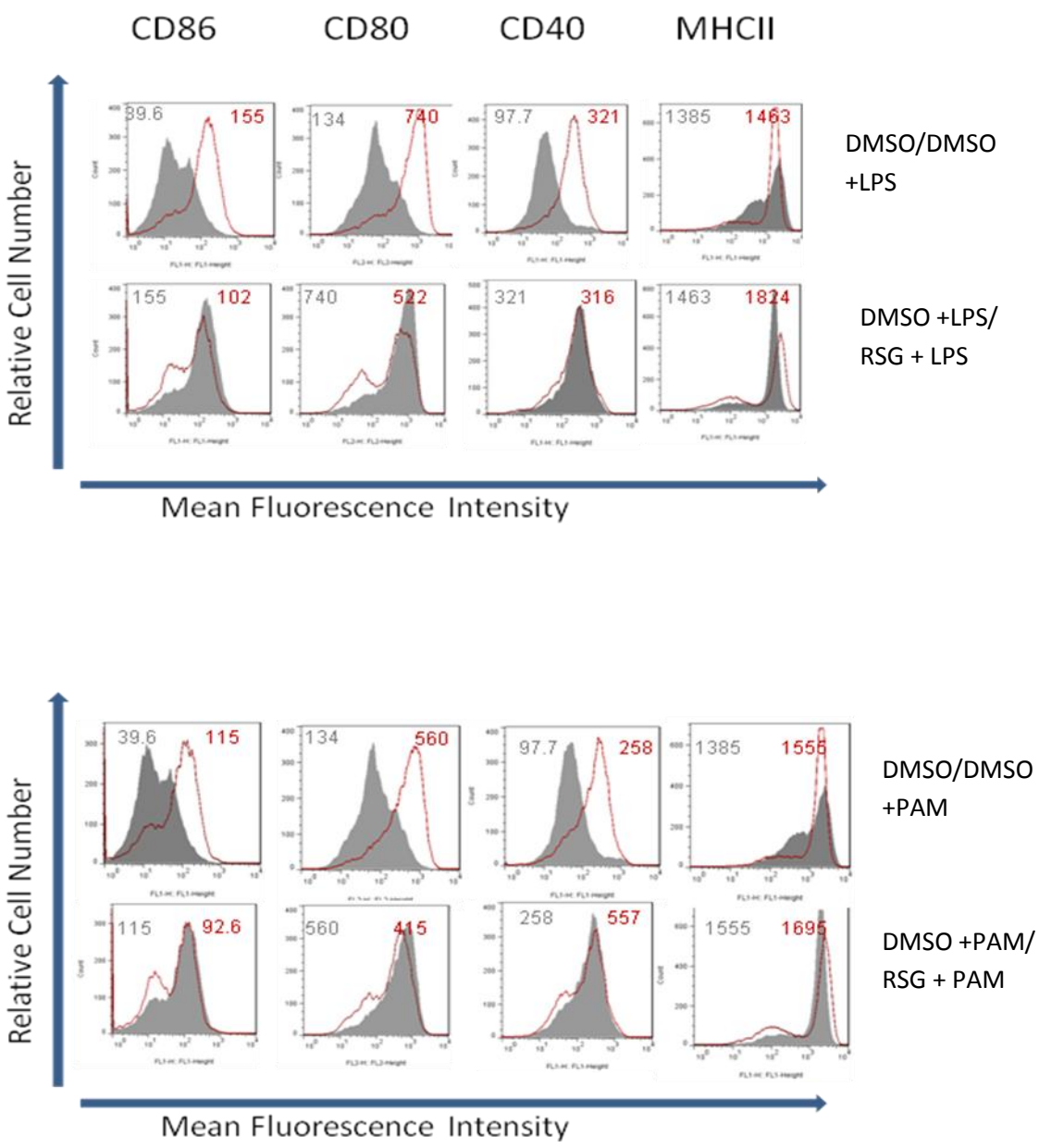


FIGURE 4.17

PPARγ activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the PPARγ agonist RSG (10µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with LPS 100ng/ml **[A]**or Pam3CSK4 (5µg) **[B]**. Row 2 represents DMSO with the TLR ligand stimulation (shaded grey) overlaid with RSG (10µM) with the TLR ligand stimulation.

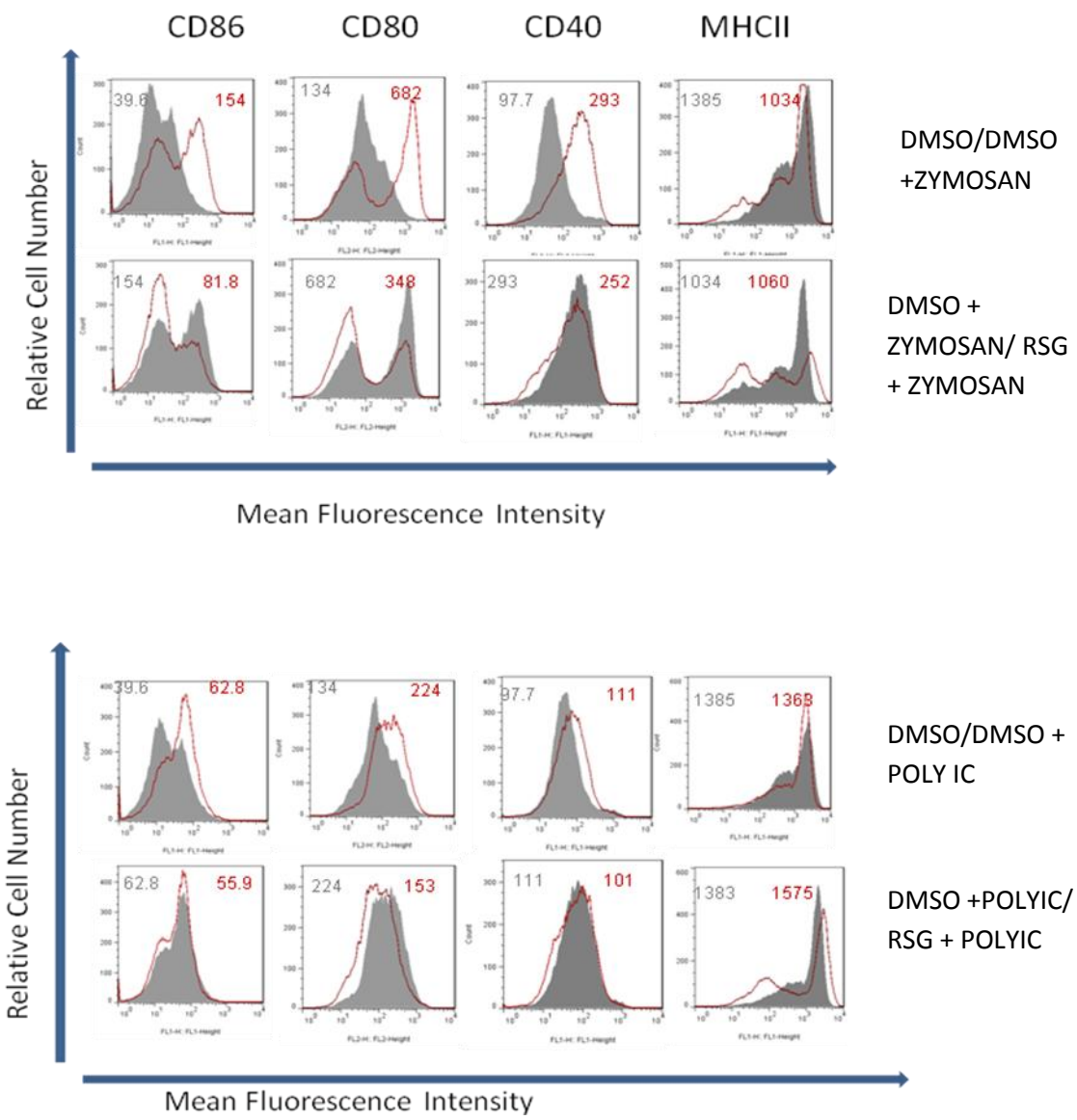


FIGURE 4.18

PPARγ activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the PPARγ agonist RSG (10µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with Zymosan (10µg/ml) **[A]** or Poly: (IC) (10µg) **[B]**. Row 2 represents DMSO with the TLR ligand stimulation (shaded grey) overlaid with RSG (10µM) with the TLR ligand stimulation.

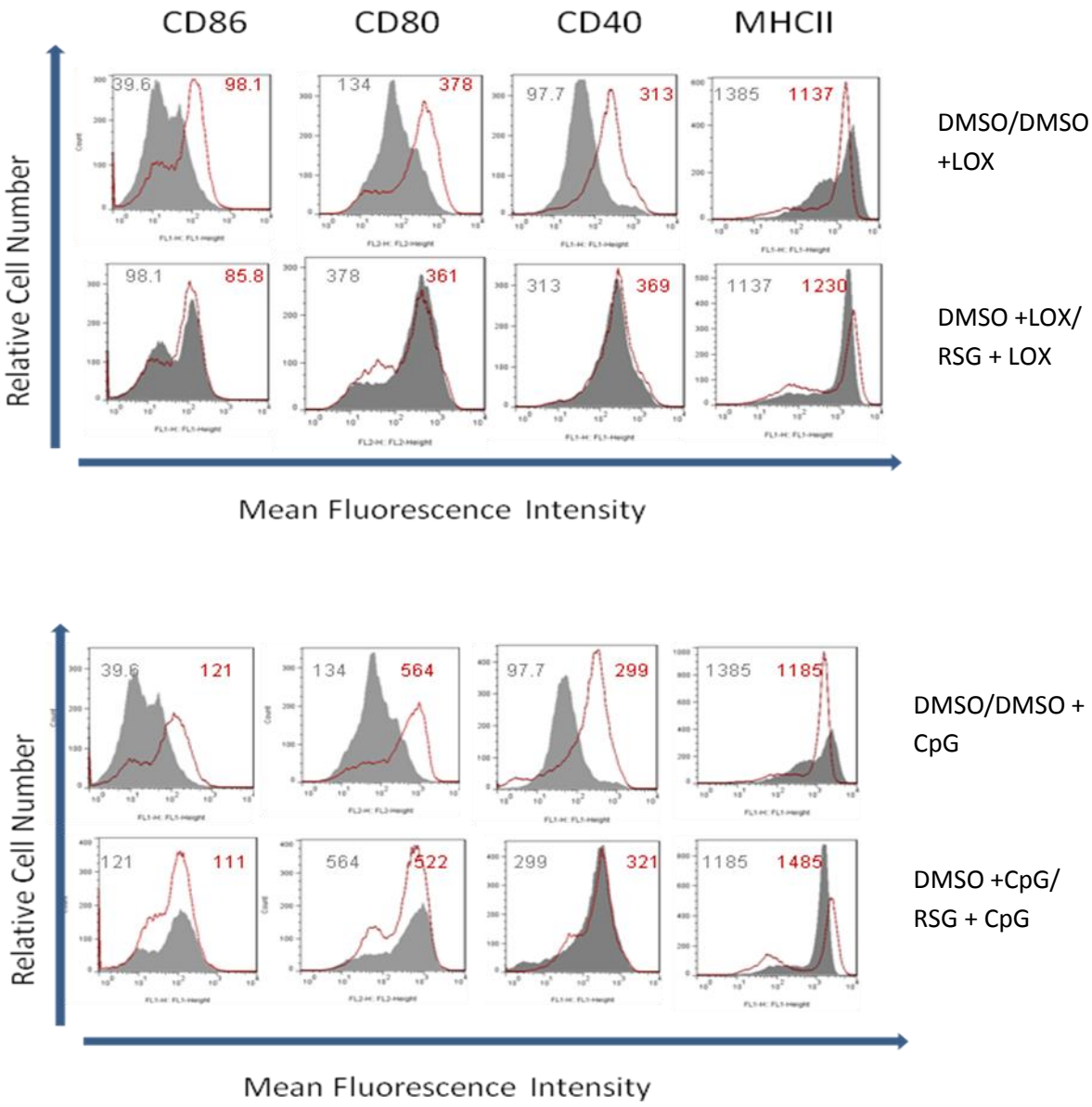


FIGURE 4.19

PPARγ activation modulates DC surface marker expression regardless of mode of TLR activation
BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the PPARγ agonist RSG (10μM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with loxoribine (1μM) **[A]** or CpG (2mM) **[B]**. Row 2 represents DMSO with the TLR ligand stimulation (shaded grey) overlaid with RSG (10μM) with the TLR ligand stimulation.

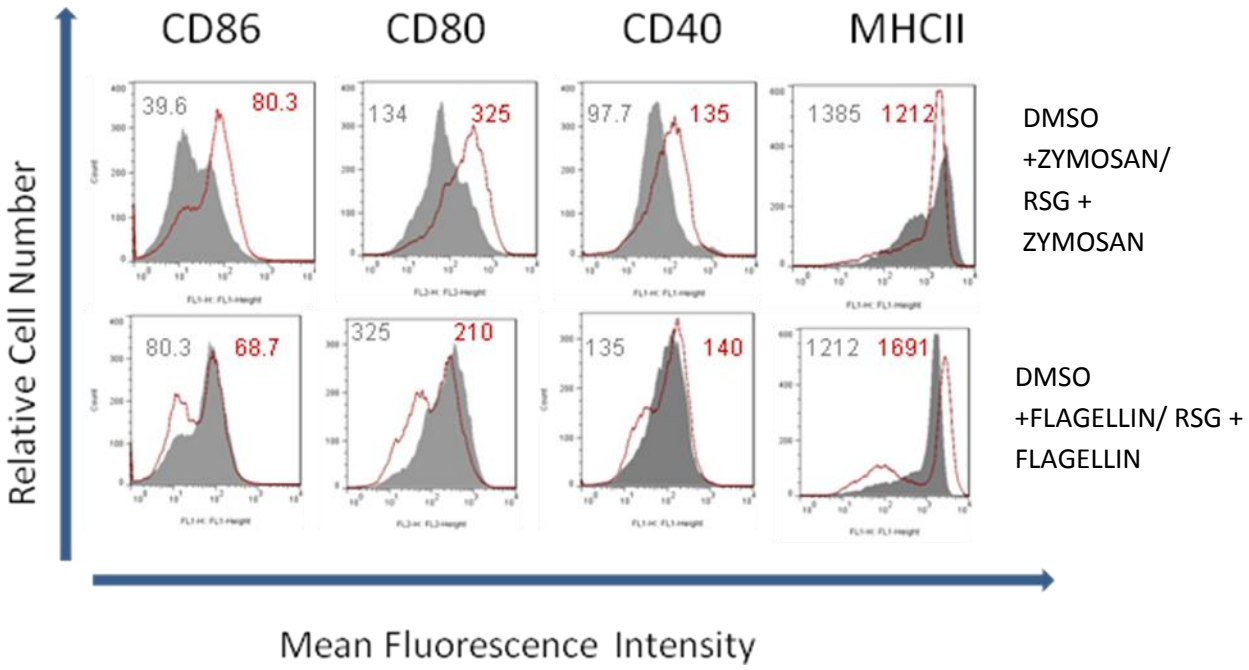


FIGURE 4.20

PPARγ activation modulates DC surface marker expression regardless of mode of TLR activation

BMDC were differentiated in GMCSF for 7 days in the presence of either DMSO or the PPARγ agonist RSG (10μM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Cells were washed and stained with antibodies specific for CD86, CD80 and CD40 and MHCII. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO stimulated with flagellin (5μg). Row 2 represents DMSO with the TLR stimulation (shaded grey) overlaid with RSG (10μM) with the TLR stimulation

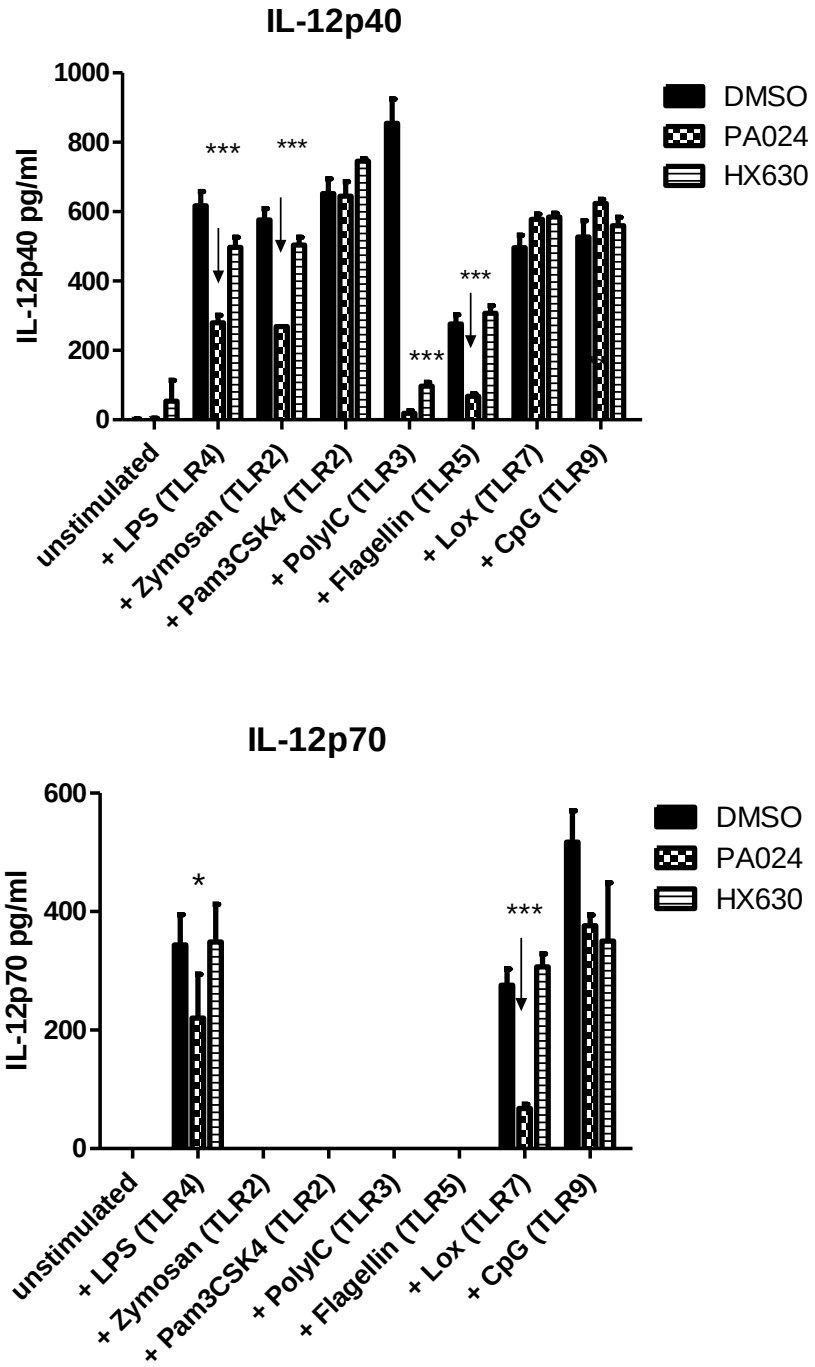


FIGURE 4.21

Activation of RXR modulates the IL-12 family of cytokines in BMDC matured with a panel of TLR ligands

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1μM) after which cells were plated and stimulated for 24hrs with a panel of TLR ligands. Supernatants were then harvested and assessed for levels of IL-12p40 and IL-12p70 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, *p<0.05 comparing DMSO/LPS vs. RXR/LPS groups as determined by one-way ANOVA test.

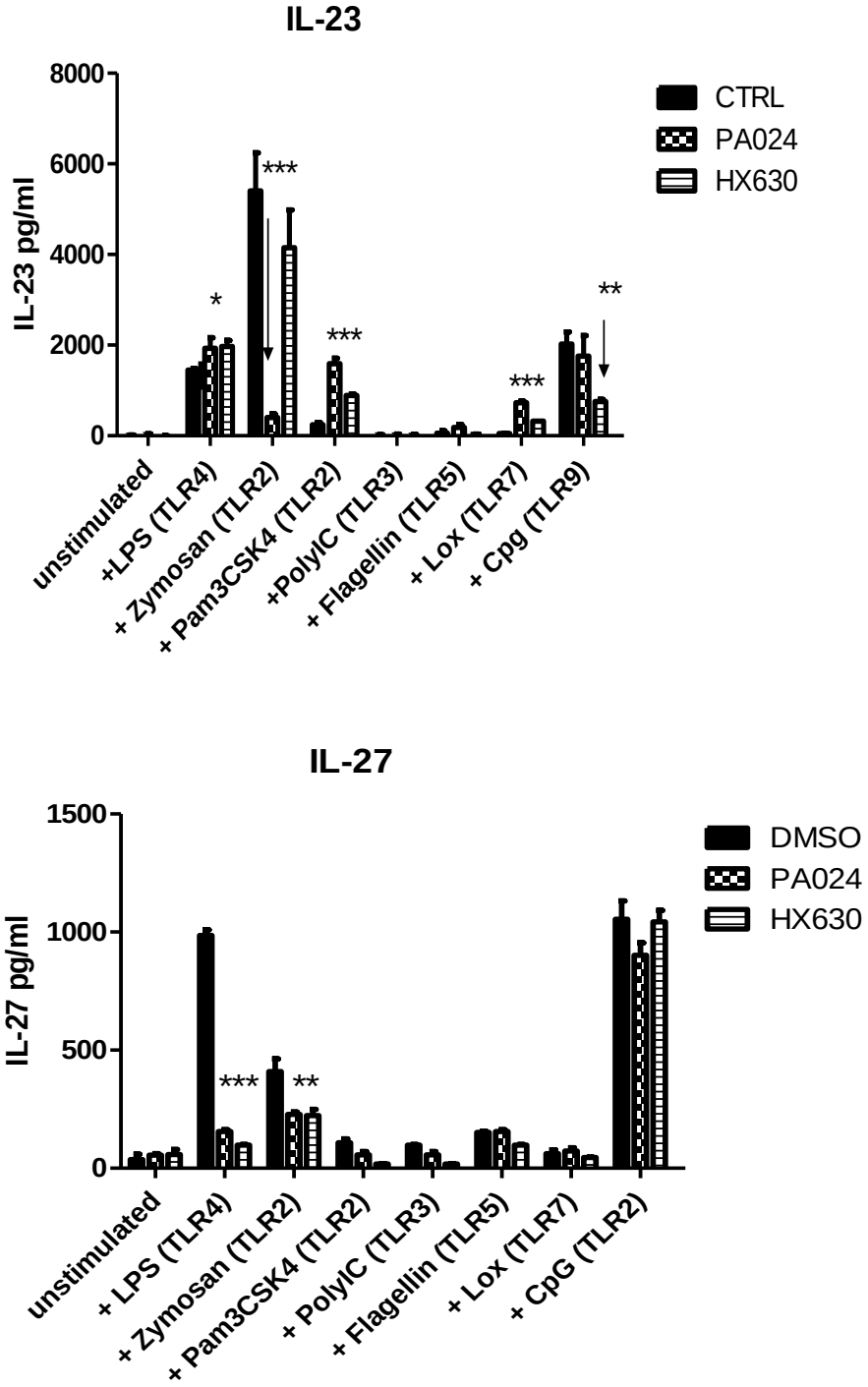


FIGURE 4.22

Activation of RXR modulates the IL-12 family of cytokines in BMDC matured with a panel of TLR ligands

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) after which cells were plated and stimulated for 24hrs with a panel of TLR ligands. Supernatants were then harvested and assessed for levels of IL-23 and IL-27 using immunospecific assays. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001, **p<0.01, *p<0.05 comparing DMSO/LPS vs. RXR/LPS groups as determined by one-way ANOVA test.

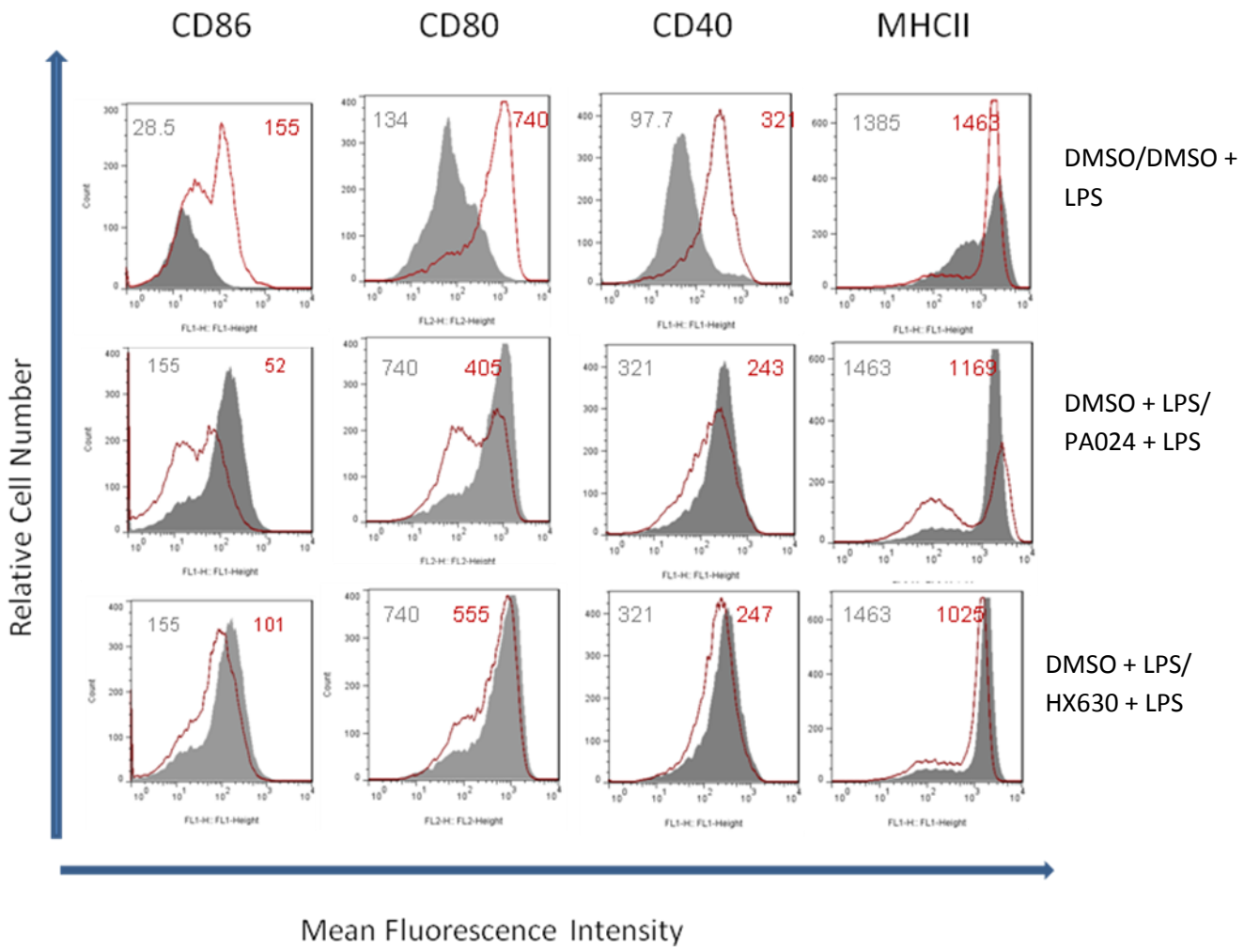


FIGURE: 4.23

RXR activation modulates DC surface marker expression regardless of mode of activation.

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with LPS 100ng/ml. Row 2 represents DMSO with LPS (shaded grey) overlaid with PA024 (1µM) with LPS and Row 3 represents DMSO + LPS (shaded grey) overlaid with HX630 (1µM) with LPS.

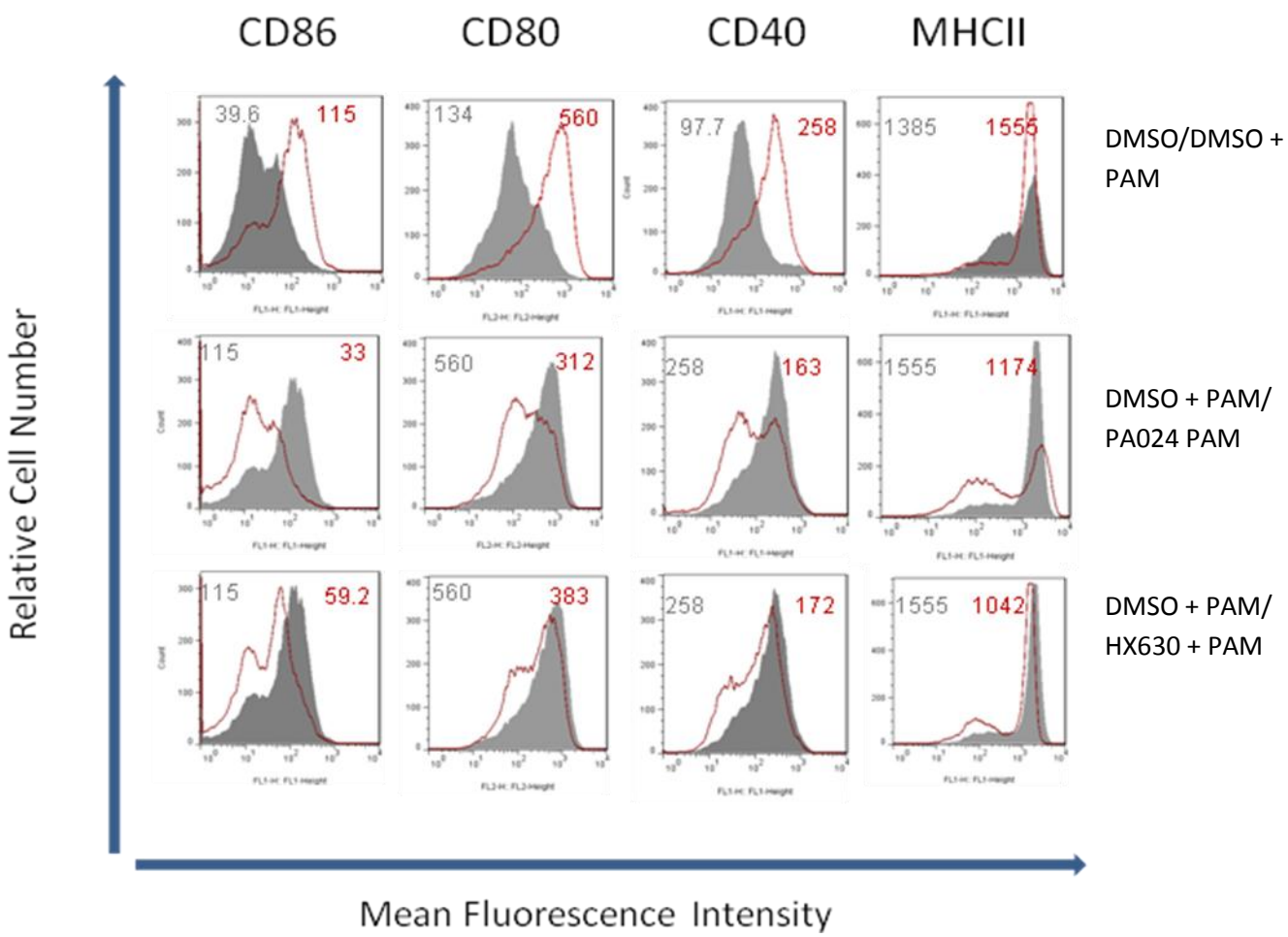


FIGURE: 4.24

RXR activation modulates DC surface marker expression regardless of mode of activation.

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with Pam3CSK4 (5µg). Row 2 represents DMSO + Pam3CSK4 (shaded grey) overlaid with PA024 (1µM) + Pam3SK4 and Row 3 represents DMSO + Pam3CSK4 (shaded grey) overlaid with HX630 (1µM) + Pam3CSK4.

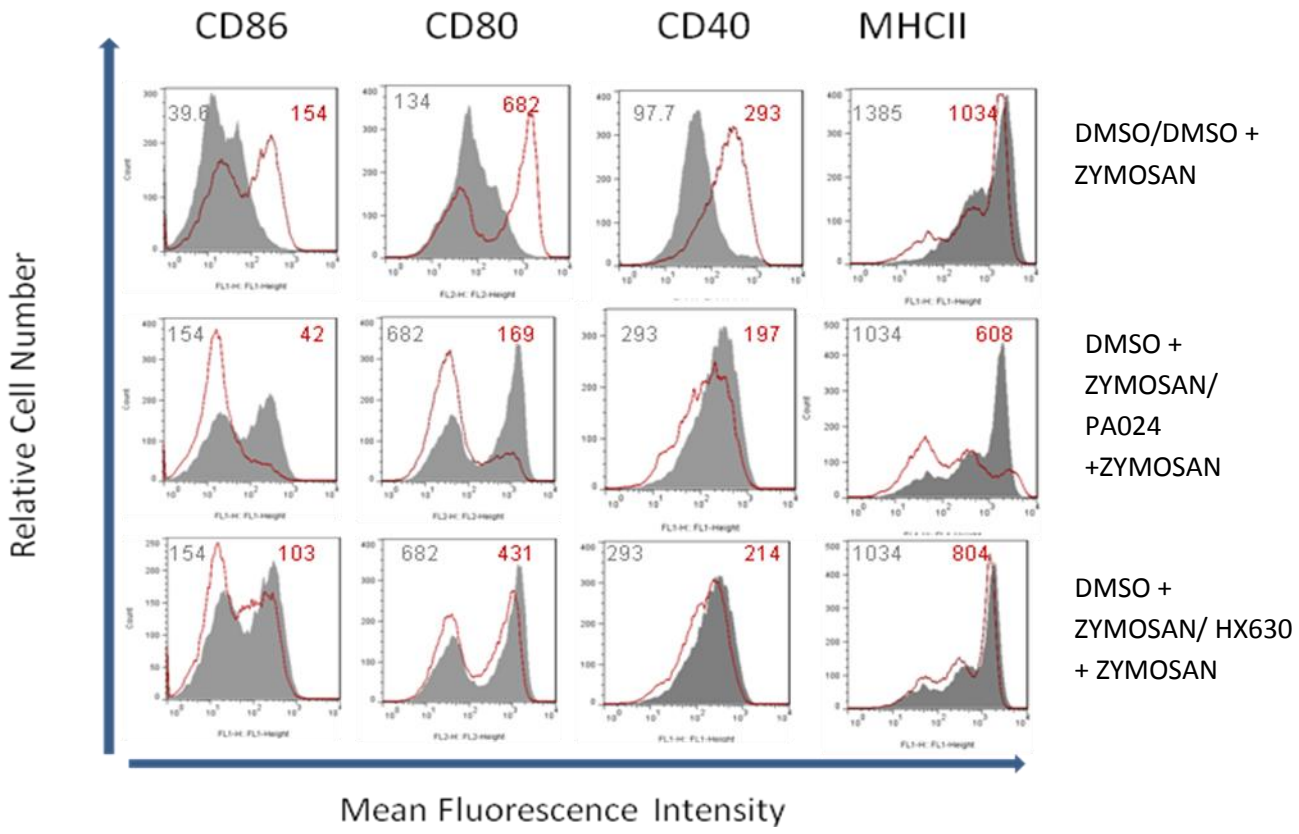


FIGURE: 4.25

RXR activation modulates DC surface marker expression regardless of mode of activation.

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with Zymosan (10µg/ml)). Row 2 represents DMSO + Zymosan (shaded grey) overlaid with PA024 (1µM) + Zymosan and Row 3 represents DMSO + Zymosan (shaded grey) overlaid with HX630 (1µM) + Zymosan.

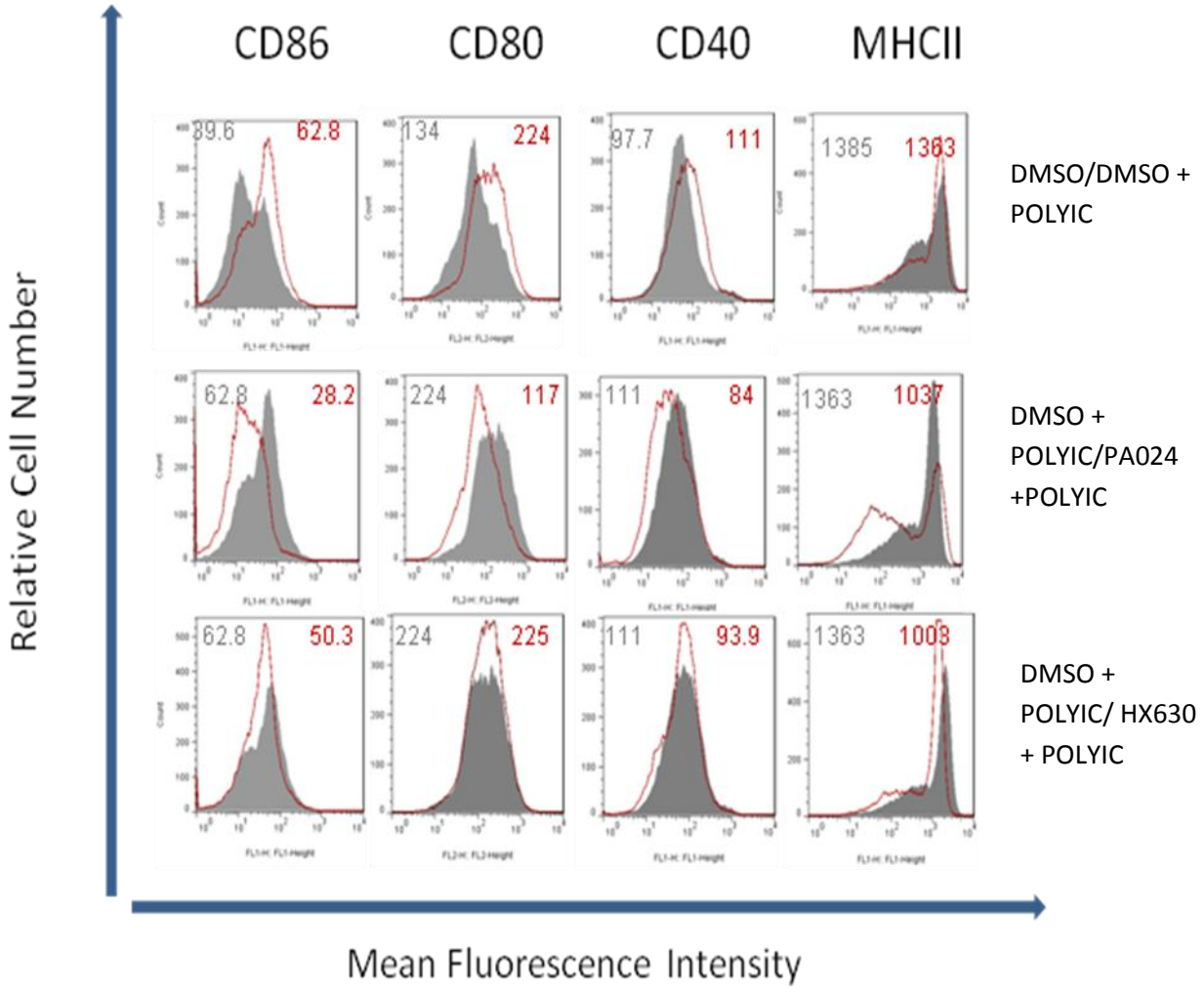


FIGURE: 4.26

RXR activation modulates DC surface marker expression regardless of mode of activation.

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with Poly:(IC) (10µg). Row 2 represents DMSO + Poly: (IC) (shaded grey) overlaid with PA024 (1µM) + Poly:(IC) and Row 3 represents DMSO + PGN (shaded grey) overlaid with HX630 (1µM) + Poly:(IC).

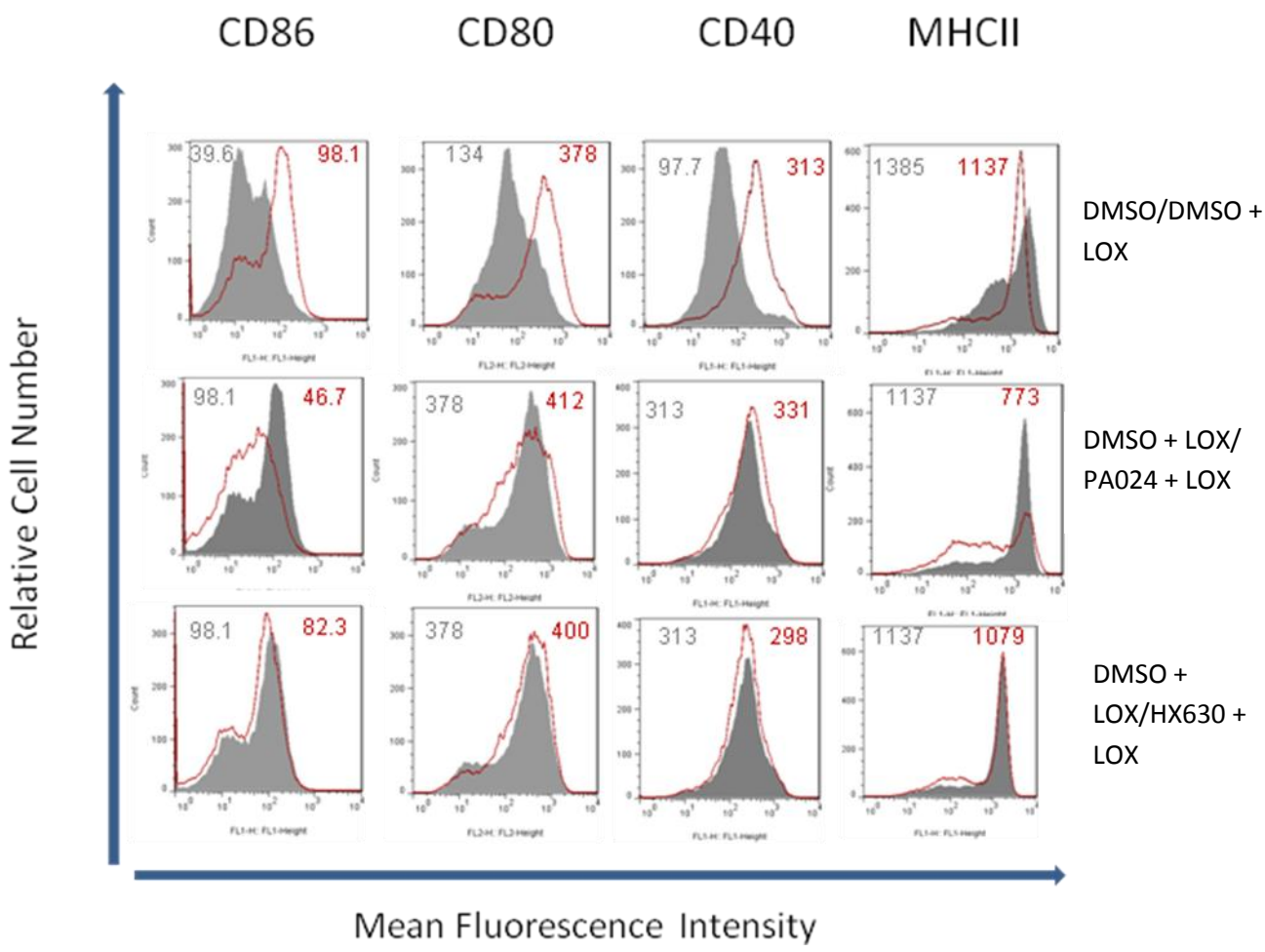


FIGURE: 4.27

RXR activation modulates DC surface marker expression regardless of mode of activation.

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with loxoribine (1µM). Row 2 represents DMSO + loxoribine (shaded grey) overlaid with PA024 (1µM) + loxoribine and Row 3 represents DMSO + loxoribine (shaded grey) overlaid with HX630 (1µM) + loxoribine.

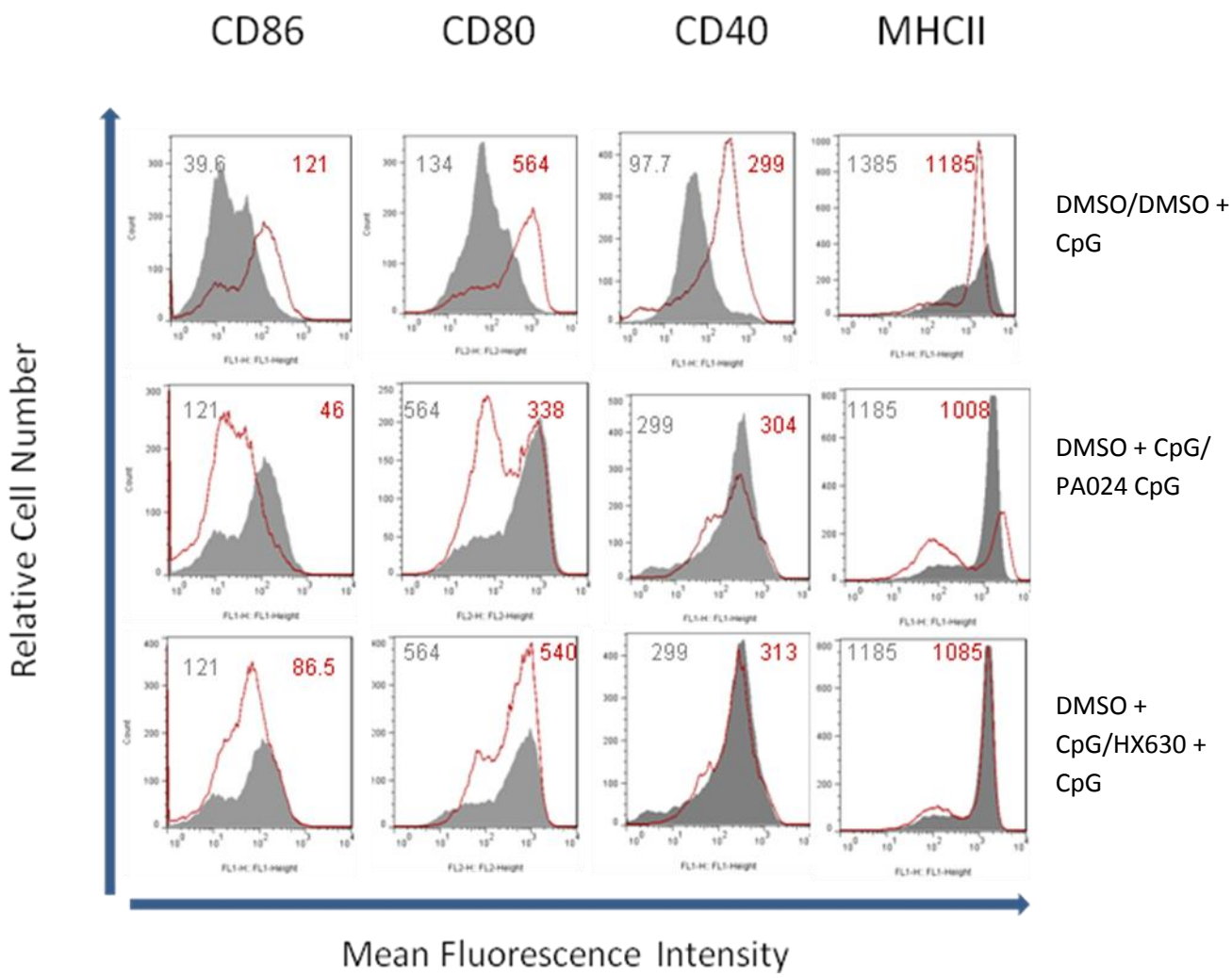


FIGURE: 4.28

RXR activation modulates DC surface marker expression regardless of mode of activation.
BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with CpG (2mM). Row 2 represents DMSO + CpG (shaded grey) overlaid with PA024 (1µM) + CpG and Row 3 represents DMSO + CpG (shaded grey) overlaid with HX630 (1µM) + CpG.

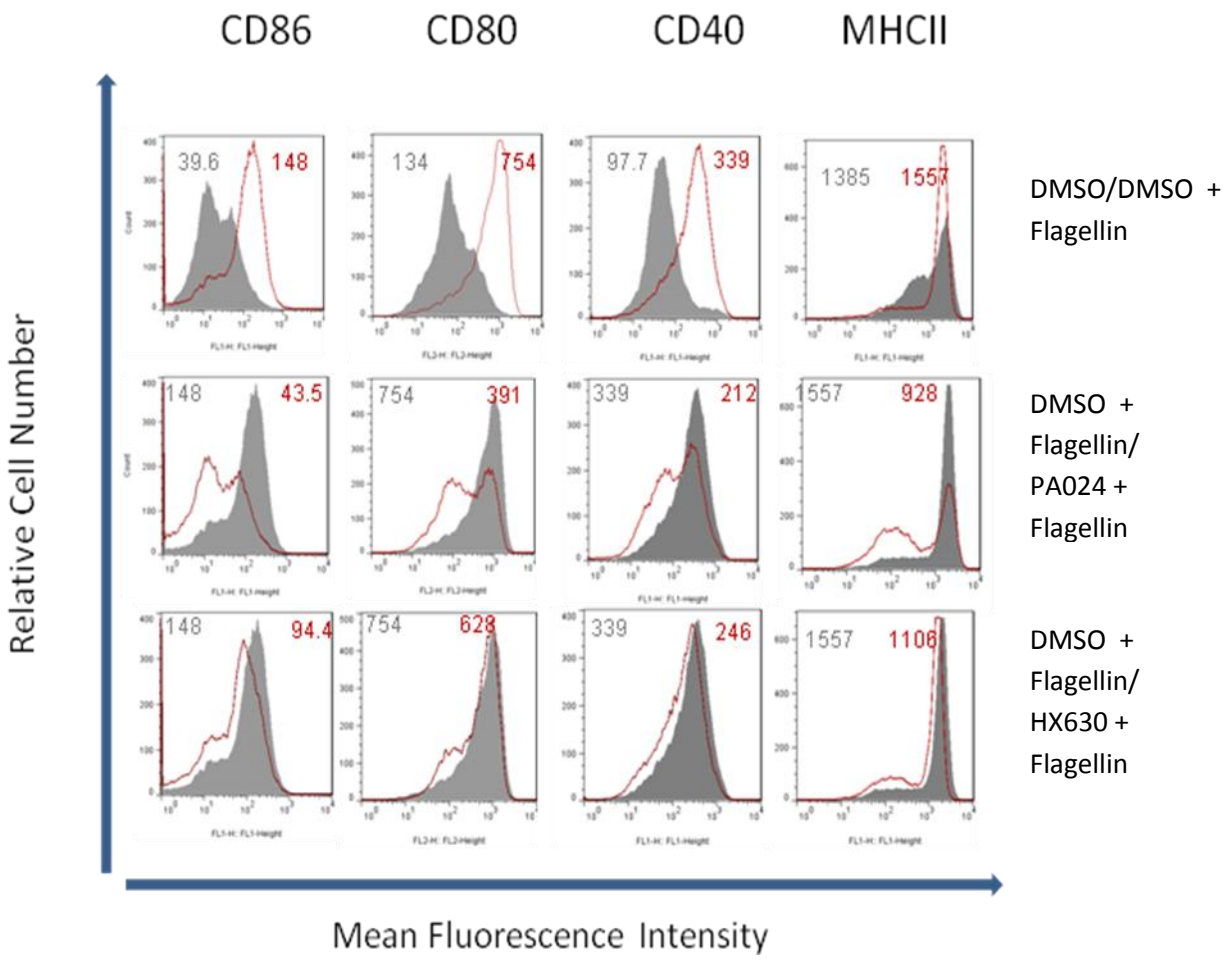


FIGURE: 4.29

RXR activation modulates DC surface marker expression regardless of mode of activation.

BMDC were differentiated in GMCSF for 7 days in the presence of the RXR agonists PA024 or HX630 (1µM) before being plated and stimulated with a panel of TLR ligands for 24hrs. Row 1 represents DMSO vehicle control (shaded grey) overlaid with DMSO vehicle control stimulated with Flagellin (5µg/ml)). Row 2 represents DMSO + Flagellin (shaded grey) overlaid with PA024 (1µM) + Flagellin and Row 3 represents DMSO + Flagellin(shaded grey) overlaid with HX630 (1µM) + Flagellin.

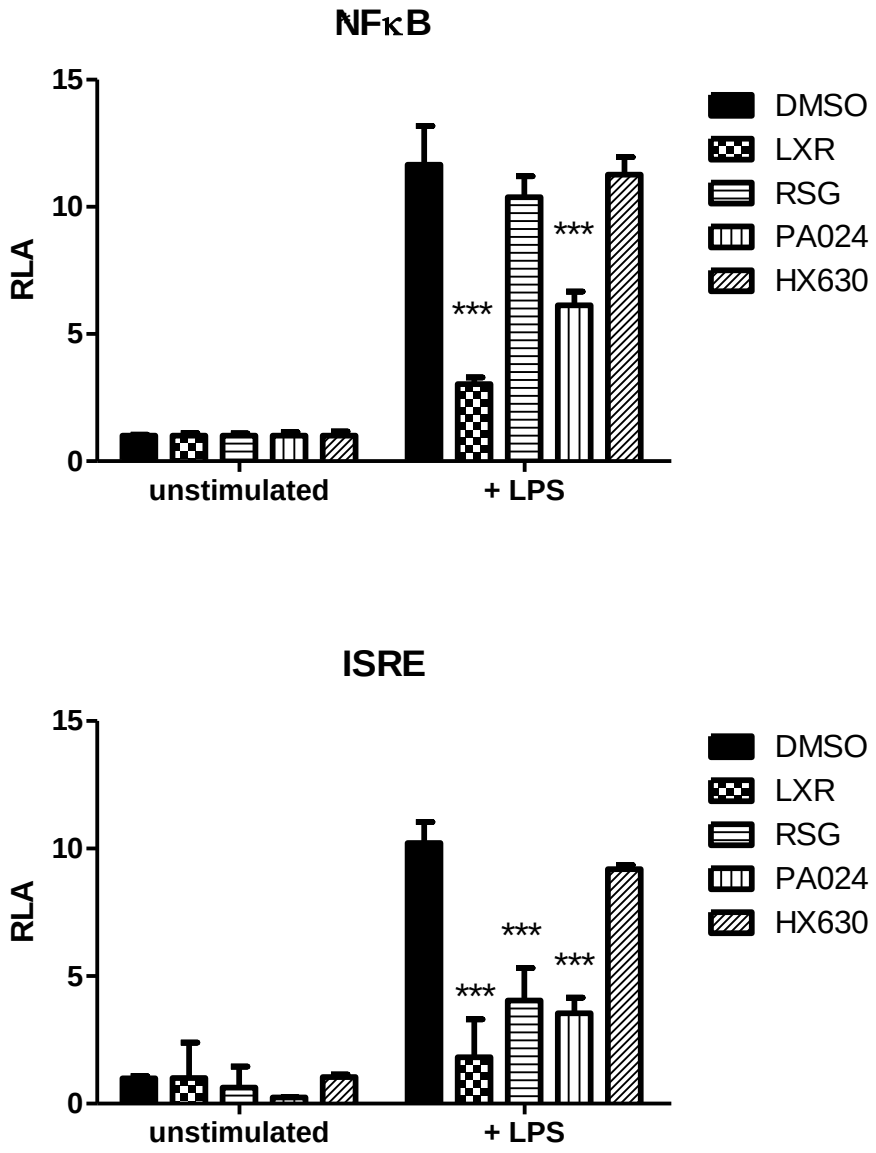


FIGURE 4.30

LXR and RXR activation suppress the induction of both NFκB and IRF dependent gene transcription

Hek293 stably expressing CD14, MD2 and TLR4 were cultured for 7 days with specific PPARγ (RSG 10μM), LXR (T0901317 2μM) and RXR (PA024, HX630 1μM) agonists. Cells were then plated and transiently transfected with either NFκB or ISRE luciferase reporter constructs for 24hrs before being stimulated with LPS (100ng/ml). Firefly luciferase activity was quantified 6 hrs after LPS stimulation and subsequently all samples were normalised to Renilla Luciferase. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001 comparing DMSO/LPS vs. Nuclear Receptor agonist/LPS groups as determined by one-way ANOVA test.

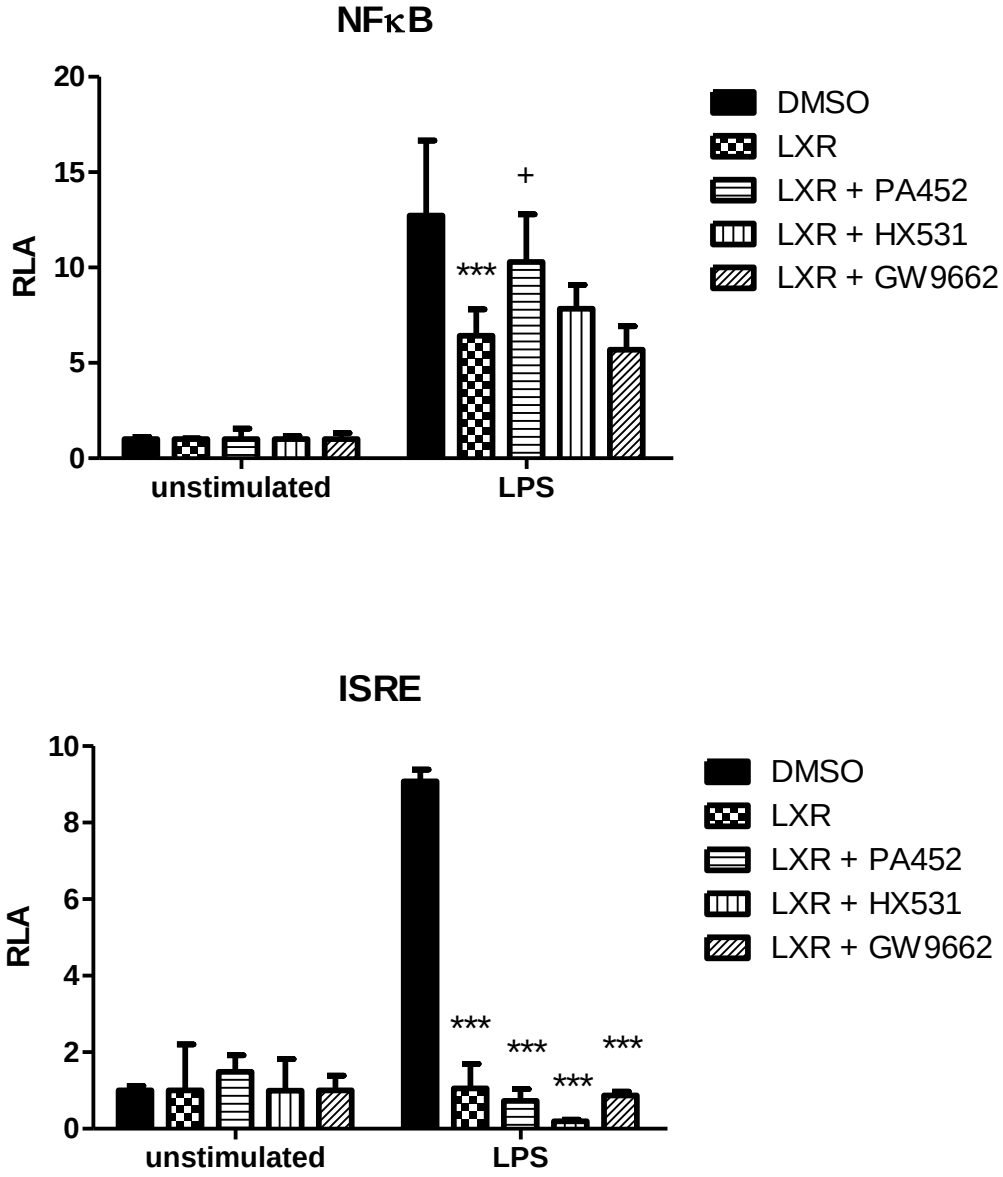


FIGURE: 4.31

The suppressive effect of LXR on NFκB is reversed in the presence of the specific RXR inhibitor PA452

Hek293 stably expressing CD14, MD2 and TLR4 were cultured for 7 days in the presence of the specific LXR agonist (T0901317 2μM) and specific PPARγ and RXR antagonists. The RXR antagonists (PA452, HX531 1μM) and the PPARγ antagonist where added 2 hours prior to LXR activation. Cells were then plated and transiently transfected for 24hrs with NFκB and ISRE luciferase constructs before being stimulated with LPS (100ng/ml). Firefly luciferase activity was quantified 6 hrs after LPS stimulation and all samples were normalised to Renilla Luciferase. Results are ± SEM of triplicate assays and represent three independent experiments. ***p<0.001 comparing DMSO/LPS vs. T0901317/LPS vs. T0901317/Antagonist/LPS groups as determined by one-way ANOVA test.

Effect of LXR	TLR4	TLR2/1	TLR2/6	TLR3	TLR7	TLR9	TLR5
↓ CD86	✔	✔	✔	✔	✔	✔	✔
↓ CD80	✔	✔	✔	✔	✔	✔	✔
↓ CD40	✔	✔	✔	✔	**x**	✔	✔
↓ MHCII	✔	✔	✔	✔	✔	✔	✔
↓ IL-12p40	✔	**x**	✔	✔	✔	**x**	**x**
↓ IL-12p70	✔	**Not produced**	**Not produced**	**Not produced**	✔	✔	**Not produced**
↓ IL-27	✔	**x**	✔	**Not produced**	✔	**x**	**x**
↓ IL-23	✔	✔	✔	**Not produced**	**x**	**x**	**Not produced**

Table 4.1: Summary of results from LXR treated cells stimulated with a panel of TLR ligands

Effect of PPARγ	TLR4	TLR2/1	TLR2/6	TLR3	TLR7	TLR9	TLR5
↓ CD86	✔	x	✔	✔	x	x	✔
↓ CD80	✔	✔	✔	✔	x	x	✔
↓ CD40	✔	x	✔	✔	x	x	x
↓ MHCII	x	x	x	x	x	x	x
↓ IL-12p40	✔	✔	✔	✔	✔	x	✔
↓ IL-12p70	✔	Not produced	Not produced	Not produced	✔	✔	Not produced
↓ IL-27	✔	x	✔	Not produced	x	✔	x
↓ IL-23	✔	x	✔	Not produced	x	✔	Not produced

Table 4.2: Summary of results from RSG treated cells stimulated with a panel of TLR ligands

Effect of PA024	TLR4	TLR2/1	TLR2/6	TLR3	TLR7	TLR9	TLR5
↓ CD86	✔	✔	✔	✔	✔	✔	✔
↓ CD80	✔	✔	✔	✔	**x**	✔	✔
↓ CD40	✔	✔	✔	✔	**x**	**x**	✔
↓ MHCII	✔	✔	✔	✔	✔	✔	**x**
↓ IL-12p40	✔	✔	**x**	✔	**x**	**x**	✔
↓ IL-12p70	✔	**Not produced**	**Not produced**	**Not produced**	✔	**x**	**Not produced**
↓ IL-27	✔	**x**	✔	**Not produced**	**x**	**x**	**x**
↓ IL-23	**x**	**x**	✔	**Not produced**	**x**	**x**	**Not produced**

Effect of HX630	TLR4	TLR2/1	TLR2/6	TLR3	TLR7	TLR9	TLR5
↓ CD86	✔	✔	✔	✔	✔	✔	✔
↓ CD80	✔	✔	✔	**x**	**x**	✔	✔
↓ CD40	✔	✔	✔	**x**	**x**	**x**	✔
↓ MHCII	✔	✔	✔	✔	✔	✔	✔
↓ IL-12p40	**x**	**x**	**x**	✔	**x**	**x**	**x**
↓ IL-12p70	**x**	**Not produced**	**Not produced**	**Not produced**	**x**	**x**	**Not produced**
↓ IL-27	✔	**x**	✔	**Not produced**	**x**	**x**	**x**
↓ IL-23	**x**	**x**	**x**	**Not produced**	**x**	✔	**Not produced**

Table 4.3: Summary of results from RXR treated cells stimulated with a panel of TLR ligands

4.3 DISCUSSION

The aim of this study was to investigate which nuclear receptor heterodimers are important for how they exert their effects and also to investigate the mechanism of action of these nuclear receptors in DC. Our results show that the ability of LXR to suppress the IL-12 family of cytokines (IL-12p40, IL-12p70, IL-23 and IL-27) is abrogated in the presence of the specific RXR inhibitor PA452. PA452 inhibits RXR when it is heterodimerised with LXR, PPARγ and other members of the nuclear receptor family. We see no reversal with the HX630 inhibitor which only inhibits RXR when it dimerises with PPARγ, thus highlighting that LXR must heterodimerise with RXR in order to inhibit the production of these cytokines. This hypothesis was established based on our understanding of these specific nuclear receptor agonists and antagonists.

Upon nuclear receptor ligation, the LBP of the receptor undergoes a conformation change – specifically altering the orientation of Helix 12 and thus sealing this LBP. This stabilizes the receptor and leads to the recruitment of coactivators which ultimately lead to a targeted response. However following the use of nuclear receptor antagonists the conformation of the receptor is left in a less compact form by displacing H12 thus preventing co-activator recruitment and subsequent downstream signalling (Perez et al. 2012). LXR activation suppresses the IL-12 family of cytokines however given that cytokine levels remain unchanged in cells in which RXR is antagonised, we propose that the formation of a LXR:RXR heterodimer is important in how LXR suppresses these cytokines.

Interestingly no other association could be identified amongst the other nuclear receptors examined suggesting that PPARγ and RXR may exert their anti-inflammatory effects through an interaction with a nuclear receptor not examined within this study e.g. FXR, or Pregnane X receptor (PXR). We also show that LXR activation can still suppress the expression of the costimulatory markers CD86 and CD40 in the presence of the RXR antagonist however CD80 expression remains unchanged upon RXR inhibition. This suggests that although LXR must associate with RXR to inhibit production of the IL-12's, this association may not be important in how LXR regulates surface marker expression.

Following on from this work we next determined if nuclear receptor activation could modulate cytokine production and cell surface marker expression in DC matured with a panel of TLR ligands. TLRs are classified into several groups depending on the particular PAMP they recognise. TLR1, 2, 4 and 6 for example recognise lipids, TLR 5 and 11 recognize protein ligands and TLR3, 7, 8 and 9 which are located intracellularly detect nucleic acids derived from viruses and bacteria (Akira, Uematsu & Takeuchi 2006). Following TLR ligation, two individual signalling pathways can be initiated. TLR 1,2,4,5,7,9 signal through the MyD88 dependent pathway where MyD88 acts as a nonredundant adaptor protein that promotes the induction of pro-inflammatory molecules through NFκB activation. In this pathway TLR 2 and TLR 4 also use the adaptor molecule known as Mal which distinguishes them from other TLRs. TLR3 and TLR4 use the adaptor molecule TRIF to mediate downstream signalling and so initiate the MyD88-independent pathway which results in the later activation of NFκB and IRF (Kawai, Akira 2010).

Our results show that in DC matured with a panel of TLR ligands, LXR activation suppressed the production of IL-12p40, IL-12p70, IL-23 and IL-27 irrespective of the mode of activation. However there are some notable exceptions to this observation. In TLR5 matured DC the levels of Il-12p40 and IL-27 remained unchanged following LXR activation. Similarly in TLR7 matured DC, LXR activation did not suppress IL-23 production however the cytokine levels of IL-12p40, IL-12p70 were significantly inhibited. Following stimulation with the TLR2/1 ligand Pam3CSK4 there was no change in either IL-27 or IL-12p40 production in LXR activated cells however there was a significant decrease in the production of IL-23. LXR activation in TLR9 stimulated cells also failed to suppress the production of these IL-12 family of cytokines.

As shown in Chapter 3, LXR activation in LPS matured DC can also downregulate the expression of costimulatory markers. Here we show that LPS stimulated LXR activated DC have reduced expression of CD86, CD80, CD40 and MHCII. This decrease is seen to varying degrees in LXR activated cells stimulated with the panel of TLR ligands. However in one case – CD40, the expression remains unchanged following TLR7 ligation. This result is surprising given that the TLR7 pathway is identical to the pathway utilized by TLR 5 and 9. Similarly the varying effects of LXR activation on the secretion of the IL-12 family of cytokines following TLR5 and TLR7 are also surprising. Therefore these results have led us to believe that LXR is unlikely to directly target a single common TLR element – but instead have differential effects depending on the stimulus used to initiate DC maturation. Indeed, if LXR activation failed to suppress these cytokines following TLR3 ligation, this would suggest a requirement for MyD88 to carry out its function. Similarly if a decrease in IL-12 secretion and costimulatory marker expression was only seen following maturation with TLR2 and

TLR4 ligands, then perhaps the adaptor Mal would be highlighted as a potential LXR target. Since this is not the case however, we concluded LXR must target an element of the TLR pathway that is common to all and is further downstream in this inflammatory pathway.

Interestingly this hypothesis also applies to the remaining two nuclear receptors investigated in this study – PPARγ and RXR. PPARγ activation suppressed IL-12p40, IL-12p70, IL-23 and IL-27 following stimulation with all TLR ligands. As seen with LXR there are three exceptions to this observation. TLR7 matured DC treated with the PPARγ agonist RSG showed no inhibition in IL-27 however there was a significant decrease in IL-12p40, IL-12p70 and IL-23 following TLR7 stimulation. TLR9 stimulated cells also showed no change in IL-12p40 production in RSG treated cells whereas TLR2/1 matured DC showed no significant change in the production of IL-23 and IL-27 following PPARγ activation.

The expression of costimulatory and cell surface marker expression was also differentially regulated in PPARγ activated DC following exposure to these TLR ligands. TLR2/1,7 5 and 9 stimulation resulted in an upregulation of CD40 whereas TLR3 and TLR2/6 stimulation caused a small upregulation in CD40 expression. This suggests that depending on how these cells are stimulated, PPARγ can effect DC maturation in very distinct ways.

At the cytokine level RXR activation affected the IL-12 family of cytokines differentially depending on the TLR stimulus used. Notably in TLR7 stimulated cells there was no change in the production of IL-12p40 and IL-27. In TLR9 stimulated cells there was also no change in IL-12p40, IL-12p70 or IL-27 production. Interestingly TLR2/6 ligation also resulted in decreased IL-23 production were previously following TLR4 maturation RXR did not affect the production of this cytokine. RXR activated

cells that were stimulated through this TLR also do not have decreased IL-12p40 expression. Stimulation with the remaining TLR ligands resulted in changes consistent with those shown in Chapter 3 following LPS maturation.

RXR activation in LPS matured DC resulted in a downregulation in CD86, CD80, CD40 and MHCII. This profile was also seen, again to varying degrees in DC matured with a panel of TLR ligands. However cells stimulated through the TLRs 3, 7 and 9 were the exceptions to this observation. Following TLR7 ligation in RXR activated DC there was an increase in CD80 and CD40 expression. Similarly in TLR9 stimulated cells there was also an increase in CD40. There was also no decrease in MHCII following exposure to the TLR5 ligand flagellin. Finally in TLR3 stimulated cells there was no decrease in CD80 and CD40 expression following exposure to the RXR ligand HX630.

The results of this TLR ligand study highlight that LXR, RXR and PPARγ do not have utilise a specific TLR adaptor molecule but instead function further downstream in this inflammatory pathway and perhaps modulate the transcription factors NFκB and IRF. To further investigate this hypothesis we examined the affect of LXR, RXR and PPARγ activation on the transcription of both NFκB and IRF dependent genes using a luciferase reporter gene assay.

Our results showed that LXR and RXR activation can significantly reduce both NFκB and ISRE activity therefore concurring with the results presented within the TLR study. It is interesting to note that of the two RXR agonists used throughout this work HX630 and PA024, only PA024 was capable of inhibiting both NFkB and IRF whereas HX630 did not significantly change either transcription factors. Work undertaken by our collaborators in Japan Kagechika *et al* has highlighted key differences between these ligands in their ability to distinguish heterodimer partner receptors. PA024 activates

both a RXR-PPARγ and RXR-LXRα heterodimers whereas HX630 only activates a RXR-PPARγ heterodimer and does not affect RXR-LXRα heterodimers (Kagechika, Shudo 2005b, Kagechika, Shudo 2005). Since we have previously shown that an LXR: RXR partnership is necessary to suppress the production of the IL-12 family of cytokines, the difference between these two RXR agonist's highlights that the inhibition of NFκB and IRF could also be via an LXR:RXR heterodimer.

To further investigate this theory we examined the effects of LXR activation on NFκB and IRF in the presence of both PPARγ and RXR antagonists. Interestingly, the ability of LXR to downregulate NFκB was abrogated only in the presence of the RXR antagonist and not the PPARγ antagonist. Even in the presence of this RXR inhibitor, LXR activated cells could still suppress IRF however thus indicating that the formation of a LXR:RXR partnership is important for inhibiting NFκB but not IRF transcription.

The work presented in this chapter is novel and to the best of our knowledge no other published study has examined nuclear receptor activation following stimulation with an entire panel of TLR ligands. We have examined both surface marker expression and cytokine production in LXR, RXR and PPARγ activated DC. Similarly no other group has examined the effect of LXR and RXR activation on the IRF and NFκB transcription factors. We have also reported for the first time that an association between LXR and RXR may be important in the regulation of the IL-12 family of cytokines.

Although most of the work shown in this chapter is extremely novel, there are certain aspects of the results presented above that concur with work previously published. One recent study undertaken by Hanley *et al* concluded (as we have) that nuclear receptors, specifically glucocorticoid receptor (GR), PPARγ and LXR could carry out their effects irrespective of their mode of activation but instead by affecting NFκB. This study showed that NR activation could inhibit TLR-activated HIV-1 transcription in primary

macrophages and this repression was reported in response to a wide range of NFκB stimulators such as TL4, TLR2/1 and TLR2/6 ligands (Hanley et al. 2010). There have also been reports suggesting that there is a reciprocal regulation between nuclear receptors and the TLRs whereby activation of TLRs can downregulate the functions of nuclear receptors but (as seen in this work) activation of nuclear receptors can downregulate TLR responses. Castrillo *et al* reported that TLR3 and TLR4 stimulation inhibit LXR activity and this inhibition was reliant on the MyD88 independent pathway (Castrillo et al. 2003). A study undertaken by Hanley *et al* in which they proved that PPARγ and LXR signalling could inhibit DC-mediated HIV1 capture reported that monocyte derived DC matured with the TLR2 ligand Pam3CSK4 and treated with the LXR agonist T0901317 had decreased MHCII, CD80 and CD86 expression (Hanley et al. 2010). This correlates with the results presented in this chapter. Although it has previously been published that LXR can inhibit NFκB activation, this is the first time an association with RXR has been reported.

The effects of RXR activation following exposure to ligands of the TLR pathway has not been explored by other groups. However consistent with our results, one published study by Zapata-Gonzalez *et al* reported that RXR activation in DC inhibited both CD86 and CD80 expression following TLR4 and TLR3 ligation (Zapata-Gonzalez et al. 2007).

Consistent with our PPARγ work, Gurley *et al* reported a decrease in IL-12p40 following PPARγ activation in microglial cells stimulated with ligands to TLR 2,4, 3, 7 and 5 (Gurley et al. 2008). However no other pro-inflammatory cytokines or surface markers were examined within this study.

The results of this study highlight that an association with LXR and RXR results in inhibition of NFκB but not IRF downstream of TLR ligation. It also provides evidence

that nuclear receptors can exert their targeted effects independent of their mode of activation. In the next chapter we aim to determine the molecular target of these LXR:RXR heterodimers upstream of NFκB.

CHAPTER 5

IDENTIFICATION OF THE LIVER X RECEPTOR'S MOLECULAR TARGET

5.1 INTRODUCTION

NFκB is a transcription factor whose activation is essential for the regulation of cellular immunity, stress responses, apoptosis and differentiation (Ruland 2011). In particular its activation in immune cells, in response to injury or infection controls the expression of pro and anti-inflammatory mediators such as cytokines. In vertebrates, NFκB consists of five members – Rel A (p65), RelB, c-Rel, NFκB1 (p50/p105) and NFκB2 (p52/p100). These members form homo or heterodimers with each other and subsequently bind to specific target sites within the genome (Siggers et al. 2011). NFκB1 and NFκB2 are synthesised as large precursors known as p105 and p100 respectively. These are partially proteolysed by the proteosome, which removes their C-terminal halves to produce active NFκBp50 and NFκBp52 (Beinke, Ley 2004). The subunits p65 and c-Rel both have strong C-terminal transactivation domains and can strongly activate transcription of target genes. RelB also contains a transactivation domain and can function as an NFκB activator when complexed with p50 or p52. NFκBp50 and NFκBp52 lack a transactivation domain and therefore can only promote transcription when heterodimerised with another Rel subunit (Amit, Ben-Neriah 2002). Under resting conditions, these NFκB dimers are bound to inhibitory proteins known as the IκB proteins. Here, IκB sequesters NFκB complexes in the cytoplasm and prevents dysregulated gene activation. However, in the presence of an activation stimulus, these IκB proteins become degraded, releasing NFκB and allowing the dimer complex to translocate to the nucleus. Degradation of IκB is initiated through phosphorylation by the IKK complex, which leads to ubiquitination and ultimately proteasomal degradation (Beinke, Ley 2004).

Within the cell, there are two main NFκB pathways known as the canonical and the alternative pathway. The canonical pathway is induced in response to activation of

cytokine receptors such as TNFα or in response to activation of pattern recognition receptors such as TLR4 and is therefore the more common of the two pathways (Ghosh, May & Kopp 1998). Each NFκB family member and indeed each dimeric complex is known to regulate a specific set of genes. The generation of mice deficient in each of these family members provided the strongest evidence for this, with each deficiency resulting in a different phenotype (Ghosh, May & Kopp 1998). While redundancies among NFκB members has made it more difficult to identify specific target genes, due to the essential role the IL-12 family of cytokines play in immunity and disease, the NFκB mechanism associated with their production has been extensively studied. The production of IL-12p40 has been linked to the formation of p65/p50 heterodimers in response to LPS (Sanjabi et al. 2000) whereas the production of its cytokine partner IL-12p35 is associated with p50/c-Rel dimer complexes (Kollet, Petro 2006). The expression of EBI3, one of the subunits of IL-27, is induced by p50/p65 heterodimers binding to the NFκB response element in the promoter of the gene (Wirtz et al. 2005). Finally IL-27p28 and IL-23p19 are produced in response to LPS by the formation of c-Rel/p65 heterodimers (Zhang et al. 2010, Carmody et al. 2007) [**Figure 5.5**].

We have previously shown in Chapter 3 the effect of LXR activation on the IL-12 family of cytokines and its ability to significantly downregulate their production. In chapter 4 we demonstrated that LXR can specifically decrease NFκB activation. Therefore in order to gain more information on the specific NFκB subunits targeted by LXR, we examined the expression of the individual cytokine subunits that make up IL-12 (p40 and p35), IL-23 (p40 and p19) and IL-27 (p28 and EBI3) which are all under the control of different NFκB subunits. It was our aim, through this experimental approach to thus identify which subunits were responsive to LXR activation and ultimately to identify a possible NFκB target.

5.2 RESULTS

5.2.1 LXR ACTIVATION IN DENDRITIC CELLS POSITIVELY REGULATES THE EXPRESSION OF ITSELF AND THE EXPRESSION OF ITS HETERODIMERISATION PARTNER, RXR.

BMDC were harvested and differentiated in GMCSF in the presence of T0901317 (2μM) or DMSO for 7 days. Cells were then stimulated over the course of 24hr with LPS (100ng/ml). Total RNA was extracted, converted to cDNA and subsequently used to assess the expression of LXRα RXRα and PPARγ. The relative levels of these transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control tissue was adjusted to 1 and served as the basal reference value throughout experiments. Results are representative of fold change within the sample.

Following LPS stimulation in DMSO control cells there was a significant increase in the expression of LXR over the course of 24hr **[Figure 5.1].** LXR activation significantly enhances this expression at 4hr ($p<0.001$), 6hr ($p<0.001$) and 12hr ($p<0.01$) post LPS stimulation **[Figure 5.1]**. in contrast the expression of RXR decreases following LPS stimulation in DMSO control cells, however LXR activation in these cells significantly increased the expression of RXR at 30 min, 2hr ($p<0.05$),4hr, 6hr and 12hr LPS stimulation ($p<0.001$). The expression of PPARγ is also decreased following LPS stimulation in DMSO cells. LXR activation does not significantly alter PPARγ expression in these cells however in unstimulated cells (0hr) there is a significant decrease in the expression of the receptor ($p<0.001$) **[Figure 5.1].**

5.2.2 LXR ACTIVATION IN DENDRITIC CELLS SPECIFICALLY TARGETS THE IL-12p40, IL-12p35 AND EBI3 CYTOKINE SUBUNITS.

BMDC were harvested and differentiated in GMCSF in the presence of T0901317 (2μM) or DMSO for 7 days. Cells were then stimulated over the course of 24hr with LPS (100ng/ml). Total RNA was extracted, converted to cDNA and subsequently used to assess the expression of EBI3, IL-12p40, IL-12p35, IL-23p19 and IL-27p28. The relative levels of these transcripts were then calculated using the ΔΔCt method, after normalizing with S18 as the endogenous control. The relative level of mRNA in untreated control tissue was adjusted to 1 and served as the basal reference value throughout experiments. Results are representative of fold change within the sample.

The expression of the IL-27 cytokine subunit EBI3 was significantly increased after 2hr LPS stimulation in DMSO cells with its expression peaking at 6hr post LPS. LXR activation in these cells significantly decreased the expression of EBI3 in unstimulated cells and cells stimulated from 30 min to 4hr ($p<0.001$), 6hr ($p<0.01$) and 12hr ($p<0.05$) **[Figure 5.2]**. Similarly the expression of IL-12p40 was also increased following LPS exposure, with changes in expression seen as early as 30min. LXR activation in these cells significantly decreased the expression of IL-12p40 in cells stimulated for 30min ($p<0.01$) and those stimulated from 1hr to 24hrs ($p<0.001$) **[Figure 5.2]**. The expression of IL-12p35 is significantly increased after 2hr LPS stimulation in DMSO cells and continued to increase over this 24hr stimulation period. LXR activation in these cells significantly inhibited IL-12p35 production after 2hr ($p<0.001$), 4hr, 6hr and 12hr LPS stimulation ($p<0.05$) **[Figure 5.3]**. LXR activation did not significantly decrease the remaining IL-12 related subunits i.e. IL-23p19 and IL-27p28 following LPS **[Figure 5.4]**. Following LPS stimulation in DMSO control cells IL-23p19 was increased

immediately after stimulation, peaking at 1hr and was back to control levels by 12hr. LXR activation did not decrease this, indeed there is a significant increase at 30min (p<0.001). Similarly the expression of IL-27p28 was also increased after LPS exposure; with its expression peaking 4hr post LPS. LXR activation in these cells significantly increases the expression of IL-27p28 after exposure to LPS for 2hr (p<0.001) however there was also a significant decrease in its expression at 12hr and 24hr (p<0.001) **[Figure 5.4]**

5.2.3 LXR ACTIVATION IN DENDRITIC CELLS SPECIFICALLY DECREASES THE EXPRESSION OF THE NFκB SIGNALING SUBUNIT p50.

Given that LXR activation decreased IL-12p40, IL-12p35 and EBI3 and these subunits are regulated by NFκBp50, this suggested that LXR may directly affect this NFκB subunit **[Figure 5.5].** We therefore examined this in greater detail. BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) or DMSO for 7 days. Cells were then stimulated over the course of 30min with LPS (100ng/ml). Lysates were generated as described in [section 2.8.1] and run on SDS gels, transferred onto nitrocellulose membranes and immunoblotted for NFκB-p50 and its precursor protein p105.

NFκBp50 was found to be expressed in both unstimulated and LPS stimulated BMDC **[Figure 5.6]**. Interestingly, following LXR activation in these cells there is a significant decrease in the expression of this protein after 10 min LPS stimulation. Densitometric analysis of these immunoblots confirmed that changes in p50 expression after 10 mins stimulation were statistically significant (p<0.05).

NFκBp105, the precursor protein of p50, is also present in unstimulated and stimulated BMDC and its expression does not significantly change following LPS stimulation.

LXR activation in these cells does not affect the expression of this protein, as there is no significant change compared to DMSO control. Densitometric analysis confirmed this observation.

In order to confirm that LXR can specifically target the p50 subunit of NFκB and not its other signalling subunits, we also examined the effect of LXR activation on NFκBp65 expression in BMDC. NFκBp65 is present in unstimulated cells and its expression is significantly increased following LPS stimulation **[Figure 5.7]**. However LXR activation in these cells does not significantly alter the expression of the p65 subunit compared to DMSO controls. Densitometric analysis also confirmed this observation.

5.2.4 LXR ASSOCIATES WITH NFκBp50 AND THIS ASSOCIATION IS INCREASED FOLLOWING LXR ACTIVATION.

Given that we have shown a decrease in NFκBp50 expression following LXR activation we next wanted to examine whether LXR could physically associate with this subunit. BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM) or DMSO for 7 days. Cells were then stimulated with LPS for 15 minutes and lysates were generated as described in [section 2.8.1] and lysates were then immunoprecipitated (IP) with an LXR antibody using A/G sepharose beads as described in section [section 2.9]. Lysates were then run on SDS gels, transferred onto nitrocellulose membranes and immunoblotted (IB) for NFκBp50. Total levels of LXR were used as a loading control.

A small amount of association is present in DMSO control cells between LXR and NFκBp50 and this association increases in response to LPS. LXR activation however significantly increases this association. **[Figure 5.8].** In both unstimulated and LPS stimulated LXR activated cells there is a significant increase in the receptors association

with NFκBp50. Densitometric analysis confirmed that these changes were significant ($p<0.01$, $p<0.001$).

5.2.5 CONFOCAL MICROSCOPY CONFIRMS AN ASSOCIATION BETWEEN LXR AND NFκBp50

In order to confirm the association between LXR and NFκBp50, we carried out confocal microscopy experiments. BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM), PA452 (1μM) or DMSO for 7 days. Cells were plated on sterilised coverslips, left overnight to adhere and subsequently stimulated with LPS (100ng/ml) for 15 min. Slides were then incubated with LXR, NFκBp50 or NFκBp65 specific primary antibodies followed by incubation with appropriate fluorescently labelled secondary antibodies. Slides were then imaged using the Zeiss LSM 710 confocal microscope.

Firstly LXR expression is increased in response to LPS in both DMSO and LXR activated cells. Colocalisation was observed between LXR and NFκBp50, as indicated by a yellow signal in merged images **[Figure 5.9].** This colocalisation can be seen clearly in unstimulated and stimulated cells, however in stimulated cells the level of colocalisation appears to be increased; particularly in LXR treated cells **[Figure 5.9 A & B].** Given that our previous chapter highlighted an important requirement for RXR in LXR signalling, we also examined if an RXR inhibitor could interfere with colocalisation of LXR with NFκBp50. As can be seen in **Figure 5.10** LXR and NFκBp50 still associate with each other in PA452 treated cells however we can see that the level of association between these two proteins appears to be decreased compared to LXR treated cells.

Next we examined if the colocalisation between LXR and NFκB was specific to the p50 subunit. We therefore examined the level of colocalisation between LXR and NFκBp65. As we can see in **Figure 5.11**, although LXR and p65 are abundantly expressed in the cell they do not colocalise with each other in untreated, LXR activated or stimulated cells.

5.2.6 LXR ACTIVATION IN DENDRITIC CELLS SPECIFICALLY INHIBITS THE TRANSLOCATION OF NFκBp50 TO THE NUCLEUS

Given that we have shown an association between LXR and NFκBp50, we next wanted to determine if this association interfered with the translocation of p50 into the nucleus.

BMDC were harvested and differentiated as previously described and treated with T0901317 (2μM), PA452 (1μM) or DMSO for 7 days. Cells were plated on sterilised coverslips, left overnight to adhere and subsequently stimulated with LPS (100ng/ml) for 15 min. Slides were then incubated with NFκBp50 or NFκBp65 specific primary antibodies followed by incubation with appropriate fluorescently labelled secondary antibodies. The nucleus was stained using a propidium iodide solution. Slides were then imaged using the Zeiss LSM 710 confocal microscope.

In DMSO unstimulated cells NFκBp50 is localised on the membrane and in the cytoplasm and following exposure to LPS for 15 min, p50 translocates to the nucleus where it is needed to signal. This is shown by the colocalisation seen in **Figure 5.12** in merged images. However, in LXR activated cells, p50 is localised mainly on the membrane in unstimulated cells and following LPS stimulation, p50 does not translocate into the nucleus and remains cytoplasmic. We also show in **Fig 5.13** that as expected NFκBp65 translocates to the nucleus following LPS exposure in DMSO

treated cells, however LXR activation does not affect the translocation of p65 as it is still present in the nucleus following LPS exposure in LXR treated cells **[Figure 5.13]**

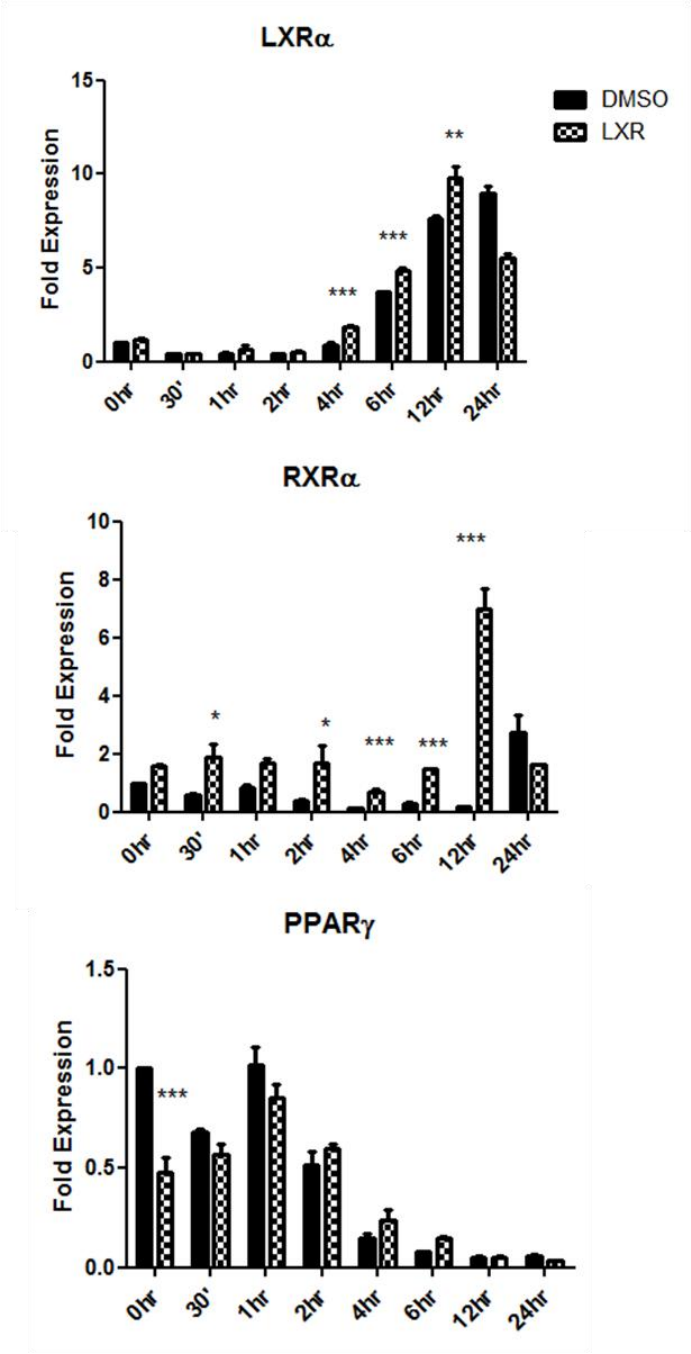


FIGURE: 5.1

The expression of LXR and its heterodimerisation partner, RXR is positively regulated following LXR activation in BMDC.

BMDC were differentiated in GMCSF in the presence of 2μM T0901317 or DMSO for 7 days and stimulated over the course of 24hrs with 100ng/ml LPS. Total RNA was isolated, converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represent 3 independent experiments ***p<0.001, **p<0.01, *p<0.05 comparing control vs. LXR treated groups as determined by one-way ANOVA test.

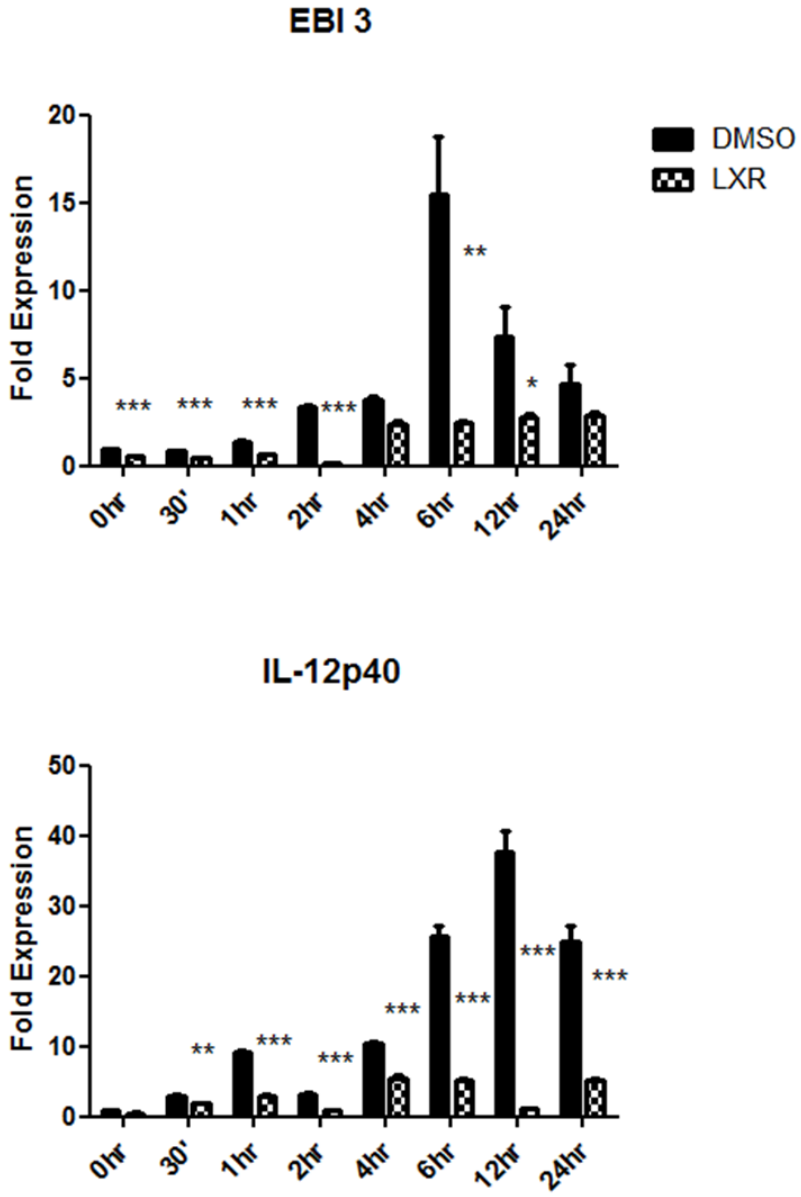


FIGURE: 5.2

The expression of IL-12p40 and EBI3 are significantly decreased following LXR activation in BMDC.

BMDC were differentiated in GMCSF in the presence of 2μM T0901317 or DMSO for 7 days and stimulated over the course of 24hrs with 100ng/ml LPS. Total RNA was isolated, converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represent 3 independent experiments ***p<0.001, **p<0.01, *p<0.05 comparing control vs. LXR treated groups as determined by one-way ANOVA test.

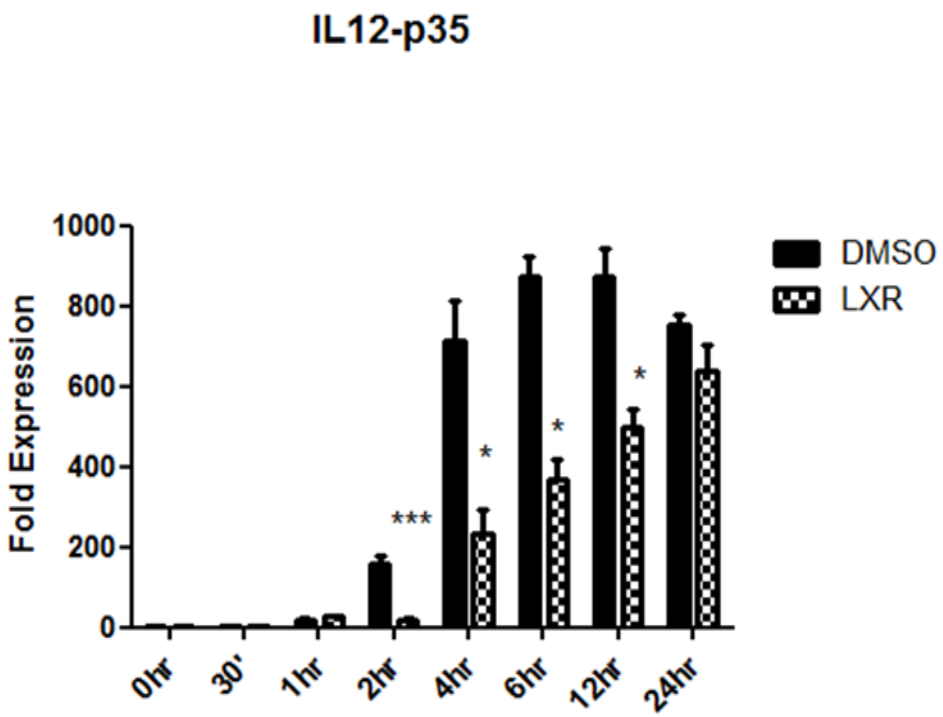


FIGURE: 5.3

The expression of IL-12p35 is significantly decreased following LXR activation in BMDC. BMDC were differentiated in GMCSF in the presence of 2µM T0901317 or DMSO for 7 days and stimulated over the course of 24hrs with 100ng/ml LPS. Total RNA was isolated, converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represent 3 independent experiments ***p<0.001, *p<0.05 comparing control vs. LXR treated groups as determined by one-way ANOVA test.

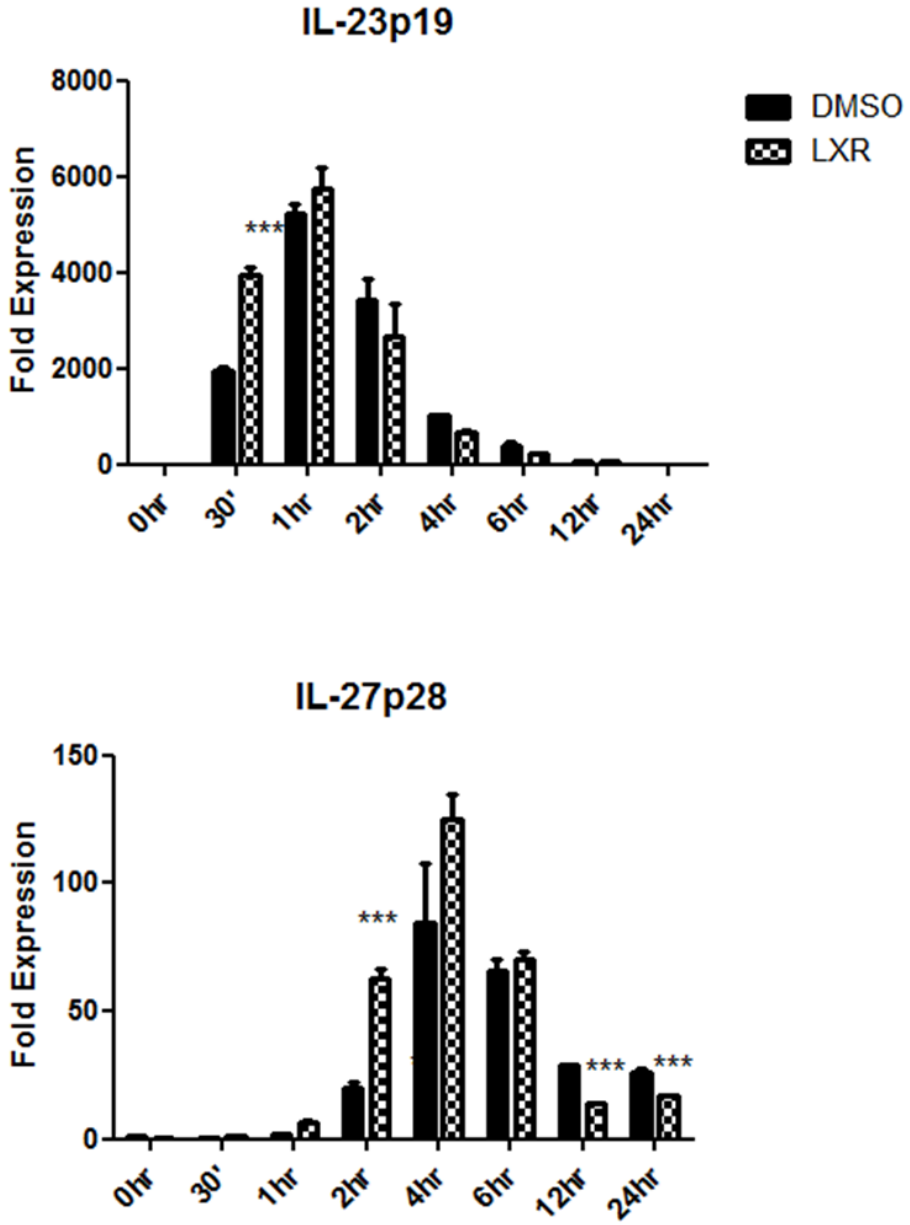


FIGURE: 5.4

The expression of the cytokine subunits IL-23p19 and IL-27p28 are differentially regulated following LXR activation in BMDC.

BMDC were differentiated in GMCSF in the presence of 2µM T0901317 or DMSO for 7 days and stimulated over the course of 24hrs with 100ng/ml LPS. Total RNA was isolated, converted to cDNA and used for subsequent RT-PCR experiments. Results are expressed as fold change after normalising to the endogenous control S18. Results are ± SEM of triplicate assays and represent 3 independent experiments ***p<0.001, *p<0.05 comparing control vs. LXR treated groups as determined by one-way ANOVA test.

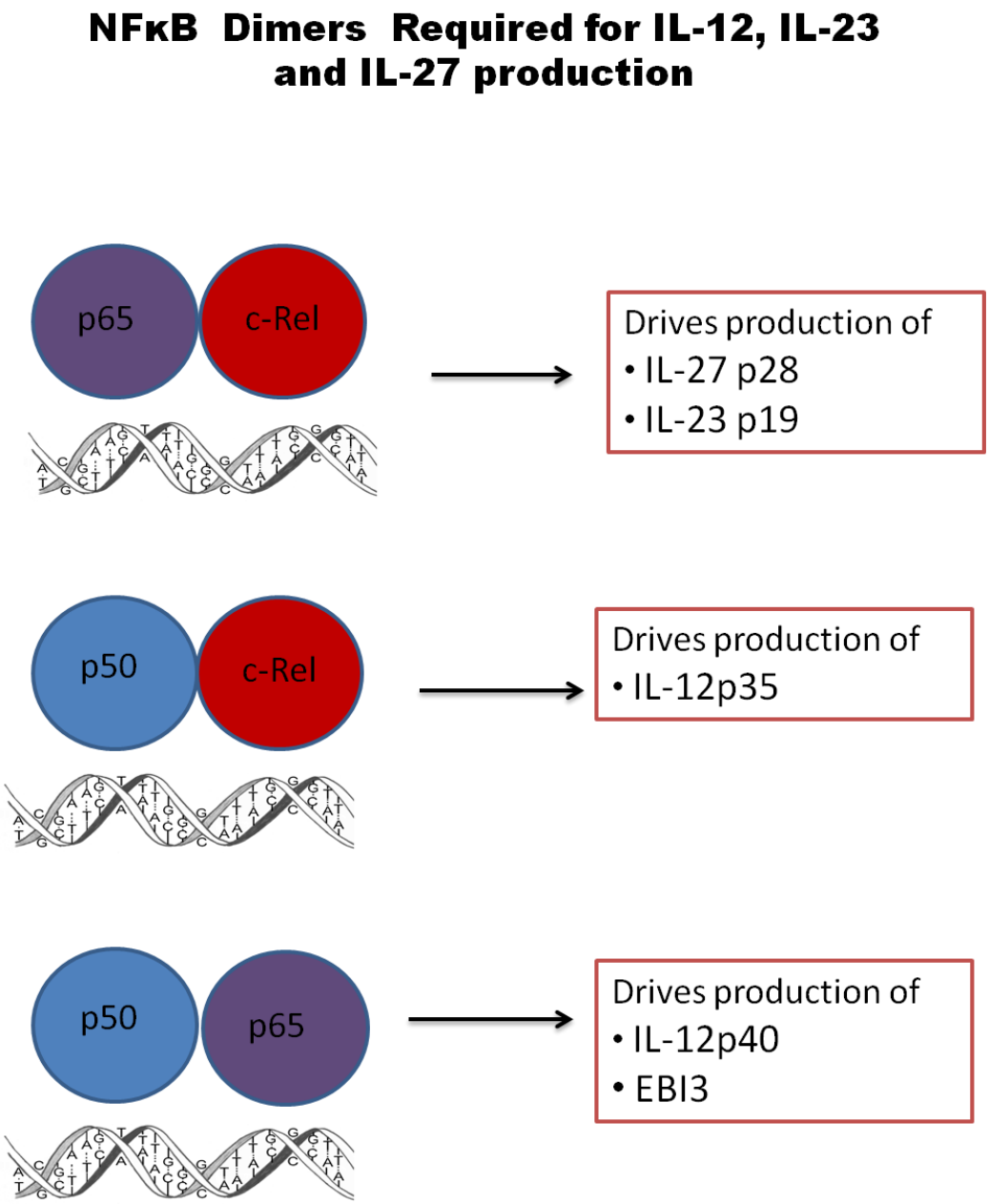


FIGURE: 5.5

The production of each IL-12 related cytokine subunit is controlled by specific NFκB dimers.

We have previously shown in Chapter 4 that LXR activation can inhibit NFκB and we also show here that LXR activation can specifically target IL-12p40, IL-12p35 and EBI3. We therefore used these observations and our knowledge of the specific NFκB dimers shown above to determine the molecular target of LXR. We hypothesised that the NFκB subunit p50 or its precursor p105 may be a direct target for LXR.

Diagram adapted from results published by (Sanjabi et al. 2000)(Kollet, Petro 2006),(Wirtz et al. 2005, Zhang et al. 2010, Carmody et al. 2007).

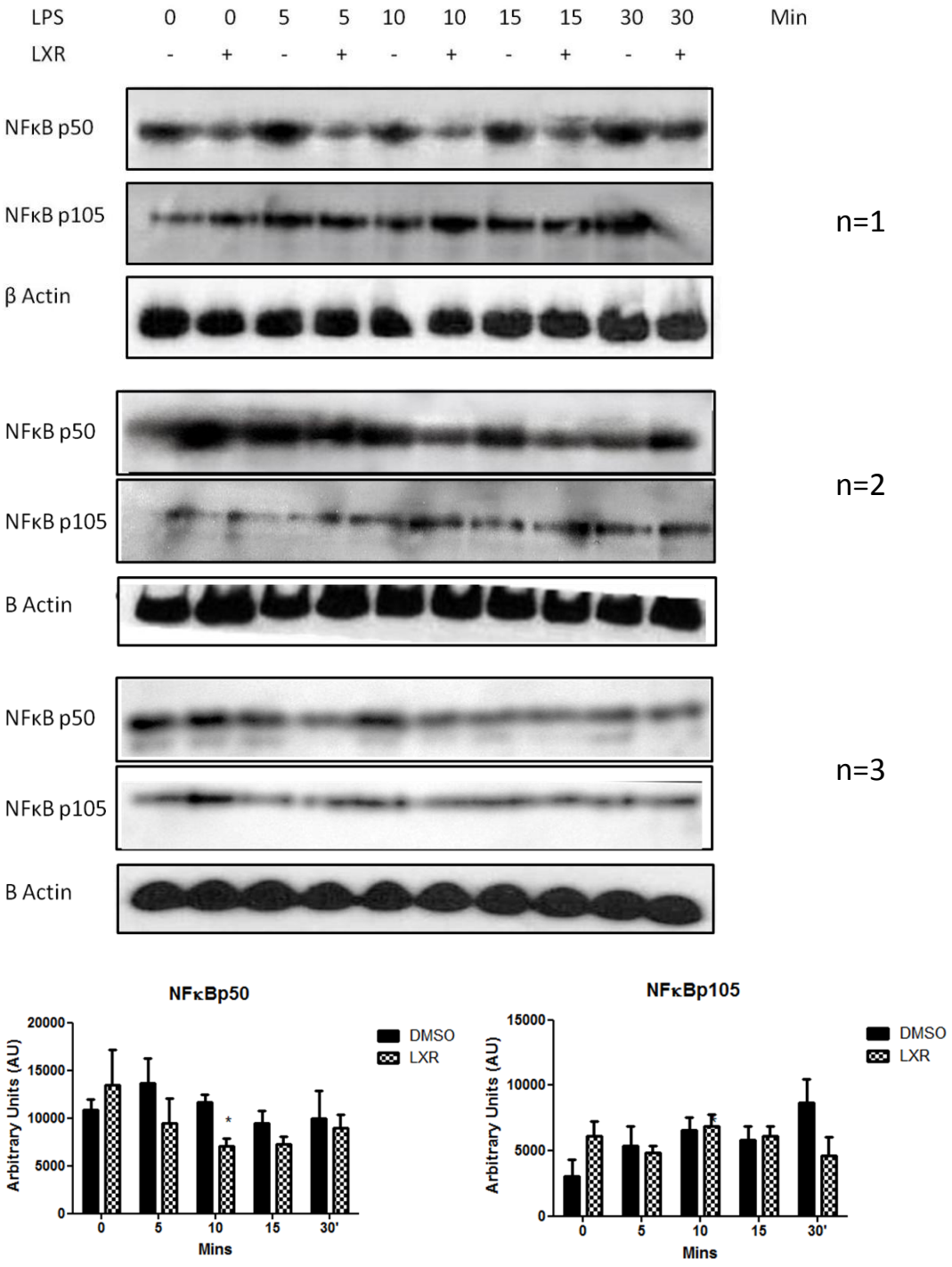


FIGURE: 5.6

LXR activation decreases the expression of NFκBp50 in BMDC.

BMDC were differentiated in GMCSF in the presence of 2μM T0901317 or DMSO for 7 days and stimulated over a course of 30 min with 100ng/ml LPS. After this time, cells were lysed and immunoblotted for p50 and p105. Total cellular levels of β actin were used as a loading control. Densitometric analysis was conducted on 3 representative immunoblots and graphical representation of NFκBp50 expression in arbitrary units (AU) is shown. *p<0.05 comparing control vs. LXR treated groups as determined by one-way ANOVA test.

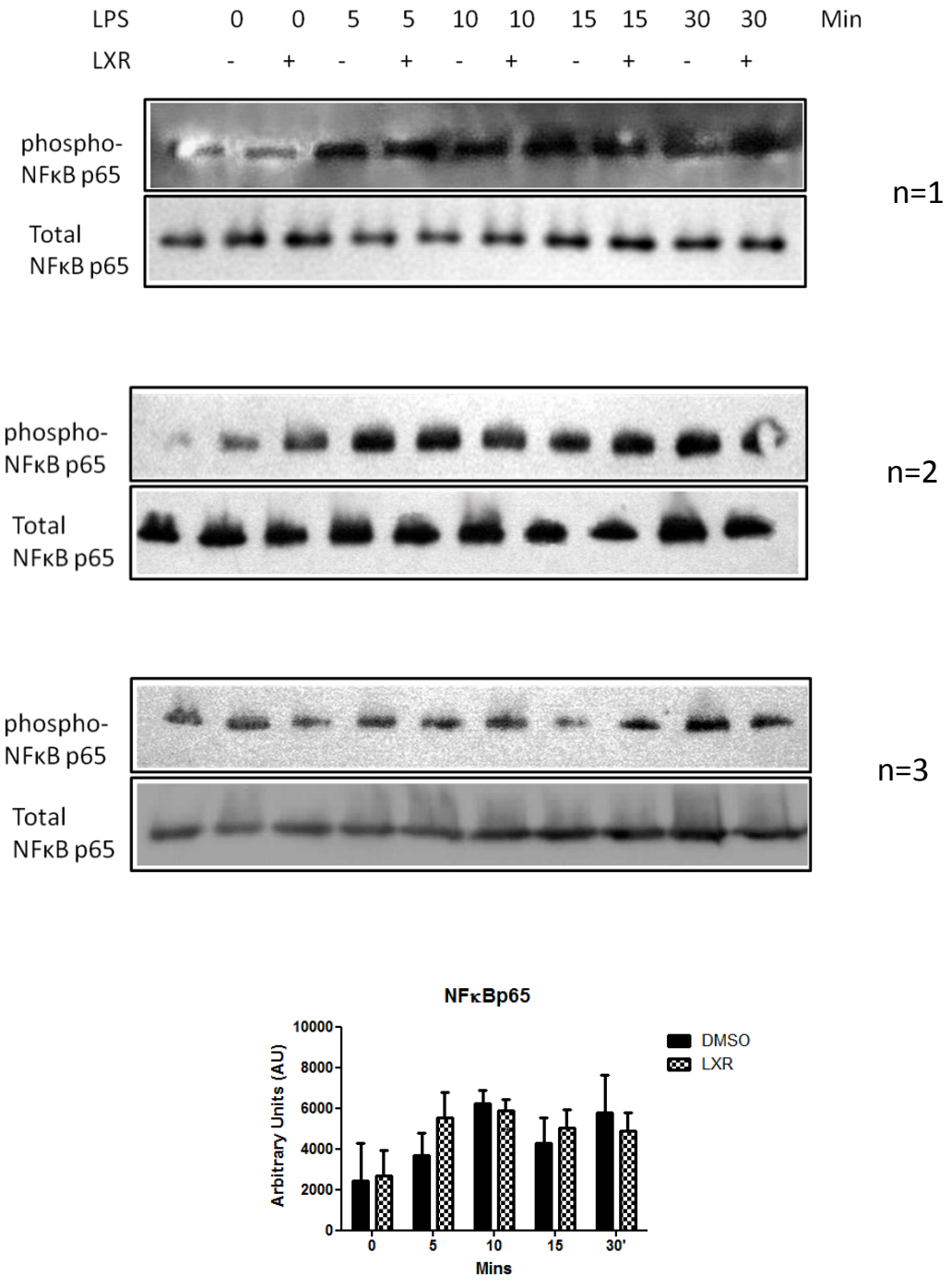


FIGURE: 5.7

LXR activation does not affect the expression of NFκBp65 in BMDC.

BMDC were differentiated in GMCSF in the presence of 2μM T0901317 or DMSO for 7 days and stimulated over a course of 30 min with 100ng/ml LPS. After this time, cells were lysed and immunoblotted for NFκBp65. Total cellular levels of total NFκBp65 were used as a loading control. Densitometric analysis was conducted on 3 representative immunoblots and graphical representation of NFκBp65 expression in arbitrary units (AU) is shown.

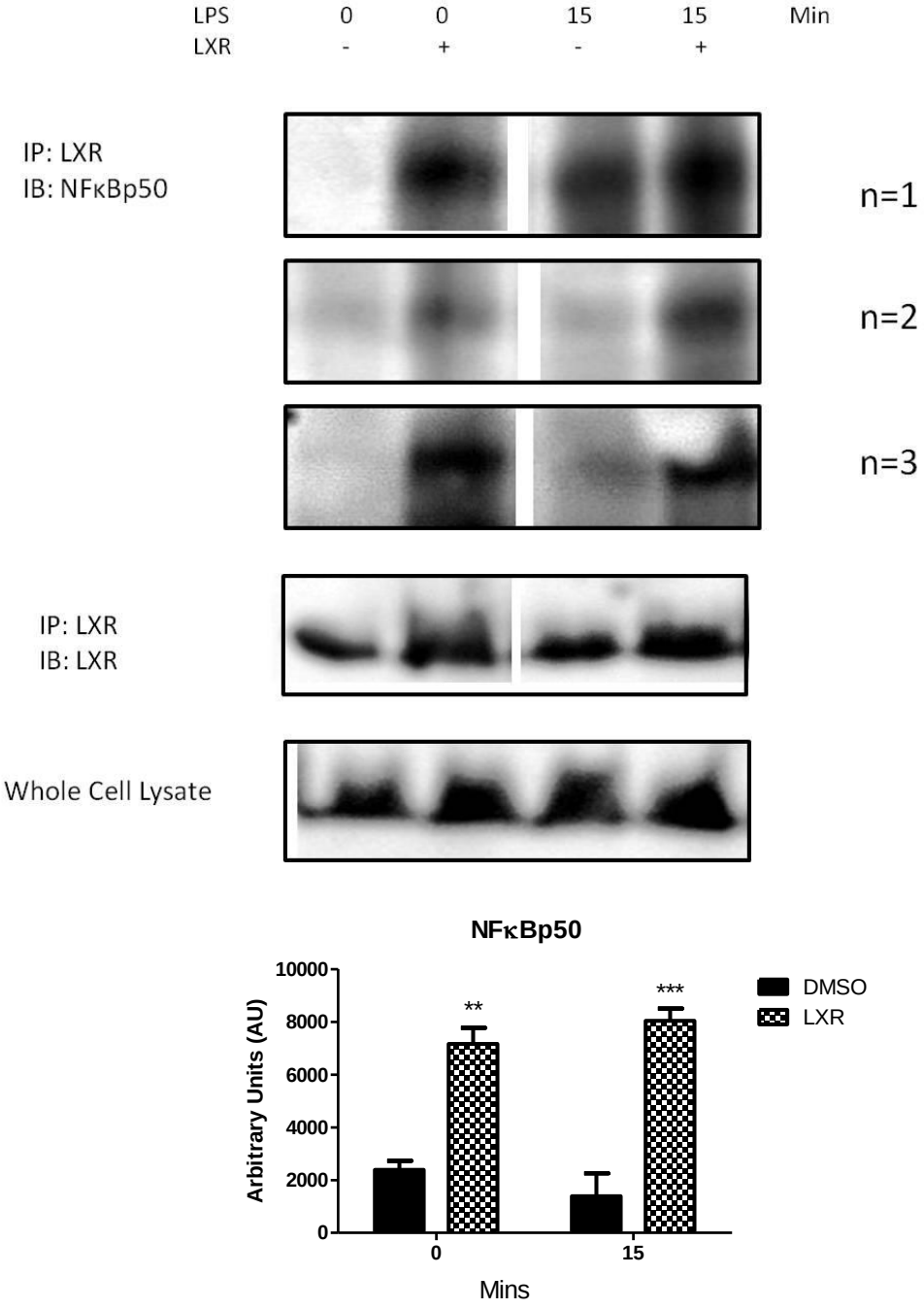


FIGURE: 5.8

LXR activation in BMDC significantly increases association of LXR with NFκBp50.
BMDC were differentiated in GMCSF in the presence of 2μM T0901317 or DMSO for 7 days and stimulated for 15 min with 100ng/ml LPS. After this time, cells were lysed, immunoprecipitated with an LXR antibody and immunoblotted for NFκBp50. Whole cell lysates were immunoblotted with LXR as a loading control. Densitometric analysis was conducted on 3 representative immunoblots and graphical representation of NFκBp50 expression in arbitrary units (AU) is shown. ***p<0.001, **p<0.01 comparing control vs. LXR treated groups as determined by one-way ANOVA test.

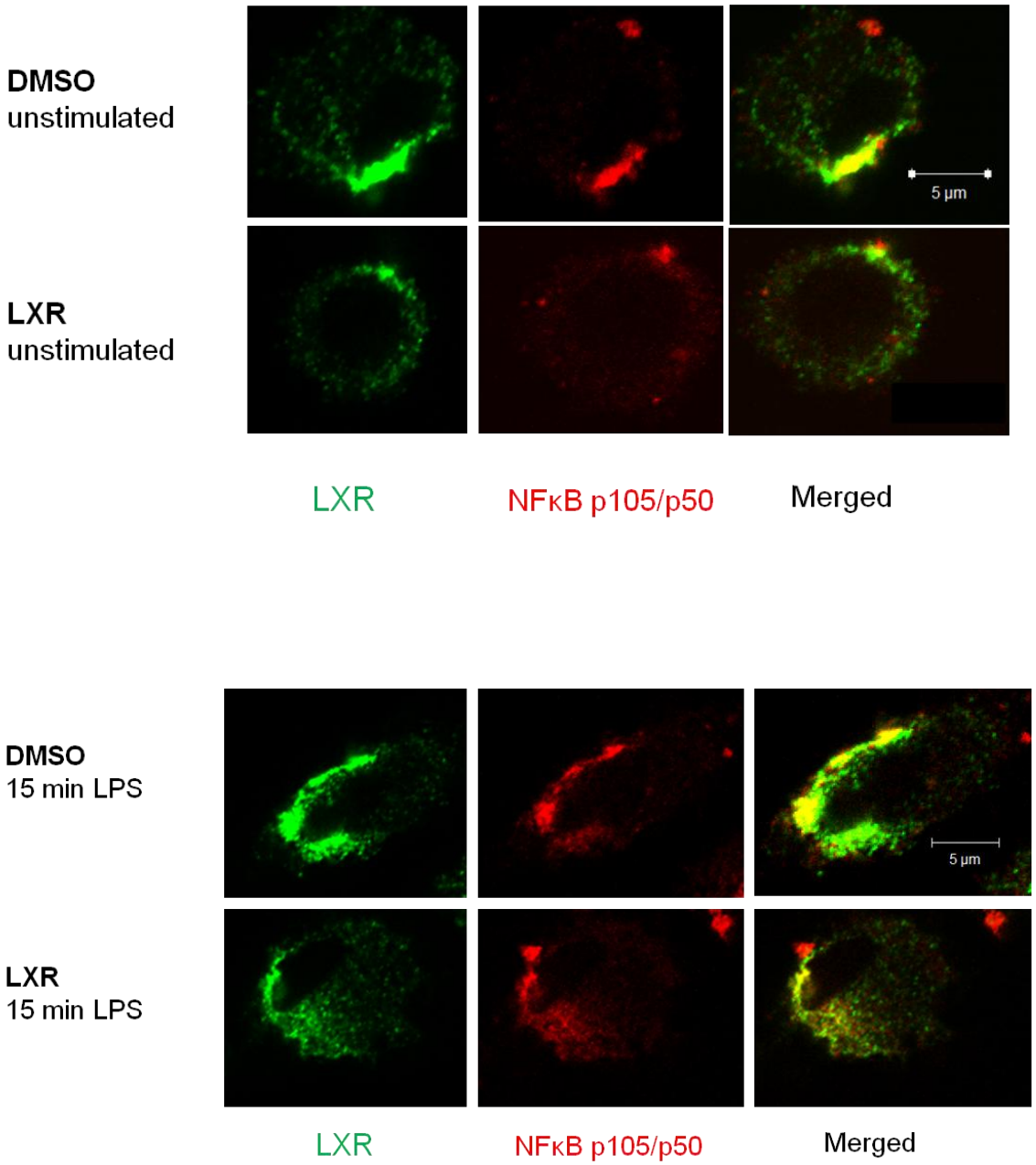


FIGURE: 5.9

LXR and NFκBp105/p50 colocalise in unstimulated and stimulated BMDC.

BMDC were differentiated in GMCSF in the presence of 2µM T0901317 or DMSO for 7 days and stimulated for 15 min with 100ng/ml LPS. Cells were subsequently stained for LXR and NFκBp105/p50 and colocalisation between these proteins was assessed by confocal microscopy. Colocalisation between LXR and NFκBp105/p50 can be seen in the merged image (yellow).

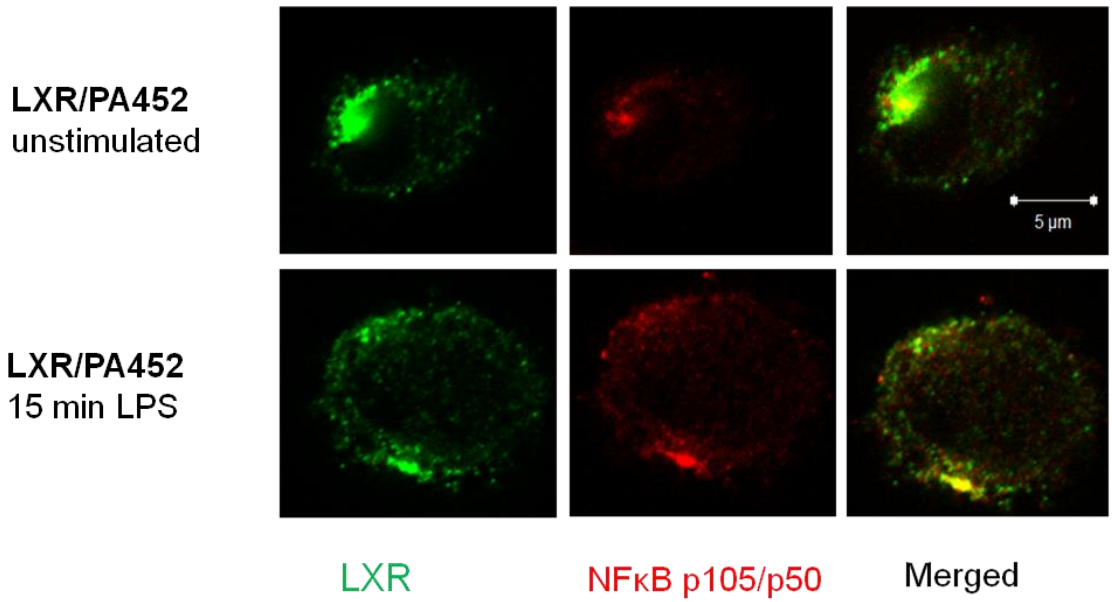


FIGURE: 5.10

LXR and NFκBp105/p50 colocalise in PA452 treated BMDC.

BMDC were differentiated in GMCSF in the presence of 2µM T0901317, 1µM PA452 or DMSO for 7 days and stimulated for 15 min with 100ng/ml LPS. Cells were subsequently stained for LXR and NFκBp105/p50 and colocalisation between these proteins was assessed by confocal microscopy. Colocalisation between LXR and NFκBp105/p50 can be seen in the merged image (yellow).

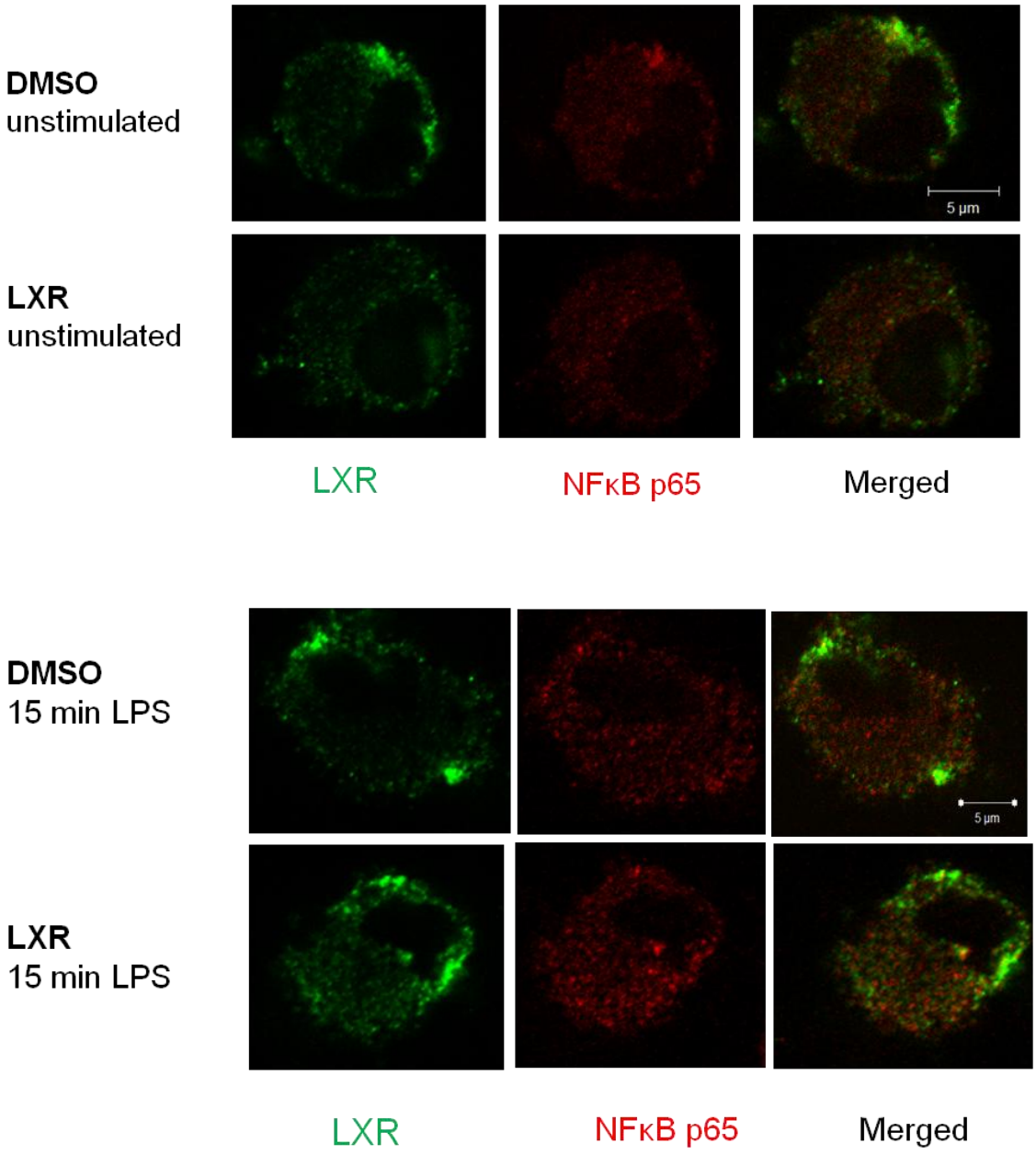


FIGURE: 5.11

LXR and NFκBp65 do not colocalise in LXR treated and untreated BMDC.

BMDC were differentiated in GMCSF in the presence of 2µM T0901317 or DMSO for 7 days and stimulated for 15 min with 100ng/ml LPS. Cells were subsequently stained for LXR and NFκBp65 and colocalisation between these proteins was assessed by confocal microscopy. Colocalisation between LXR and NFκBp65 is not present in these cells (lack of yellow signal).

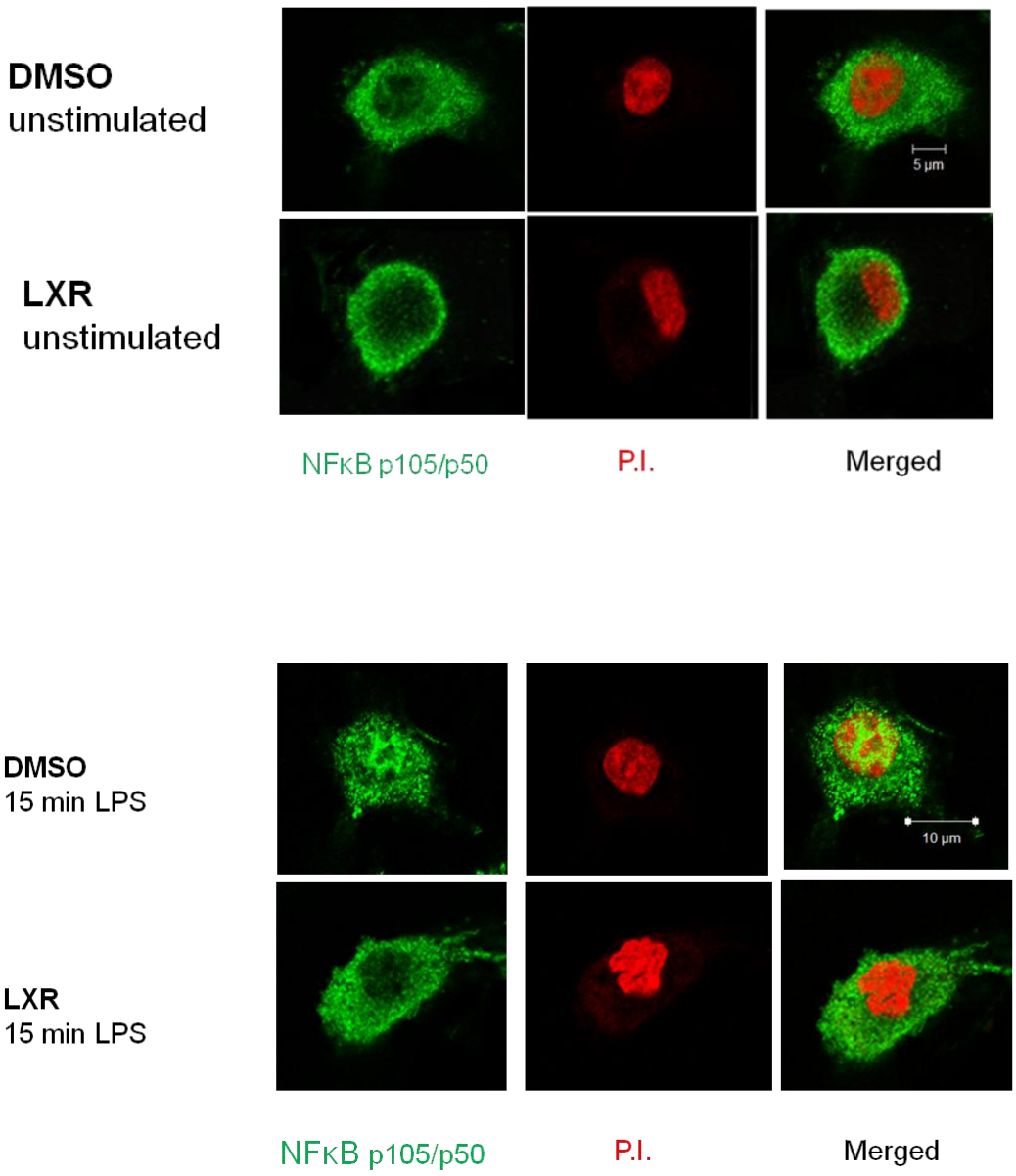


FIGURE: 5.12

LXR activation in LPS matured BMDC alters the translocation of NFκBp50 to the nucleus.

BMDC were differentiated in GMCSF in the presence of 2µM T0901317 or DMSO for 7 days and stimulated for 15 min with 100ng/ml LPS. The nuclei of cells were subsequently stained using P.I. and cells were also stained for NFκBp50. The translocation of NFκBp50 to the nucleus was assessed by confocal microscopy.

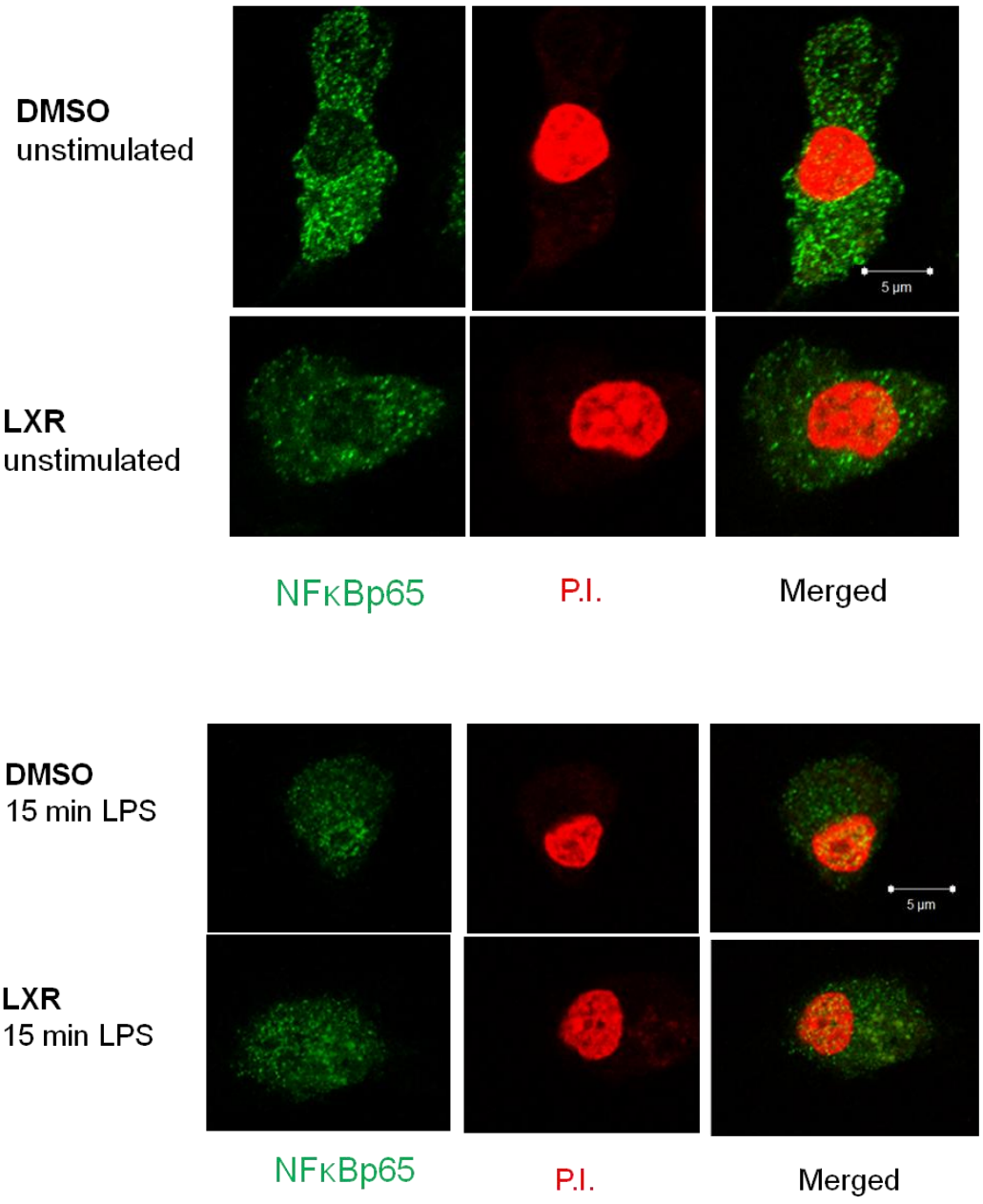


FIGURE: 5.13

LXR activation in LPS matured BMDC does not affect the translocation of NFκBp65 to the nucleus.

BMDC were differentiated in GMCSF in the presence of 2µM T0901317 or DMSO for 7 days and stimulated for 15 min with 100ng/ml LPS. The nuclei of cells were subsequently stained using P.I. and cells were then stained for NFκBp65. The translocation of NFκBp65 to the nucleus was assessed by confocal microscopy.

5.3 DISCUSSION

In this chapter, we examined the effect of LXR activation on nuclear receptor expression and on the expression of each of the IL-12 cytokine subunits. The results from this study lead to the identification of the molecular target downstream of LXR activation which is necessary for the receptor to exert its anti-inflammatory effects. Our results show that the expression of LXR, RXR and PPARγ are regulated in response to TLR4 activation and are altered in response to LXR activation. The expression of LXR increases over the course of 24hrs in response to LPS whereas the expression of both PPARγ and RXR decreases over this period. Consistent with this result, in chapter 3 we also see that the expression of LXR is increased during acute inflammation in DSS induced colitis. It has previously been reported that there may be a reciprocal relationship between the TLR pathway and the nuclear receptor pathway, in that activation of TLRs can inhibit or enhance the expression of nuclear receptors or indeed as we have shown, activation of nuclear receptors can inhibit the TLR pathway (Ogawa et al. 2005). The functional consequences of these effects have not yet been established however given the wide variety of genes each nuclear receptor may regulate, and their need to heterodimerise with each other it seems likely that their expression may be positively and negatively regulated at different stages of inflammation.

Upon activation LXR can directly regulate and induce its own expression, therefore as expected we saw a significant increase in LXRα expression following treatment with the LXR agonist (Glass, Ogawa 2006). Given that the LXRα gene is direct target of the LXR receptor, it is important to note that this result also highlights the specificity of the LXR agonist used throughout these experiments and its ability to efficiently activate the LXR receptor. Interesting, we also see a significant increase in RXRα expression

following LXR activation, whereas there is no significant increase in PPARγ expression. This result emphasises the findings we presented in Chapter 4, where the formation of LXR:RXR heterodimers and not LXR:PPARγ heterodimers were important in how LXR exerted its effects. LXR activation in these cells may thus positively regulate the expression of its heterodimerisation partner, as seen by a positive increase in RXRα expression and not PPARγ.

We also examined the effect of LPS and LXR activation on the expression of the IL-12 cytokine subunits. Our results showed that IL-23p19 was rapidly induced following LPS stimulation with a peak in expression seen after just 1hr stimulation. After 2hr LPS stimulation the expression of both IL-27p28 and IL-12p35 peak whereas the expression of EBI3 and IL-12p40 were highest at 6hr and 12hr post stimulation. It is clear that the kinetics of IL-12, IL-23 and IL-27 mRNA vary considerably amongst family members; however it is possible that this variation is due to different signalling complexes required for different cytokine subunits. LXR activation in these cells significantly decreases the expression of EBI3, IL-12p40 and IL-12p35 over the course of stimulation without significantly affecting IL-23p19 and IL-27p28. Since we have previously shown a decrease in IL-12, IL-23 and IL-27 secretion by DC following LXR activation, these results have highlighted that the reduction shown in IL-12 is a result of decreased IL-12p40 and IL-p35 expression; the reduction shown in IL-23 is a result of decreased IL-12p40 expression and finally the reduction shown in IL-27 is a result of decreased EBI3 expression. The specific expression of these subunits has also been implicated in disease. IL-12p35 KO mice were reported to develop significantly milder experimentally induced arthritis compared to WT (Vasconcellos et al. 2011) while increased expression of EBI3 has been described in patients suffering from ulcerative colitis (Gehlert, Devergne & Niedobitek 2004). IL-12p40 has also been widely

implicated in numerous inflammatory diseases such as psoriasis IBD, RA and MS (Fuss et al. 2006) (Lee et al. 2004).Therefore directly targeting these subunits through LXR activation could prove beneficial in the treatment of these diseases.

Upon further examination of the NFκB dimer complexes that control IL-12, IL-23 and IL-27 production, NFκBp50 was highlighted as a potential LXR target. The formation of p65:c-Rel complexes are necessary for the production of both IL-23p19 and IL-27p28. However as we have shown in this chapter, LXR activation does not significantly decrease the expression of these subunits. LXR activation does however significantly affect the production of EBI3, IL-12p40 and IL-12p35 which are all under the transcriptional control of either p50:p65 or p50:c-Rel NFκB complexes. We therefore hypothesised that LXR must target the NFκBp50 signalling subunit in order to exert its effects on the IL-12 family of cytokines.

Our data shows that LXR activation in DC can significantly decrease the level of NFκBp50 without affecting the levels of p105. NFκBp105 is now known to have two functions within the NFκB signalling system. First, NFκB activation can lead to the proteolytic degradation of p105 to p50. Furthermore p105, similar to IκB proteins can associate with other members of the NFκB family in the cytoplasm and prevent nuclear translocation of these subunits (Martinez-Micaelo et al. 2012). Our data shows that LXR activation did not affect p105 expression. This suggests that the cells capability to sequester the NFκB subunits that are not required for signalling, away from the nucleus remains unchanged. However LXR activation can significantly decrease the levels of NFκBp50, consequently affecting the levels of the NFκB dimers required for IL-12 production i.e. p65:p50 and p50:c-Rel. Increased levels of p50 in addition to the IL-12 family of cytokines has also been implicated in disease. Visekruna *et al* reported an

increase in the capacity of proteasomes isolated from Crohns disease patients to process p105 to p50 where increased levels of p50 correlated with disease severity (Visekruna et al. 2006). Furthermore in a mouse model of MS, a deficiency in p50 was sufficient to block the induction of disease (Hilliard et al. 1999). Therefore targeting this NFκB subunit could prove to be beneficial in targeting the IL-12 cytokines and ameliorating disease. Interestingly, our results also demonstrate that LXR specifically affects the p50 subunit and not other NFκB subunits, as no significant change in NFκp65 was observed in LXR activated cells.

We also show by co-immunoprecipitation experiments and visually using confocal microscopy that LXR associated with NFκBp105 and NFκBp50 in resting, stimulated and LXR activated cells. Co-immunoprecipitation experiments confirmed that LXR activation significantly increased the level of association between NFκBp50 and LXR in these cells. Interestingly, the level of LXR: p50 association in these cells also appeared to be less but not abrogated in cells treated with the specific RXR inhibitor. This indicates that although LXR:RXR heterodimers are important in the ability of LXR to exert its effects this association may not control LXR ability to target the p50 subunit of NFκB. No association between LXR and p65 was observed in resting, stimulated or LXR activated cells emphasising once more the specificity of LXR for p50 and not other NFκB subunits.

Following efficient processing of p105 to p50, the p50 signalling subunit translocates to the nucleus, associates with its specific dimerisation partner, binds to NFκB response elements and initiates transcription (Ruland 2011). We hypothesised that given LXR ability to associate with p50; this association could inhibit the translocation of p50 to the nucleus, thus inhibiting transcription. Our results showed that as expected, upon

NFκB activation, p50 translocates to the nucleus to initiate transcription of target genes. However following LXR activation in these cells, p50 is retained in the cytoplasm and is prevented from translocating to the nucleus. This event is specific for p50 as LXR activation does not affect the translocation of p65 to the nucleus.

While aspects of the results presented in this chapter concur with previous studies in the literature; we have proposed a novel mechanism to explain the anti-inflammatory effects LXR exerts in DC. The expression of nuclear receptors following TLR4 activation has previously been examined and the data presented here concurs with these results. Welch *et al* also reported a decrease in PPARγ expression in LPS stimulated macrophages after 6hrs while Castrillo *et al* showed that the expression of LXRα is increased in peritoneal macrophages following *E.Coli* infection (Castrillo et al. 2003, Welch et al. 2003). The expression of RXRα has previously been reported to decrease in the kidney and liver following LPS administration (Berczi 1998, Feingold et al. 2008). However, this is the first time that the kinetics of nuclear receptor expression over the course of 24hr LPS exposure in DC has been reported. Similarly this is also the first time the expression of RXR and PPARγ has been reported in response to LXR activation. A study undertake by Schuetze *et al* in which the expression of IL-12p40, p35, EBI3, IL-12p28 and IL-23p19 were examined following TLR4 activation correlated with the mRNA profile we have reported within this chapter (Schuetze et al. 2005). Although it has previously been reported that LXR activation can decrease the expression of IL-12p40 (Myhre et al. 2008), here we present for the first time a comprehensive analysis of LXR activation on all other members of the IL-12 family of cytokines.

We also present for the first time a novel mechanism to describe the anti-inflammatory effects LXR exerts in DC. However a link between nuclear receptors and NFκB has previously been described. Chen *et al* demonstrated a physical association between PPARγ and NFκBp65 in colon cancer cells resulting in a decrease in NFκB transcriptional activity (Chen et al. 2003). The glucocorticoid receptor, once activated has been reported to increase the nuclear export rate of Rel A, therefore reducing the duration of an NFκB transcriptional response (De Bosscher et al. 1997). The VDR, upon activation has been reported to reduce the phosphorylation of p65 and its subsequent translocation to the nucleus (Penna et al. 2007). There have also been previous reports to suggest a possible LXR: p65 related mechanism in which LXR activation in DC prevented the association of p65 to a set of NFκB target gene (Hanley, Viglianti 2011). However it is important to note that although LXR activation in this study inhibited a p65 response, no association between LXR and p65 was reported. Therefore it is difficult to determine if LXR activation has a direct effect on NFκBp65 or if the effect reported is a result of an interaction with p65's heterodimerisation partner i.e. p50 or c-Rel. We therefore propose a model (illustrated below) whereby LXR activation in DC results in the formation of LXR:RXR heterodimers where LXR physically associate with NFκBp50, preventing its translocation to the nucleus. Inhibited p50 translocation subsequently decreases the transcription of EBI3, IL-12p40 and IL-12p35 expression leading to a decrease in the production of IL-12 family of cytokines.

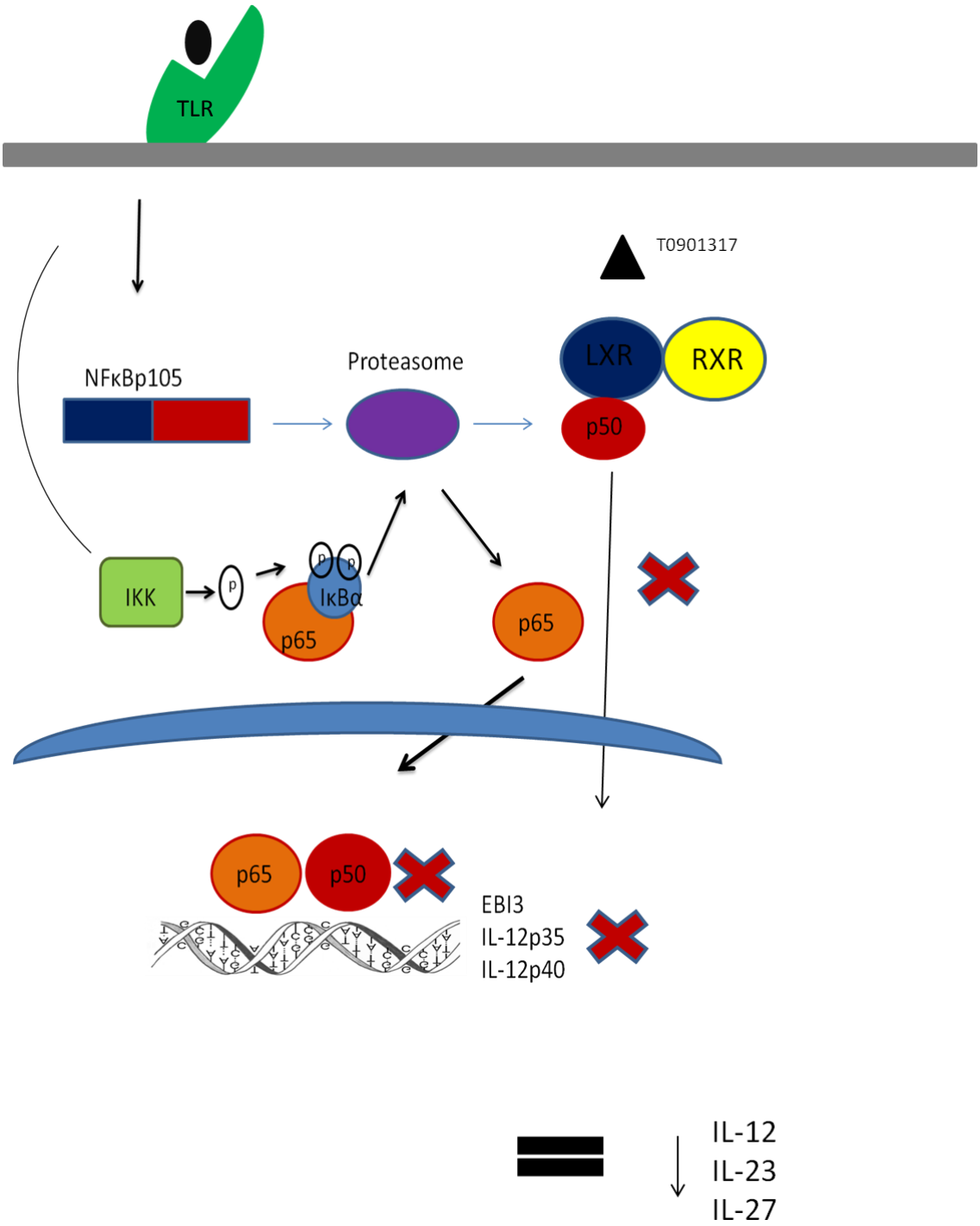


FIGURE: 5.14

Proposed mechanism of action of LXR in BMDC. LXR activation in DC results in the formation of LXR:RXR heterodimers where LXR physically associate with NFκBp50, preventing its translocation to the nucleus. Inhibited p50 translocation subsequently decreases the transcription of EBI3, IL-12p40 and IL-12p35 expression leading to a decrease in the production of IL-12 family of cytokines.

CHAPTER 6

GENERAL

DISCUSSION

CHAPTER 6 GENERAL DISCUSSION

Nuclear receptors are a large unique family of ligand- regulated transcription factors that are involved in almost every aspect of development, physiology and disease (Venteclef et al. 2011). RXR, for example can be detected in almost every tissue in the body, highlighting the importance of these receptors in cell signalling (Szeles et al. 2010). In recent years, our understanding of these receptors and their signalling and transcriptional capabilities has improved immensely. The PPAR subset of nuclear receptors are best known for their ability to regulate lipid metabolism, adipocyte differentiation and glucose homeostasis. LXR is commonly referred to as a cholesterol sensor and is known to play a pivotal role in regulating cholesterol metabolism (Rigamonti, Chinetti-Gbaguidi & Staels 2008). FXR, a bile acid sensor was originally identified as a regulator of glucose, lipid and bile acid homeostasis (Lefebvre et al. 2009). The RXR receptor is however considered the master regulator in the nuclear receptor family. Considering activation of this receptor has been reported to control cell growth, differentiation, survival and death, and given that RXR can also heterodimerise with a large number of other nuclear receptors to control their effects; the network of genes regulated by RXR is vast (Germain et al. 2006). Despite the unique and diverse roles these receptors play in key physiological processes once their expression was discovered in a number of immune cells, a possible role for these receptors in inflammation began to emerge. Interestingly, expression profiling experiments have documented the expression of 28 members of the nuclear receptor family in primary mouse monocytes alone, highlighting an important role these receptors may have in inflammation.(Barish et al. 2005).

Inflammation is a normal defence mechanism that the body uses to protect itself from invading microorgansims and tissue injury. It is characterised by increased blood flow, vascular permeability and the induction of chemokines, cytokines and adhesion molecules which all aid in the recruitment and infiltration of effector cells to the site of infection/injury (Serhan et al. 2007). Once the infection has been cleared; tissue remodelling processes are activated, leading to a resolution of the inflammatory response and a return to normal. However if this response becomes dysregulated, excessive inflammation can lead to extensive tissue damage, acute and chronic inflammatory disease and even death (Andreakos, Foxwell & Feldmann 2004). Given the fundamental roles cytokines play in the development and pathogenesis of many inflammatory diseases, there has been extensive research focused on identifying novel compounds to inhibit proinflammatory cytokines or indeed enhance antiinflammatory cytokine production.

DC are essential antigen presenting cells which link both the innate and adaptive immune response. Recognition of invading pathogens through PRR on their surface initiates a distinct maturation programme, ultimately leading to increased cytokine production and upregulated cell surface and costimulatory marker expression (Janeway, Medzhitov 2002). Mature DC can subsequently interact with and differentiate naive Th cells, thus initiating the adaptive immune response. Although Th cells are essential in clearing intra and extracellular infections they have also been widely implicated in inflammatory disease (Palmer, Weaver 2010). The aim of this study was to examine the effect of nuclear receptor activation on DC maturation and determine if targeting these receptors could alter cell surface marker expression and cytokine production from these cells - thus having potential downstream effects for Th cell differentiation and disease.

Our results showed that activation of LXR, RXR and PPARγ resulted in a significant decrease in the IL-12 family of cytokines. Interestingly, while activation of LXR or PPARγ resulted in a decrease in IL-12 (p70 and p40), IL-23 and IL-27, RXR activation resulted in decreased IL-12 (p70 and p40) and IL-27 production without decreasing the production of IL-23. Therefore, although there are structural similarities between these nuclear receptors, it is likely that each receptor controls a distinct set of target genes. Indeed, it is also possible that the activation of these receptors is regulated in a tissue or cell specific manner. It has previously been reported that the GR displays potent anti-inflammatory properties in immune cells, however upon activation in the liver, it can positively regulate the expression of acute phase response (APR) proteins (Wang et al. 2001). The selectivity of these nuclear receptors offers a great advantage in treating inflammatory diseases. Following RXR activation the specific decrease in IL-12 and IL-27 could prove beneficial in specifically targeting Th1 mediated disease, given that both these cytokines are necessary for the generation of a Th1 response.

There is a significant body of evidence demonstrating the role of IL-12, IL-23 and IL-27 in the pathogenesis of inflammatory disease. Murphy *et al* reported, through the use of IL-12p35 and IL23p19 specific KO mice that the production of IL-23 was essential in mediating autoimmune inflammation in the joints (Murphy et al. 2003). In humans, overexpression of the IL-12p40 and IL-12p35 was detected in the intestinal tissue of patients with Crohns disease (Berrebi et al. 1998).Indeed, we ourselves report in this study that the expression of individual IL-12 cytokine subunits is significantly increased in both chemically induced and infection induced models of colitis in mice. Ustekinumab and briakinumab represent two therapeutic monoclonal antibodies that target the p40 subunit of both IL-12 and IL-23. Use of these mAb has proved effective in the treatment of psoriasis and psoriatic arthritis in Phase III clinical trials (Leonardi et

al. 2008). Ustekinumab has also showed benefits in the treatment of Crohns disease, especially in patients that failed to respond to the TNFα targeting drug, Inflixamab (Sandborn et al. 2008). However the cost in generating and ultimately using these drugs is extremely high. Therefore it is necessary to continually discover new therapeutic targets for the treatment of these diseases. Given that LXR, RXR and PPARγ can significantly and specifically alter the production of the IL-12 family of cytokines, there is promising potential for targeting these receptors in disease. The therapeutic potential for these receptors is further increased with the identification of LXRs heterodimerisation partner, RXR. In this study we showed that LXR activation, through a specific interaction with RXR could decrease the IL-12 family of cytokines. It has previously been reported that activation of both receptors in the nuclear receptor dimer resulted in more potent affects than activation of a single receptor (Perez et al. 2012). This result highlights the potential of using combination treatments to activate both LXR:RXR heterodimers. Indeed Desreumaux *et al* reported that co-administration of both PPARγ and RXR agonists to mice with chemically induced colitis significantly reduced disease severity (Desreumaux et al. 2001). Combinational therapy may not only be beneficial because of its synergistic effects, it is likely that lower doses of the respective agonists would be needed to activate the heterodimer, thus decreasing the possibility of off target effects and undesirable side effects. Interestingly in this study we also showed that the expression of these nuclear receptors is regulated throughout the course of DSS induced colitis, further suggesting that activation of these receptors can regulate inflammation. Specifically, the expression of LXR and not RXR or PPARγ is increased in response to both an acute inflammatory stimulus (*in vitro*) and also in the acute stages of DSS induced colitis (*in vivo*). This suggests that LXR expression and subsequent activation may limit the extent of inflammation in autoimmune diseases.

Indeed, Xu *et al* reported that treatment of EAE mice with specific LXR agonists significantly reduced disease severity while a study undertaken by Park *et al* highlighted that activation of LXR in CIA mice could suppress the onset of disease while reducing inflammation and joint destruction (Xu et al. 2009, Park et al. 2010). These reports further highlight a potential therapeutic role for LXR in autoimmune diseases.

LXR, RXR and PPARγ activation not only resulted in a decrease in cytokine production, a downregulation in the expression of the costimulatory markers CD40, CD80 and CD86 was also observed. Costimulation between these markers and coreceptors (CD40L and CD28 respectively) on the surface of naive T cells is necessary to drive specific T helper cell responses (Palucka, Banchereau 1999). Numerous reports have also shown increased expression of these surface markers in disease. Increased expression of CD40 in the lamina propria of patients with Crohns disease has been reported while the expression of CD86 was also increased in the inflamed mucosa of IBD patients (Polese et al. 2003, Rugtveit, Bakka & Brandtzaeg 1997). Therefore targeting these costimulatory markers may be of therapeutic interest as it provides the means to terminate or repress Th cell responses and ameliorate disease. The production of T cell costimulatory blockers such as Abatacept has thus far proven beneficial in the treatment of RA (Chen 2010). Also in three separate murine models of colitis the use of anti-CD40L therapy has shown promising results and treatment with specific CD80 and CD86 inhibitors also prevented the initiation of EAE in mice (Stuber, Strober & Neurath 1996, Nishikawa, Matsuo 1999). These reports therefore support our evidence that targeting these nuclear receptors in disease may have therapeutic potential as a downregulation in costimulatory marker expression is seen following their activation.

It is clear that LXR, RXR and PPARγ display potent anti-inflammatory effects in DC and negatively affect DC maturation. However, in order to elucidate this mechanism, we examined the TLR pathway in greater detail. LPS signals through TLR4, and was the main receptor utilised in this study. However there is a large family of TLR receptors which have overlapping but distinct signalling pathways. Upon TLR occupancy by its ligand, specific adaptor molecules are recruited to the receptor to mediate downstream signalling (Moynagh 2005). We therefore examined the effects of nuclear receptor activation following DC maturation with a panel of TLR ligands in order to identify a possible TLR specific target. Interestingly our results highlighted that within the TLR signalling pathway, these nuclear receptors did not exclusively utilise a specific TLR adaptor or signalling protein. Instead the effects of these receptors were mediated through a common element within all TLR signalling pathways – NFκB and IRF3. Our data shows that activation of these receptors can inhibit NFκB and IRF3 activation however, upon further examination we highlighted that LXR:RXR heterodimers were implicated in the suppression of NFκB and not IRF3. Previous reports have highlighted a link between the TLR pathway and the nuclear receptor pathway, whereby activation of one can negatively affect the response of the other (Hanley et al. 2010). This emphasises again a role for these receptors in the regulation of the inflammatory response. This study also examines for the first time markers of DC maturation in response to entire panel of TLR ligands, providing further insight into the specific anti-inflammatory properties they can display in DC *in vitro*.

Given that the role of LXR in DC biology has been relatively unexplored, and that the findings of our initial studies indicated the formation of LXR:RXR heterodimers to inhibit NFκB activation, and suppress IL-12, we therefore focused the remainder of our study on LXR and the identification of its specific molecular target. Our results show

that LXR activation specifically targets the IL-12p35 subunit of IL-12, the IL-12p40 subunit of both IL-12 and IL-23 and the EBI3 subunit of IL-27. However, the question still remained how activation of LXR could specifically affect the transcription of these three subunits and not IL-27p28 or IL-23p19. Given the importance of the IL-12 family of cytokines in disease and Th cell responses, numerous studies in the past ten years have focused on understanding how the production of these cytokines is regulated. Through the use of specific KO mice and luciferase transfection systems the mechanism by which IL-12p40, IL-12p35, IL-27p28, IL-23p19 and EBI3 are regulated in immune cells, in particular DC has been identified. The production of IL-12p40, IL-12p35, and EBI3 all require the involvement of the NFκB signalling subunit p50. Therefore we hypothesised that LXR may directly affect NFκBp50 in order to specifically target the expression of individual IL-12 subunits. Our results show that LXR activation can significantly reduce the expression of this subunit without affecting other NFκB subunits such as p65. Furthermore, we have also shown that LXR associates with p50 and subsequently sequesters NFκBp50 in the cytoplasm, preventing its translocation to the nucleus thereby inhibiting the transcription of NFκBp50 dependent genes. Interestingly, the effect of LXR on NFκBp50 may not directly depend on an association with RXR. As we have shown in this study, even in the presence of an RXR inhibitor, the association between LXR and NFκBp50 is still present, however it appears that the level of association is reduced. To date, numerous studies have identified the specific heterodimer combinations required in the regulation of specific proteins and indeed there have also been a number of studies indicating that activation of one particular nuclear receptor results in a physical interaction with transcription factors such as NFκB or AP-1. However the exact mechanism in which heterodimerisation affects this association still remains elusive. We therefore hypothesize that the formation of

LXR:RXR heterodimers control the production of the IL-12 family of cytokines where LXR directly associates with NFκBp50 in order to exert its affects. Although it was not examined in this study it may be possible that RXR targets p50's heterodimerisation partner i.e. c-Rel or p65 to have a combined affect on the transcription of their target genes.

The ability of LXR to directly target NFκBp50 in DC and ultimately inhibit IL-12 production highlights the potential of targeting this receptor in disease. It has previously been shown that while p50 is essential in EBI3 transcription in DC, the expression of this IL-27 subunit is not controlled by p50 in B cells (Wirtz et al. 2005). This finding suggests that activation of LXR could target the production of the IL-12 family of cytokines specifically released from the DC and not other immune cells. Therefore activation of LXR could directly target cytokines important for the differentiation of Th cells without compromising the ability of other immune cells to respond normally to infections. Given that the current treatments for inflammatory diseases can leave patients immunosuppressed, targeting LXR in these diseases could improve on the therapies that are already available.

Although the maintenance of appropriate levels of NFκB activity is critical for immunological homeostasis and normal cellular proliferation, excessive activation of this pathway can also lead to disease (Yamamoto, Gaynor 2001). NFκB has been reported to be highly activated in RA, MS, IBD, asthma and atherosclerosis (Tak, Firestein 2001). The association between NFκB and disease is also highlighted in animal models of inflammation. In experimentally induced colitis in mice, NFκB activation was enhanced in the colonic epithelium (Yang et al. 1999). Similarly in collagen induced arthritis in mice; increased NFκB activity was reported in the

synovium (Han et al. 1998). Therefore the development of drugs targeting the NFκB pathway shows promise in the treatment of these diseases. Tacrolimus (or FK-506) is an approved immunosuppressive agent that is used after organ transplantation to prevent graft-versus host disease. It has been shown that FK-506 can inhibit the NFκB pathway by preventing the translocation of c-Rel from the cytoplasm to the nucleus (Venkataraman, Burakoff & Sen 1995). Similarly the use of terpenoids, small secondary metabolites released from plants, in NFκB inhibition is currently being explored. Pinene and Lycopene are two members of this family which have been indicated in the treatment of arthritis and atherosclerosis by affecting the translocation of p65 to the nucleus (Zhou et al. 2004, Heber, Lu 2002). Interestingly, ablation of p50 in mice has reduced detrimental affects compared to other NFκB subunits, for example NFκBp65 deficient mice die in utero, while RelB deficient mice develop normally but suffer from severe disorders ranging from splenomegaly to chronic microbial infections (Sha et al. 1995). Therefore targeting p50 through activation of LXR may suppress IL-12 production with minimal unwanted side effects and therefore represents a potential therapeutic target in disease. However it has been reported that p50 deficient mice are more susceptible to intracellular and extracellular Gram positive bacterial infections (Sha et al. 1995). It is therefore essential that activation of LXR is limited to situations with increased p50 expression or activation as is the case with numerous inflammatory diseases. However, targeting this receptor in the treatment of these diseases shows substantial potential and represents a means to directly target the IL-12 cytokines via NFκBp50 without having off target effects.

In the future, the design of specific LXR agonists which promote the preferential partnership between one nuclear receptor over another will be instrumental in targeting a specific subset of genes. Similarly, the production of tissue specific agonists would

also be beneficial in the treatment of inflammatory disorders characterised by site specific inflammation. Kaneoko *et al* described the first such agonist YT-32,whereby the expression of ABCA1, ABCG5 and ABCG8 were regulated in the intestine following oral administration of YT-32 without any effect on the expression of these genes in the liver (Kaneko et al. 2003). More recently Wyeth has developed a tissue specific LXR agonist known as WYE-672 which has been shown to modulate functions in the kidney but not the liver (Hu et al. 2010). Developments such as these are essential in maximising the full beneficial potential of LXR activation in disease while ultimately inhibiting unwanted adverse side effects in the patient.

CHAPTER 7

APPENDIX

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CELL CULTURE MEDIA

COMPLETE RPMI 1640	500ml
5% Heat inactivated Foetal Calf Serum (FCS)	25ml
Penicillin/streptomycin/L-glutamine Culture Cocktail	10 ml
(Gives a final concentration of 2 mM L-glutamine,	
100 μg/ml penicillin and 100 U/ml streptomycin)	

COMPLETE DMEM	500 ml
5% Heat inactivated Foetal Calf Serum (FCS)	25ml
Penicillin/streptomycin/L-glutamine Culture Cocktail	10 ml
(Gives a final concentration of 2 mM L-glutamine,	
100 μg/ml penicillin and 100 U/ml streptomycin)	

10X PHOSPHATE BUFFERED SALINE (PBS)

$Na_2HPO_4.2H_2O$ (8 mM)	23.2 g
KH_2PO_4 (1.5 mM)	4 g
NaCl (137 mM)	160 g
KCl (2.7 mM)	4 g

Make up to 2 L pH to 7.4

10 X TRIS BUFFERED SALINE (TBS) pH 7.6

NaCl	48.4 g
Trizma Base	160 g

Dissolve in 2 L dH_2O pH to 7.6

2N H_2SO_4

H_2SO_4 (36 N)	11.1 ml
dH_2O	88.9 ml

FACS BUFFER

2%FCS

0.05% NaN_3

PBS

10X TAE Buffer

Tris Base	242g
Glacial Acetic Acid	57.1ml
.5M EDTA	100ml
dH_20	750ml

Make up to 1L and pH to 8.5

5X SAMPLE BUFFER

125 mM Tris	6.25 ml 1M Tris HCl pH 6.8
10 % Glycerol	5 ml
2 % Sodium dodecyl sulphate (SDS)	10 ml (10 % (w/v) SDS)
0.05 % (w/v) Bromophenol Blue	0.01 g
dH_2O	28.75 ml
0.25 M Dithiothreitol (DTT)*	250 µl 1 M DTT S

* Added to 1 ml 5X Sample Buffer just before use

SEPARATING GEL (10 % (v/v))

33% w/v Bisacrylamide (30% stock)

1.5M Tris-HCl pH8.8

1% w/v SDS

0.5% w/v Ammonium persulpate

dH_2O

0.1% v/v TEMED

STACKING GEL

6.5% v/v Acrylamide/Bisacrylamide (30% stock)

0.5M Tris-HCl pH6.8

1% w/v SDS

0.5% w/v Ammonium persulphate

dH_2O

0.1% v/v TEMED

ELECTRODE RUNNING BUFFER

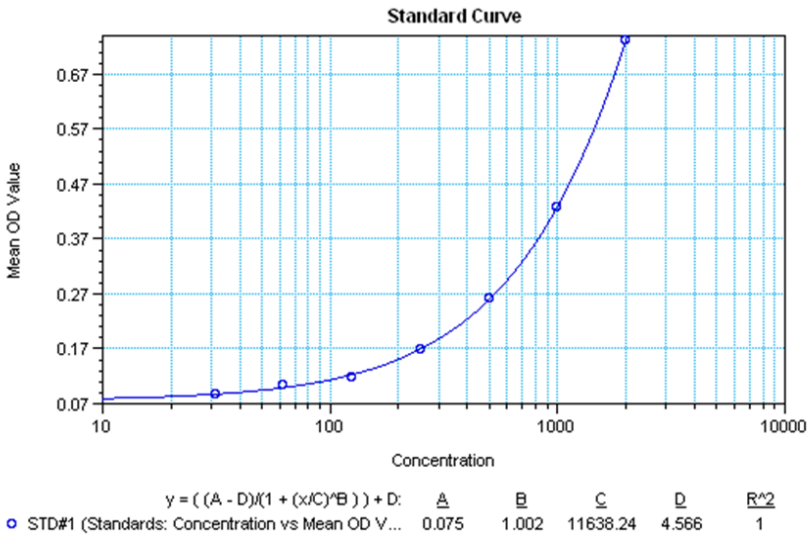
25mM Tris base

200mM Glycine

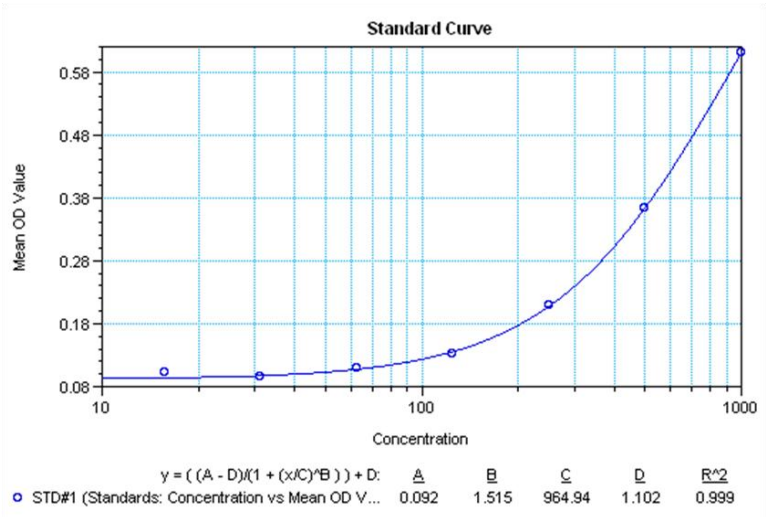
17mM SDS

ELISA STANDARD CURVES

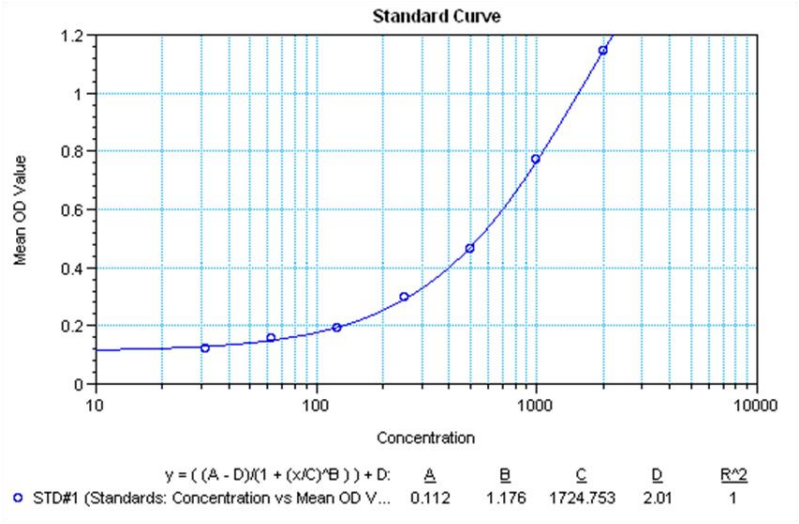
IL-12p40



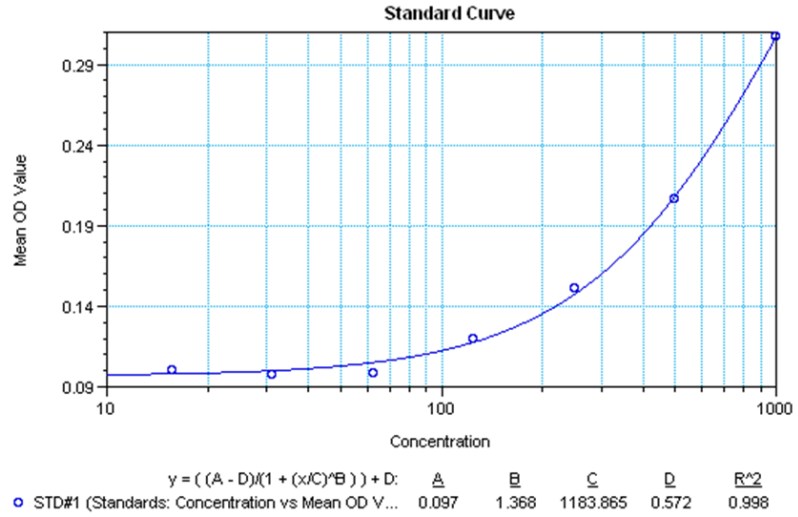
IL-23



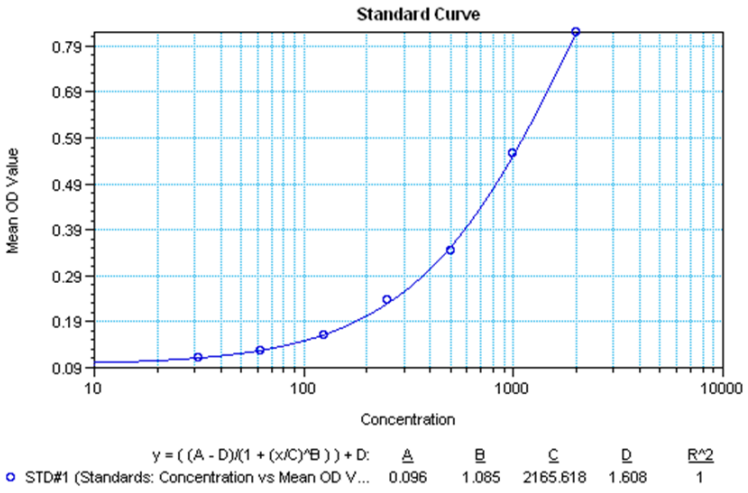
IL-12p70



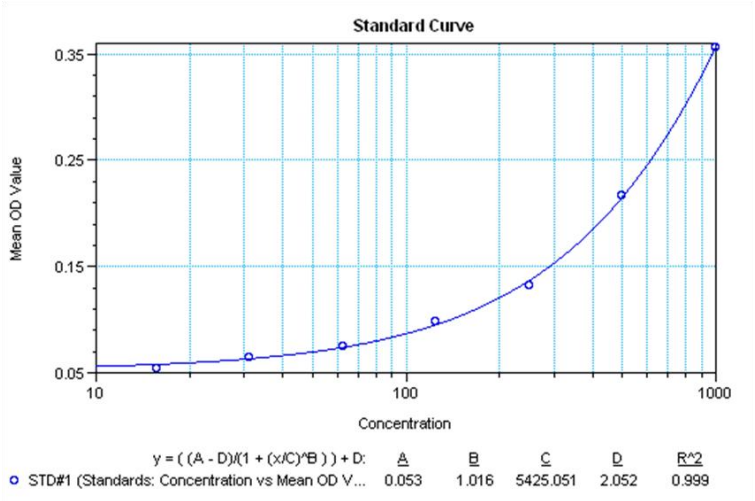
IL-27



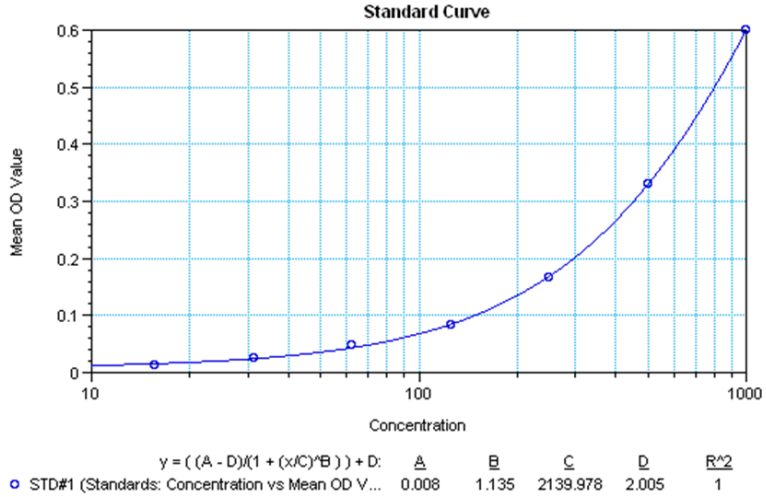
TNFα



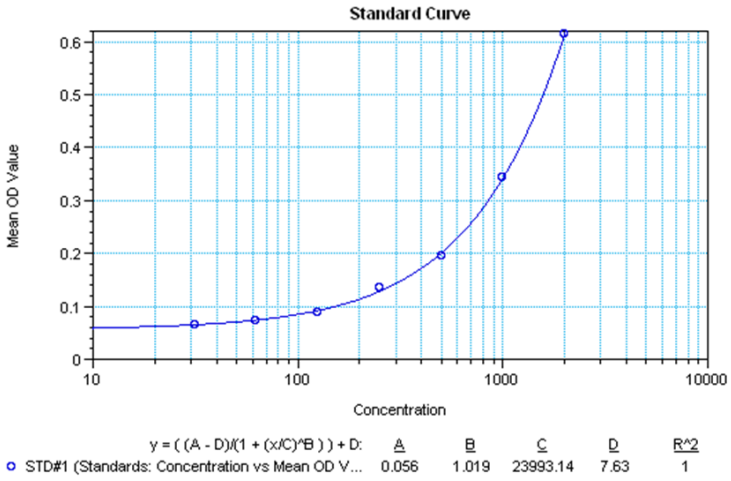
IL-1β



IL-6



IL-10



	y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
○	STD#1 (Standards: Concentration vs Mean OD V...	0.056	1.019	23993.14	7.63	1

CHAPTER 8

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