Negative Feedback of Inflammation of Macrophages:

Transcriptional Cross-talk of the LPS and IL-10 Signalling Pathways

Michael Luke Ridley

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Abstract

The classical picture of interleukin 10 (IL-10) is of a powerful anti-inflammatory cytokine, which suppresses many functions of myeloid cells, including antigen presentation and pro-inflammatory cytokine expression. IL-10 acts via the Janus-activated kinase JAK1 to activate the transcription factor Signal transducer and activator of transcription 3 (STAT3). The actions of IL-10 are essential for prevention of excessive inflammatory responses, particularly in gut tissues that are constantly exposed to potential pathogens. Many of the anti-inflammatory effects of IL-10 have been attributed to the inhibition of the transcription factor Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). However, this simplistic picture of IL-10 is becoming increasingly under challenge, with emerging evidence of immune-stimulatory properties, and actions that cannot be attributed to NF-κB inhibition.

In this thesis I used monocyte-derived macrophages from healthy human donors to investigate the effects of IL-10 on responses to lipopolysaccharide (LPS), a pathogen associated molecule that strongly activates macrophages. Such a transcriptome-wide analysis has not been previously reported in primary human macrophages. Microarray analysis revealed that of the several hundred genes robustly induced by LPS less than half were inhibited by IL-10. Surprisingly, IL-10 cooperated with LPS to enhance the expression of several genes. Both the inhibitive and cooperative regulation of gene expression by IL-10 occurred at the level of transcription, and were dependent on STAT3. Using a pharmacological inhibitor, it was shown that the suppressive effects of IL-10 at the transcriptome level did not correlate with inhibition of NF-κB. Many NF-κB dependent genes were either insensitive to IL-10-mediated inhibition or cooperatively regulated by LPS and IL-10, and many IL-10 inhibited genes were not dependent on NF-κB.

To investigate cooperative regulation of gene expression by LPS and IL-10 I focused on the gene TNF-superfamily member 9 (*TNFSF9*) which was cooperatively regulated by LPS and IL-10 at the mRNA and protein level. Although *de novo* protein synthesis was required for the inhibitory effect of IL-10 on *TNF* expression, it was dispensable for the induction of *TNFSF9*. An evolutionary conserved region (ECR) upstream of the *TNFSF9* gene was shown to possess potent enhancer activity, and mediate cooperative transcriptional induction by LPS and IL-10. Analysis of the ECR identified putative NF-κB and STAT3 binding sites, and its cooperative regulation by IL-10 and LPS was dependent on JAKs and NF-κB. Finally a STAT3 reporter construct was shown to mediate cooperative transcriptional activation by LPS and IL-10. This response was dependent on JAKs and mitogen-activated kinase p38, but independent of NF-κB. This suggests that a convergence between the JAK and p38 signalling pathways may contribute to the cooperative gene regulation by LPS and IL-10.

Together, these findings shed new light on the mechanisms by which IL-10 inhibits pro-inflammatory gene expression. They also indicate surprisingly broad and powerful gene-specific cooperative interactions between LPS-induced and IL-10 induced signalling pathways, which are likely to contribute to the subtle immune-modulatory properties of IL-10.

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“Come forth into the light of things, let nature be your teacher.”

-*William Wordsworth*

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List of Abbreviations

ac	Acetylation
bp	base pair
BSA	Bovine Serum Albumin
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CH25H	Cholesterol 25-Hydroxylase
ChIP	Chromatin immunoprecipitation
CLR	C-type leptin receptors
CMV	Cyclomegalovirus
C_t	Threshold cycle number
CXCL	Chemokine (C-X-C motif) ligand
DAMPS	Damage associated molecular patterns

DC	Dendritic cell
DHS	DNase hypersensitive site
DMEM	Dulbecco's modified eagle's media
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
ds	Double stranded
DUSP	Dual specificity phosphatase
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMP	Erythromonocyte progenitor
FFAR	Free Fatty Acid Receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GO	Gene Ontology
H3K27	Histone
H3K4	Histone
HBSS	Hanks balanced salt solution
HIFCS	Heat-inactivated fetal calf serum
HOMER	Hypergeometric optimisation of motif enrichment
HRP	Horse radish peroxide
IBD	Inflammatory bowel disease
IFN	Interferon
IKK	IκBα kinase
IL-10	Interleukin 10
IL10RA	IL-10 receptor subunit A
IL10RB	IL-10 receptor subunit B
IRAK	Interleukin-1 Receptor-Associated Kinase
IRF	Interferon regulatory factor
IκBα	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Alpha
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KO	Knockout out
LBP	LPS binding protein
LPS	Lipopolysaccharide
LRR	leucine rich region
MAPK	mitogen activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MD2	Myeloid differentiation factor 2
MDM	Monocyte derived macrophages
me	mono-methylation
me3	Tri-methylation
MEM	Modified eagle's media
MKK	MAPK kinase
M.O.I	Multiplicity of Infection

mRNA	messenger RNA
Myd88	Myeloid differentiation protein 88
NFR	Nucleosome free region
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like-receptors
NR	Nuclear receptor
p-	phospho-
PAMPS	pathogen associated molecular patterns
PBS	Phosphate buffered saline
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PRR	Pattern recognition receptor
PT-QPCR	Primary Transcript quantitative polymerase chain reaction
PVDF	Polyvinylidene difluoride
QPCR	Quantitative polymerase chain reaction
RELA	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A
RHD	Rel homology domain
RLR	RIG1-like-receptors
RNA	Ribonucleic acid
RNA Pol II	RNA polymerase II
RPMI 1640	Roswell park memorial institute 1640
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
ss	Single stranded
STAT	Signal transducer and activator of transcription
TAB	TAK binding protein
TAD	Transactivation domain
TAK	TGF-Beta Activated Kinase
Taq	*Thermus aquaticus*
TE	Tris-EDTA
TF	Transcription factor
TH1	T helper cell (type 1)
TH2	T helper cell (type 2)
TH17	IL-17 producing T helper cell
TIRAP	Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TNFAIP	TNF, alpha-induced protein
TNFSF	Tumour Necrosis Factor Superfamily
TNIP	TNFAIP3 interacting protein
TRAF	TNF Receptor-Associated Factor
T_{reg}	Regulatory T cell
TSS	Transcription start site

Chapter 1 – Introduction

1.1 – The Immune System

In order to protect the body from pathogen invasion and infection the immune system has evolved both broad and adaptable strategies to detect and remove these organisms. Most organisms from bacteria to Homo sapiens have a system of biochemically detecting and responding to parasitic or pathogenic attack, varying in complexity; from the specific cleavage of DNA sequences in bacteria, to the adaptive and long lasting immune system of vertebrates.

The vertebrate immune system is a complex multicellular network and can be further categorised into two further systems: The adaptive immune system and the innate immune system.

Adaptive immunity is the more recently evolved of the two systems, having arisen after the divergence of jawed (Gnathostomes) and jawless vertebrates (Agnathans). The adaptive immune response involves the development of antigen-specific lymphocytes through the rearrangement and mutation of genetic material, which can then be developed into long lasting immunological memory.

The adaptive immune response is a potent defence against pathogens, it is however a slow process to initiate, taking between 4-7 days to initially take effect. Innate immunity is a much more rapid and broader response against invasion or damage than the adaptive immune system. On contact with pathogens or signals indicating damage, cells of this system release cytokine or chemokine factors to recruit more cells to the locality in order to destroy the pathogen and initiate the formation or reactivation of the adaptive immune response (Murphy, 2007).

1.1.1 – Cells of the Immune System

In humans, the cells which make up the immune system has evolved a number of cell types to defend the body in a number of ways. These cells can be broadly categorised into lymphocytes and myeloid cells. Lymphocytes include: B cells, T cells and, natural killer (NK) cells, while the myeloid lineage consists of a multitude of cell types, including neutrophils, dendritic cells and macrophages (Murphy, 2007).

Both lymphocytes and myeloid cells can be formed from haematopoietic stem cells (HSC) in the bone marrow and diverge into distinct lineages early in haematopoiesis (differentiation of multiple haematopoietic cell lineages is shown in Figure 1.1). Myeloid cells differentiate from the common myeloid progenitor cells (CMPs) and lymphoid cells derive from the common lymphoid progenitor cells (CLPs) (Kondo, 2010, Iwasaki and Akashi, 2007).

1.1.1.1 – Lymphoid Cells

Lymphoid cells are derived exclusively from haematopoietic stem cells in the bone marrow, which differentiated into common lymphoid progenitor cells (CLP). From this the three main classes of lymphoid cells are derived: B cells and T cells generate antigen specific cell surface receptors during maturation, while innate lymphoid cells (ILCs) do not respond to specific antigens and instead react to presented cell surface ligands on the target cell (Murphy, 2007).

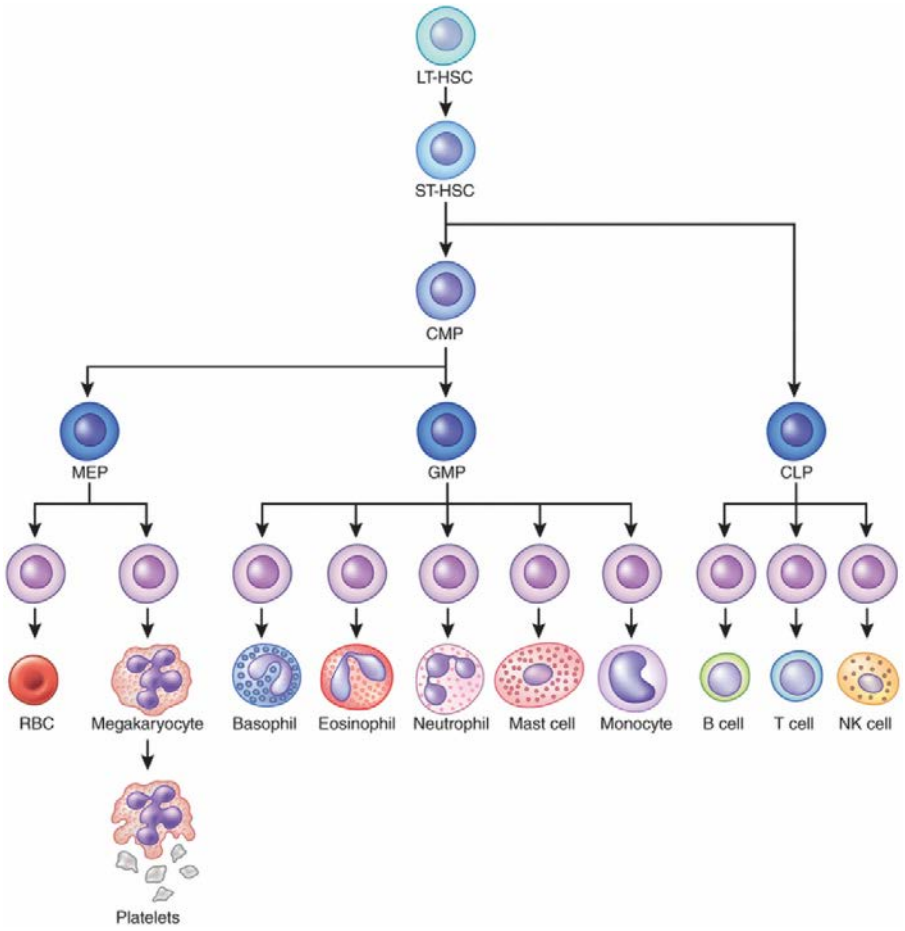

Figure 1.1 – The Differentiation of the Haematopoietic System from HSCs in adult humans

Long-term HSC (LT-HSC) residing in the bone marrow are able to differentiate into short-term HSC (ST-HSC) which can further differentiate into common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) cells. These 2 cell types are restricted in the further differentiation their daughter cells are able to undergo. CLPs are the progenitor cells of the lymphoid lineage are able to differentiate into: B cells, T cells and, innate lymphoid cells (ILCs). CMPs can further differentiate into: megakaryocyte-erythroid progenitors (MEP) or granulocyte-myeloid progenitors (GMP). MEPs further differentiate into red blood cells (RBC) or into megakaryocytes, which produce platelets. GMP cells produce a multitude of innate immune cells: such as basophils, monocytes and neutrophils. Taken from Sankaran and Weiss (2015).

1.1.1.1.1 – B cells

B cells are generated and mature in the bone marrow. After committing to become a B cell, CLP cells begin recombining the gene segments in the Immunoglobulin (Ig) locus, to form an antigen receptor. After stimulation, B cells proliferate to form a germinal centre in the lymph node follicle. These cells can then differentiate into one of two cell types; plasma cells and memory B cells. Plasma cells secrete large concentrations of soluble antibody during the course of infection, while memory B cells are less active and persist in the organism, past the initial infection, in order to activate specific humoral immunity (Pieper et al., 2013).

1.1.1.1.2 – T cells

After initial generation in the bone marrow, T cells migrate to the thymus where the rest of the stages of maturation occur. In this organ, the immature T cells undergo positive and negative selection to reduce the number of self-reactive cells and differentiate into CD4+ helper cells or CD8+ cytotoxic T cells. Cytotoxic T cells recognise and kill cells infected with virus or intracellular bacteria and CD4+ T helper cells (TH) assist in controlling the adaptive immune response. CD4+ T cells are able to interact with antigen presenting cells (APCs) which express major histocompatibility complex (MHC) class II proteins (MHC-II). The human version of this gene family is known as human leukocyte antigen II and is encoding by 6 paralogues known as: HLA-DP/DM/DOA/DOB/DQ/DR depending on the combination of subunits).

MHC-II is complex of 2 membrane-bound proteins (MHC-II α and β) that is able to present a single molecule of antigen (not larger than 9 amino acids in length) to the antigen receptor on a T cell (TCR), as well as bind the molecule CD4 in an antigen-independent manner. The binding of the TCR to an antigen bound by MHC-II at the same time as interacting with CD4, allows the T cell to become activated, when co-stimulatory receptors on the T cell (such as CD28) are bound by ligands on the APC (such as CD80 or CD86) (Murphy, 2007). On activation by TCR and co-stimulations, CD4+ T cells, are able to produce a variety of cytokines and the particular cytokines these cells produce allow them to be subcategorised into subsets of cells. There are several T helper cell subsets including: T_H1, T_H2, T_H17 and T_{reg} (Luckheeram et al., 2012).

T_H1 cells assist in activating macrophages that have ingested pathogens which persist in the phagocytic vesicles. TH2 cells assist in B cell activation, proliferation and antibody class switching (Shinomiya et al., 1989). T_H17 are observed in the mucosal barriers (such as intestine) and assist in the recruitment of neutrophils and other inflammatory cells in response to bacteria or fungus(Ouyang et al., 2008). T_{reg} cells act as key negative regulators of the immune response and inhibit potential auto-immune responses, through a number of mechanisms including the secretion of anti-inflammatory cytokines and inhibition of co-stimulatory molecules (Corthay, 2009).

1.1.1.1.3 – Innate Lymphoid Cells

Unlike the other cells of the lymphoid lineage the innate lymphoid cell (ILC) lineage does not express antigen-dependent receptors in order to become activated. Instead this family of cells is dependent on myeloid- or epithelial-derived cytokines. These cells have many roles including: lymphoid organ development and providing anti-cancer immunity (Artis and Spits, 2015, Klose and Artis, 2016). These cells can be further sub-classified based on their transcription factor expression, as well as whether the cells are able to induce cytotoxicity. The natural killer (NK) cell is the sole member of the cytotoxic ILC group. These cells interact with the ubiquitous antigen presentation complex MHC class I (MHC-I) and other cell surface proteins which suppress the release of inflammatory cytokines, Granzymes and the pore-forming protein, Perforin (Topham and Hewitt, 2009). The non-cytotoxic compartment of ILCs are segregated according to differences in cytokine production and the expression of transcription factors. These include: Type 1 ILCs (which are T-bet+ and can produce IFN-y and TNF on activation), Type 2 ILCs (which express the transcription factor GATA3 and produce IL-4/5/9 on stimulation) and, Type 3 ILCs (which can produce IL-17, IL-122 and, GM-CSF). Type 3 ILCs express the transcription factor RORyt but can also express T-bet, which causes the type 3 ILCs to produce TNF and IFNy.

1.1.1.2 – Myeloid cells

After diverging from the lymphoid lineage, the CMP further differentiate into a granulocyte/macrophage progenitor cell (GMP), prior to diverging into granulocytes

(neutrophils, eosinophils, basophils) or monocytes, dendritic cells and macrophages (Alvarez-Errico et al., 2015, Murphy, 2007).

Neutrophils and, eosinophils are short-lived blood circulating immune cells, which have a key role clearing infection by bacteria or parasites, respectively. All of these cells remain in the blood under normal conditions, but during inflammation and infection these migrate into affected tissues. Neutrophils have a potent phagocytic and bactericidal ability and are vital in controlling the bacterial load, following bacterial invasion. However, unlike macrophages and dendritic cells, they lack the capacity to effectively present antigen to adaptive immune cells (Kolaczkowska and Kubes, 2013).

Eosinophils, while also carrying some bactericidal activity, are thought to mainly target parasitic infections due to their increased numbers in circulation, during a parasitic infection (Rothenberg and Hogan, 2006). Basophils lack the ability to ingest or kill pathogens and instead have histamine granules in their cytoplasm, which (on secretion of the granules) acts to increase circulation to inflamed tissue, allowing immune cells to enter into the tissue (Voehringer, 2013).

1.1.1.2.1 – Dendritic cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) with an essential role in activating and controlling T-cell immune responses. From studies of the mouse immune system, it is apparent that DCs are a heterogeneous group of cells with multiple lineage (can be derived from both CLP and CMP cells) and functions which are heavily influenced by the

tissues in which they are found (Ardavin, 2003, Geissmann et al., 2010). In tissues these cells are quiescent until activated by innate immune signals and loaded with antigen. Once activated these cells subsequently migrate through the lymphatic system to the draining lymph node for the tissue. There they are exposed to naive and memory T-cells and able to stimulate the adaptive immune response, on recognising the recognition of the antigen they present to a cognate T-cell (Merad et al., 2013).

1.1.1.2.2 – Monocytes

Monocytes are blood circulating phagocytic myeloid cells. These cells are differentiated in the bone marrow from HSC cells which normally circulate the blood, spleen and, bone marrow (Yona et al., 2013, Swirski et al., 2009). Under inflammatory conditions, these cells are able to migrate into tissue in response to chemokines (Auffray et al., 2009, Zimmermann et al., 2010, Geissmann et al., 2010). Once inside the tissue, these cells are able to differentiate into macrophages and can assume a similar function to existing tissue macrophages (van de Laar et al., 2016).

In humans blood there are two phenotypic subsets of monocytes segregated on the basis of the expression of the cell-surface proteins: CD14 and CD16 (Geissmann et al., 2003). CD14+ CD16-/+ monocytes (which in many respects correspond to Ly6C+ monocyte subset in mice) are strong producers of pro-inflammatory cytokines in response to bacterial cell products whereas, the $CD14_{dim}$CD16+ subset (corresponding to the LY6C- subset in mice) responds more to viruses and nucleic acids (Cros et al., 2010, Ingersoll et al., 2010).

As well as having differential preference for stimuli, these cells also have been shown to exhibit differences in gene expression and cellular function in different diseases (Geissmann et al., 2003, Auffray et al., 2009, Zimmermann et al., 2010).

1.1.1.2.3 –Macrophages

Macrophages are tissue resident cells of the innate immune system. Macrophages have important roles in maintaining tissue homeostasis, modulating the adaptive immune response, as well as initiating and eventually resolving the inflammatory response (Wynn et al., 2013, Murray and Wynn, 2011). In mammals these cells originate from three 3 distinct sources, during development:

1) The Yolk sac during embryogenesis

2) The foetal liver during initial haematopoiesis

3) The bone marrow (pre-natally or post-natally depending on the species).

Initially macrophages originate during embryonic haematopoiesis, from yolk sac blood islands at E8.5 (in mice) and colonise the embryo on the establishment of circulation from the yolk sac (shown in Figure 1.2) (Gomez Perdiguero and Geissmann, 2013, Schulz et al., 2012). These macrophages can persist throughout adult life and have the potential for self-renewal to maintain the population of macrophages in that tissue (Hashimoto et al., 2013).

After mammalian foetal haematopoiesis has been established in the liver, populations of monocytes (determined by the expression of the transcription factor MYB) are able to migrate into some tissues and differentiate into macrophages (Schulz et al., 2012). This process is then

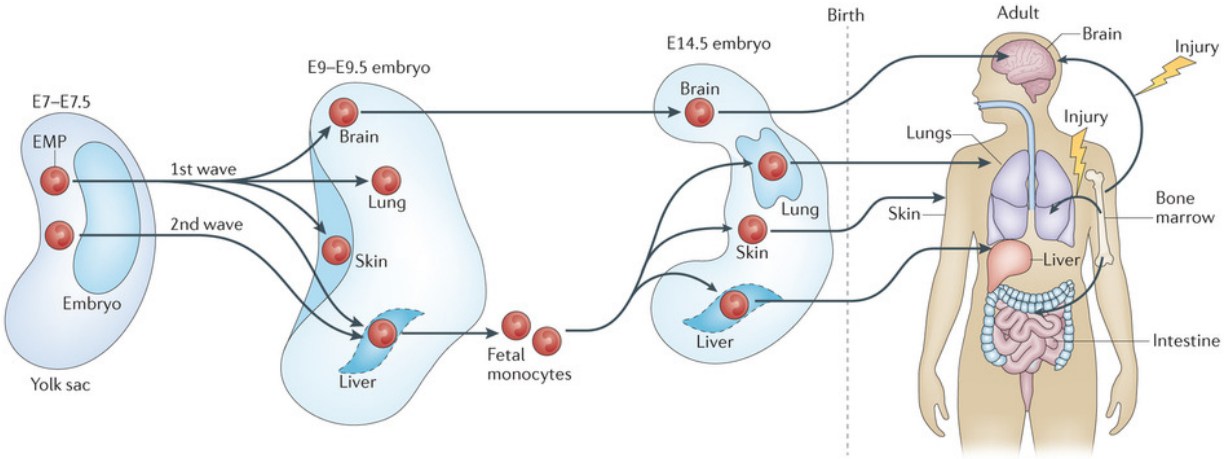

Figure 1.2 – Ontogeny of Tissue macrophages

Taken from Lavin et al. (2015a). The dissemination of tissue macrophages across an embryo occurs in mice between E7 and 7.5 from erythomeyloid progenitor cells (EMPs) in the yolk sac into the various developing tissues such as the brain (where they develop in to microglia), the lung (where they become alveolar macrophages), the skin (where they differentiate into Langerhans cells) and the liver (where they become Kupffer cells).

moved to the bone marrow, post-natally in mice, but prenatally in humans (Gordon and Taylor, 2005). There are several sub-types of macrophages, classified according to their tissue localisation, which differ in terms of function and gene expression (Lavin et al., 2015b). These subsets include:

- Microglia, located in the central nervous system (CNS)
- Osteoclasts, located in and on bones
- Kupffer cells, located in the liver
- Alveolar macrophages which reside in the lungs
- Intestinal macrophages, which are located under the intestinal endothelium.

1.1.1.2.3.1 – Microglia

Microglia are a highly distinct population of tissue macrophages which inhabit the CNS which are derived from the yolk-sac and under homeostatic conditions are not replenished by monocytes and instead retain the ability to proliferate (Lawson et al., 1992, Ginhoux et al., 2013). These cells are responsible for the maintenance of the neuronal network, able to assist in the survival of neurons, but also assist in apoptosis and the removal of debris. Microglia also sample the local tissue for antigen and are morphologically dendritic in order to survey the local tissue. On detection of a pathogen or inflammatory stimulus they release pro-inflammatory molecules and initiate antigen presentation (Nayak et al., 2014). The activation

of these cells by inflammatory stimuli has been highlighted as a driver of adverse pathology in multiple CNS disease, such as Alzheimer's and Parkinson's disease (Nayak et al., 2014).

1.1.1.2.3.2 – Osteoclasts

Osteoclasts are multi-nucleated cells which are able to resorb mineral bone. These cells are first observed in the developing bones, resorbing bone deposited inside bones by osteoblasts, which allows for the growth of the bone during development (Gilbert, 2000).

These cells are the result of multiple fusions of progenitor cells (Boyle et al., 2003, Miyamoto and Suda, 2003). In adults: osteoclast precursors from the bone marrow traffic to sites of bone damage or growth and begin to fuse with other osteoclast precursors, in the presence of the cytokines M-CSF and RANKL (Yasuda et al., 1998) (Udagawa et al., 1990). These precursor cells also arise during embryogenesis where they are detectable after establishment of circulation with the yolk sac (Thesingh, 1986).

1.1.1.2.3.3 – Kupffer cells

Since the liver provides many functions which are vital to the long term health and survival of chordates, it stands to reason that maintaining the health and integrity of the organ is also critical. Kupffer cells are the resident macrophages of the liver and play a vital role in detecting and responding to pathogens that pass through the liver in the blood. These cells also play a vital role in clearing apoptotic hepatocytes and recycling of damaged red blood cells (Bilzer et al., 2006). These cells are initially derived from yolk sac blood islands and under normal conditions do not require supplementation from monocytes. However, under inflammatory

conditions (or extensive injury) monocytes are able to extravasate from the blood into the liver (David et al., 2016).

1.1.1.2.3.4 – Alveolar macrophages

Due to the anatomy of the lung, the alveoli would be a chink in the body's defences, given the close proximity of the capillaries to the alveolar space. Alveolar macrophages are therefore key in maintaining a front-line defence against pathogen invasion(Morales-Nebreda et al., 2015).

Monocytes derived macrophages do not constitute a major population in the lung in healthy conditions but are required to control infection and repair damage to tissue (Hussell and Bell, 2014). This macrophage compartment originates from the yolk-sac but is able to be reconstituted by bone marrow-derived cells (van de Laar et al., 2016).

1.1.1.2.3.5 – Intestinal macrophages

Unlike many macrophage populations previously mentioned, macrophages which reside in the intestinal tract are supplemented and replaced by monocytes from the blood. These cells persist in multiple locations throughout the tissue and are almost constantly exposed to pathogens, microbial agents and, potential antigens (Bain and Mowat, 2014a, Bain and Mowat, 2014b, Smith et al., 2011b). Therefore unlike other macrophage populations, these cells are constantly replenished over the course of a lifetime, by circulating monocytes (Bain et al., 2014).

It was previously thought that tissue resident macrophages constitute a large proportion of the total macrophage population after birth and were replenished and eventually replaced by circulating monocytes from the blood, which are able to differentiate into macrophages through cytokine stimulation (M-CSF and GM-CSF). This replacement of embryonically derived macrophage is now only thought to be the case in the presence of inflammatory lesions or tissues that are continually exposed to pathogens such as the intestine (Ginhoux and Jung, 2014, Varol, 2015).

Macrophages in different tissues exhibit both differential function and a differential pattern of gene expression. These diverse patterns in gene expression are caused predominantly by different cytokines and constituents of the tissue in which they reside. These distinctive conditions enable recruited macrophage progenitors to assume a phenotype typical of existing resident macrophages and enforce cellular responses, appropriate to the tissue (Lavin et al., 2015b, van de Laar et al., 2016, Gosselin et al., 2014). Whether fully differentiated tissue macrophages are able to retain sufficient plasticity for tissue transplantation is a matter of debate (Lavin et al., 2015b, van de Laar et al., 2016). Evidence from van de Laar et al. (2016) indicates that fully differentiated tissue macrophages are not able to acquire a new tissue macrophage phenotype after transplantation, unlike any of the progenitor populations.

1.1.1.2.3.6 – Functional classification of macrophages

Previous attempts to classify macrophage phenotypic traits *in-vitro/vivo* lead to a model similar to T cell subtypes (Mills et al., 2000). This model proposed that there were two

phenotypic extremes of macrophages: M1 (expressing markers such as: IL-12, iNOS and, CD80) and M2 macrophages (expressing markers such as CD163, ARG1 and, IL-10) (Edin et al., 2012, Kigerl et al., 2009). M1 macrophages were described as an inflammatory macrophage subtype analogous to T_{H1} cells and previously designated as "classically activated" macrophages. At the other extreme were the M2 macrophages, described as an anti-inflammatory cell similar to T_H2 cells and previously described as "alternatively-activated macrophages" (Gordon, 2003). However, in recent years this model has proved to be restrictive to its description of macrophage responses, otogeny and, heterogeneity (Mosser and Edwards, 2008, Martinez and Gordon, 2014, Murray et al., 2014). This paradigm is now being replaced in more recent studies by a "spectrum" model of macrophage activation as well as more specific studies on tissue macrophage subsets (Mosser and Edwards, 2008, Xue et al., 2014).

1.2 – Inflammation

Inflammation is a response of vascularised tissue to pathogen invasion, or physical damage. In the inflammatory process, blood vessel walls in the vicinity of damage or immune challenge become permeable and allow leukocytes, lymphocytes and proteins from the blood stream to enter the local tissue to clear the damage or pathogen and repair the tissue. This process leads to the classical signs of inflammation; swelling, redness, heat and pain.

The redness and the heat of an inflamed area, stems from increased blood flow to the area due to vascular permeabilisation, while the pain and swelling is caused by fluid from the blood, containing leukocytes, lymphocytes and protein leaving the blood stream and accumulating in

the area of the tissue damage. Pain also arises from activation of pain receptors by soluble factors released at the site of inflammation.

The egress of leukocytes from the blood stream to the tissue is a tightly controlled process. Firstly the leukocytes can only migrate through vascular endothelium that has been activated by inflammatory cytokines. This activation causes them to express integrin ligands on their vascular facing surfaces, which is consequently bound by leukocytes. This causes them to adhere to and roll along the vascular wall, until they reach an endothelial cell-junction. It's at this point the leukocytes are then able to migrate through the cell wall, into the tissue.

The normal inflammatory response has two distinct stages, the initial inductive phase and the resolution phase. The inductive phase involves the production of inflammatory mediators (such as TNF and IL-6) to activate and/or recruit other immune cells as well as permeablising the local vascular endothelium, to enable the influx of immune cells to the tissue.

The resolution phase of inflammation involves the release of anti-inflammatory molecules, such as glucocorticoid hormones and anti-inflammatory cytokines (Opal and DePalo, 2000, Morand and Leech, 2001, Basil and Levy, 2016, Serhan and Savill, 2005). These molecules actively inhibit the production and release of pro-inflammatory signals, as well as inhibiting the function of adaptive immune cells in the tissue and restore the integrity of the vascular endothelium. With the inflammatory process dampened, macrophages and stroma can initiate repairing the tissue damage caused by inflammation (Ariel, 2012, Koh and DiPietro, 2011).

This resolving phase, is a vital part of the normal inflammatory response. Without suppression of the inflammatory response, inflammation becomes chronic, damaging or potentially

destroying normal tissue function and potentially leading to a systemic alteration in immune cell function and generation(Schuettpelz and Link, 2013).

1.3 – Pattern recognition receptors

Pattern recognition receptors are able to detect molecules known as pathogen associated molecular patterns (PAMPs). PAMPs are a diverse array of molecules ranging from cell wall constituents to large conserved proteins (flagellum), which usually are required for the life cycle or survival of the pathogen and therefore conserved against the evolutionary pressure formed by their recognition by the innate immune system (Taylor et al., 2004, Gordon, 2002).

There are several major classes of PRRs: Toll like receptors (TLRs), retinoid-inducible gene 1-like receptors (RLRs), nucleotide-binding oligomerization domain-like-receptors (NLRs) and C-type lectin receptors (CLRs) (Takeuchi and Akira, 2010). These receptors are expressed across many cell types, both in professional immune cells and non-immune cells. This range of expression allows the detection of pathogens where resident immune cells (such as macrophages or DCs) are not present, supressed or, unable to be infected (in the case of viral pathogens).

1.3.1 – Toll-like Receptors

One of the most keenly studied PRR families is the TLR family. These membrane bound receptors were initially discovered on the basis of their homology to the *D. melanogaster*

protein Toll (also known as Toll-1), which when knocked out in this insect, fatally compromises the immune defence against fungal infection (Lemaitre et al., 1996).

The two main characteristic features of TLR family are the leucine rich repeat (LRR) domain which form the binding surface of the receptor and the cytoplasmic Toll-IL1R (TIR) domain which allows the receptor to initiate cell signalling (Medzhitov, 2001). Differences in these domains between TLR family members determine which PAMPS the receptors are able to recognise as well as the signalling pathways initiated by receptor activation.

Currently 10 members of the TLR family are recognised in humans of the TLR family in humans, 6 of which are expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10), while the remainder are localised to the endosome (TLR3, TLR7, TLR8 and, TLR9)(O'Neill and Golenbock, 2013). The endosomal TLRs are able to bind to nucleic acid polymers within the endosome (such as: dsRNA, ssRNA and CpG rich DNA sequences) and their activation leads to the production of type I IFNs through phosphorylation of interferon regulatory factors (IRFs) 3 or 7(Newton and Dixit, 2012, Uematsu and Akira, 2007).

The cell surface TLRs are able to bind to microbial cell components (such as flagellin or zymosan) as well as viral envelope proteins (RSV) and upregulate multiple inflammatory cytokines (Chaturvedi and Pierce, 2009).

1.3.2 – LPS:TLR4 signalling pathway

Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria which has been shown to act as a trigger for septic shock, through its initiation of inflammatory signalling

through the receptor TLR4 (this signalling pathway is summarised in Figure 1.3) However, for the interaction to occur LPS must first bind to LPS binding protein (LBP) in order for the LPS to be transferred to CD14, which facilitates the binding of LPS to TLR4. After binding to the CD14:LPS, TLR4 then binds to myeloid differentiation factor 2 (MD2). MD2 is able to bind two TLR4:CD14:LPS complexes, in close enough proximity to initiate the intracellular signalling cascade (Park et al., 2009).

The intracellular signalling pathway utilised by TLR4 on activation is dependent on whether the receptor is activated on the cell surface, or in an endosome. When TLR4 is activated on the cell surface, an adaptor protein complex of TIRAP and MYD88 binds to the TIR domain, which in turn allows the IL-1 receptor associated kinase 4 (IRAK4) to be recruited to the complex. On binding to MYD88, IRAK4 is activated and is able to phosphorylate TRAF6. TRAF6 on phosphorylation is then able to ubiquitinated with a K67-linked polyubiquitin chain, which initiates the formation of a complex of TRAF6 with TAK-1, TAB1, TAB2 and TAB3. This complex is then able to phosphorylate 3 notable substrates to effect changes in gene expression; MAPK Kinase (MKK) 3, MKK7 and, the IKK complex. The signalling pathways initiated by LPS are summarised in (Andreakos et al., 2004, Lu et al., 2008)

If activated in the endosome, activated TLR4 recruits the adaptor proteins: TRIF and TRAM. This complex is then able to phosphorylate TRAF3 and TRAF6 and TBK1 which activates their kinase function (Fitzgerald et al., 2003a). This complex then activates the transcription factors NF-κB and IRF3/7 which initiates alterations in gene expression (Fitzgerald et al., 2003b). The activation of the of IRF3/7 induces the activation of the type I interferon response due to its

induction of the *IFNB1* gene (Ohmori and Hamilton, 2001, Fitzgerald et al., 2003b, Jiang et al., 2005, Uematsu and Akira, 2007).

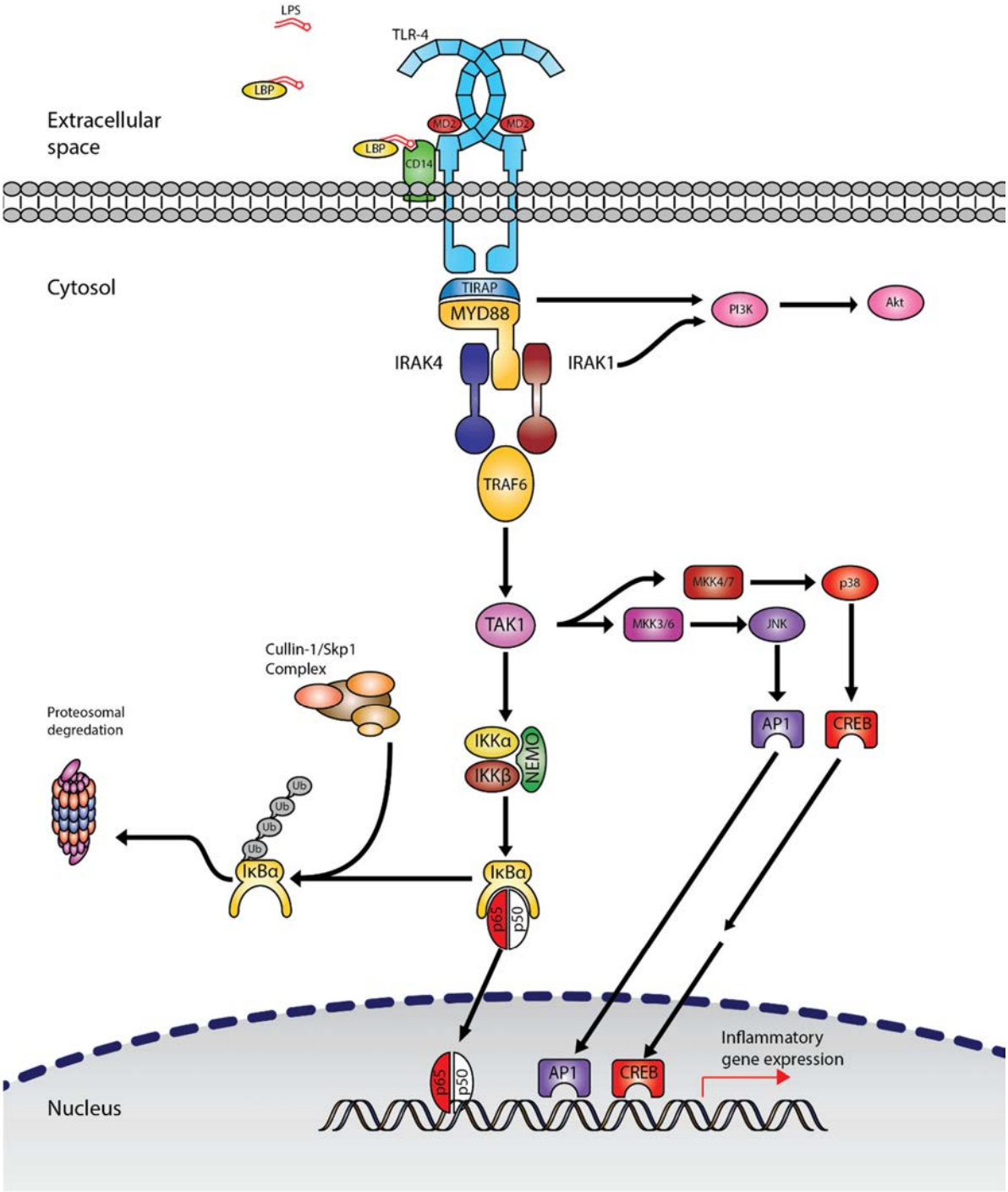
LPS
TLR-4
LBP
Extracellular space
MD2
MD2
LBP
CD14
Cytosol
TIRAP
MYD88
PI3K
Akt
IRAK4
IRAK1
TRAF6
MKK4/7
p38
TAK1
MKK3/6
JNK
Cullin-1/Skp1 Complex
AP1
CREB
Proteosomal degredation
IKKα
IKKβ
NEMO
Ub
Ub
Ub
Ub
IκBα
IκBα
p65
p50
Inflammatory gene expression
p65
p50
AP1
CREB
Nucleus

Figure 1.3 – LPS-induced signalling cascade

Summary of the LPS activation of the NF-κB, JNK, p38 MAPK and PI3K pathways. Presentation of LPS by CD14 (catalysed by LBP) to TLR4 and MD2, causes the dimerization of TLR4 and forms a binding surface for the signalling adaptor proteins TIRAP and MYD88 to bind. This association allows the binding of IRAK1/4, on binding IRAK1/4 are able to auto-phosphorylate, enabling the recruitment of TRAF6. Once TRAF6 binds and is phosphorylated by the receptor complex, it is then able to phosphorylate TAK1. TAK1 has 3 main substrates MKK3/6, MKK4/7 and the IKK complex. Phosphorylation of MKK3/67 leads to the phosphorylation of p38 MAPK, which in turn indirectly activates the transcription factor CREB. Activation of MKK4/7 leads to the activation of the kinase JNK, which in turn phosphorylates and enables the activation of the transcription factor AP-1. Phosphorylation of IKKα causes the activation of the IKK complex kinase activity and initiates the phosphorylation of IκBα.This phosphorylated IκBα is recognised by the Cullin-1/Skp-1:β-RTrCP complex and is subsequently poly-ubiquitinated. This ubiquitination leads to IκBα being transported to the proteasome and degraded, which allows the NF-κB subunits bound to it to be transported into the nucleus and activated transcription.

1.3.2.1 – LPS-induced activation of mitogen activated protein kinase

The MAPK signalling cascade is a multi-layered system of specific serine/threonine kinases, which ultimately modulates cellular function at the level of transcription (through the modification of transcription factors), mRNA stability (Mahtani et al., 2001, Clement et al., 2011) as well as altering the function of pre-existing proteins in the cell. These signalling cascades are activated under a number of circumstances, including osmotic stress and cytokine stimulation. The MAPK pathways consist of a cascade of at least 3 kinases, generally classified as a MAP kinase (MAPK), MAP kinase kinase (MKK or MAPK2) and, MAP kinase kinase kinase (MKKK or MAPK3) (Cargnello and Roux, 2011).

The phosphorylation of MKK4/7 by TAK1 causes the protein to subsequently phosphorylate c-Jun N-terminal kinase (JNK) which in turn phosphorylates the transcription factor AP-1.

The phosphorylation of MKK3/6 by the TLR4 signalling pathway activates the kinase activity of this protein, which is able to directly phosphorylate p38 mitogen activated protein kinase (P38 MAPK), this protein has a large number of substrates which it can phosphorylate including other MAPK proteins. Phosphorylation of the kinases MSK1 and MSK2 leads to the activation of the transcription factor CREB (Wiggin et al., 2002, Mayer et al., 2013).

As well as activating CREB to induce transcription, p38 MAPK also has the ability to influence mRNA through its ability to phosphorylate MAPK-activated protein kinases 2 (MK2) , which in turn inactivates the mRNA destabilising protein TTP through phosphorylation (Bode et al.,

2012, Clark and Dean, 2016). This protein is able to destabilise the mRNA of many genes upregulated by LPS, including the inflammatory cytokine TNFα (Brooks and Blackshear, 2013).

1.3.2.1 – LPS-induced activation of NF-κB

The IKK complex is a large trimeric group of proteins containing two protein kinase subunits (IKK-α and β) and one regulatory subunit (IKK-γ or NEMO). Activation of the IKK allows it to phosphorylate IκBα on residues Ser32 and Ser36 (Traenckner et al., 1995, Finco et al., 1994). IκBα (the protein transcribed from the NFKBIA gene) is a cytoplasmic protein with multiple Ankyrin repeat domains that allow it to bind to the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family of transcription factors (Viatour et al., 2005). When bound to IκBα, NF-κB subunits are sequestered in the cytoplasm due to IκBα masking the nuclear localisation signal (NLS) required for the NF-κB dimer to be transported to the nucleus (Tak and Firestein, 2001).

The phosphorylation of IκBα allows the protein to be targeted by a complex of Cullin-1:Skp1:βTrCP for poly-ubiquitination (Strack et al., 2000). The ubiquitination of IκBα causes the protein to be shuttled to the proteasome and broken down, liberating the NF-κB subunits it was bound to. This pathway of NF-κB activation is known as the “canonical” NF-κB pathway.

1.4 – NF-κB

The NF-κB family of transcription factor is a highly conserved family with orthologues found in a wide range of extant organisms (including *Drosophila melanogaster*). In humans the NF-κB family contains 5 family members (gene names shown in brackets): p65 (RELA), p50/p105 (NFKB1), p52/p100 (NFKB2), RELB (RELB) and, c-Rel (RELC).

These 5 proteins, all contain a characteristic N-terminal Rel homology domain (RHD), which enables dimerization of members of the family and DNA binding. Three of these family members (p65, RelB, c-Rel) contain c terminal transactivation domains, which are required to recruit accessory protein complexes such as positive transcription elongation factor b (PTEFb) in order to activate transcription (Barboric et al., 2001). The other two members (p50/105 and p52/100) lack these transactivation domains and require additional proteins in order to activate transcription(Oeckinghaus and Ghosh, 2009).

p50 and p52 also have distinct precursor and processed forms. The larger precursor forms of these proteins, which are translated from the mRNA, can be partially processed by the proteasome into smaller forms (p52 and p50 respectively) either immediately following translation or in a cell signalling dependent manner(Christian et al., 2016). The precursor forms (p105, 100) contain a number of Ankyrin repeat domains, which are degraded after processing. These domains act similarly to IκBα, sequestering NF-κB family members and retaining them in the cytoplasm, by masking the NLS. The cell-signalling dependent processing of p105 is

associated with the activation of a small number of cell-surface receptors (known as the "non-canonical NF-κB pathway") and has a key role in lymphoid organogenesis(Matsushima et al., 2001).

In order to bind DNA, members of the NF-κB family must first dimerise with each other. The constituents of these dimers dictate both the ability of the dimer to induce transcription, as well as the consensus motif it is able to bind to on DNA (Siggers et al., 2012, Wong et al., 2011). All subunit combinations bind the motif "GGGNNTTTCC" (consensus motifs shown in Figure 1.4) with some dimer combinations able to bind unorthodox motifs such as the p65 homodimer motif "AGGAANTTC(T/C)C" (Wong et al., 2011).

In LPS:TLR4 signalling, the p65:p50 heterodimer is the predominant combination. Unlike the other members of the family, p52 and p50 lack a transactivation domain in the protein and dimers of these subunits have little transcriptional activity without accessory proteins (Cao et al., 2006, Collins et al., 2014). However these dimers are thought to act competitively for NF-κB motifs, preventing the binding of more active NF-κB dimers (Lernbecher et al., 1993).

The activation of NF-κB has also been shown to aggravate pathology or severity in a number of diseases, including chronic-inflammatory disease and cancer(Tak and Firestein, 2001, Karin, 2009). The activation of this pathway upregulates the expression of a number of inflammatory mediators such as TNF-α and IL-6. A considerable proportion of the primary response to TLR4 activation in macrophages has also been shown to be heavily dependent on NF-κB (Tong et al., 2016, Hargreaves et al., 2009).

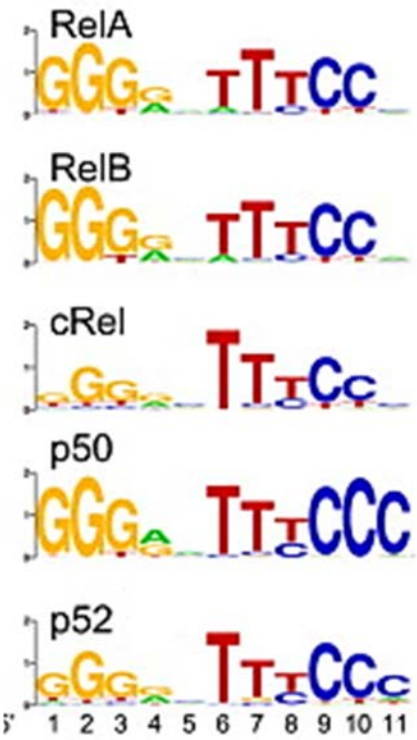

Figure 1.4 – NF-κB consensus motifs

Example consensus binding motifs of each NF-κb subunit homodimers taken from Zhao et al (2014).

1.5 – Control of gene transcription

In order to adapt protein expression to signals from the tissue environment the transcription (or processing) of specific genes have to be modulated. This can include: modifying the chromatin architecture surrounding genes and enhancers and modulating the transcriptional initiation or elongation.

1.5.1 – Chromatin structure

Chromatin is the complex of DNA and protein which resides in the nucleus of the eukaryotic cell and is amorphous prior to the prophase of cell division (where the chromatin condenses into discrete chromosomes). In chromatin, DNA is wound around an octamer complex of histone proteins: H2A, H2B, H3 and H4. This complex is able to bind approximately 146 nucleotide bases pairs of DNA around the exterior surface of the complex, forming a subunit known as a nucleosome (Alberts B, 2002).

Chemical modifications of the histones by enzymes regulate their interaction with the DNA, regulating the accessibility of the DNA to other proteins. In the case of the acetylation of residues in the histone tails, the acetyl-modification weakens the interaction between DNA and the histone complex due to its negative charge which repels the negatively charged phosphate groups in the DNA backbone. This looser interaction allows proteins to access the DNA backbone and bases in order to bind. Other histone modifications such as methylation have been shown not to affect the structure of nucleosomes but instead regulate the

interaction of other proteins with the nucleosome (Zentner and Henikoff, 2013, Tessarz and Kouzarides, 2014).

These modifications are dynamic and are altered during cellular differentiation or as a response to stimulus, and are associated with gene regulation. The acetylation of lysine 27 on H3 (H3K27ac) is associated with actively transcribed gene transcription start sites (TSS) and regulatory regions. Similarly the trimethylation of lysine 4 on the same histone subunit (H3K4me3) correlates with promoter elements, both active and poised for signal induced activation (Heinz et al., 2015).

1.5.2 – Gene transcription

The induction and regulation of gene expression in response to cellular signalling, is a highly complex multi-staged process. The process of transcribing a gene into mRNA can be split into three distinct stages: initiation, elongation and termination (Medzhitov and Horng, 2009).

Initiation consists of the recruitment of Pol II to the TSS of the gene to be transcribed and the assembly of the pre-initiation complex (PIC).

Once Pol II has been bound to the TSS, Poll II is phosphorylated on the serine 5 of the carboxyl-terminal domain (CTD) heptapeptide repeat (consisting of the peptide sequence “YSPTSPS”). Elongation occurs when Poll II escapes the PIC and moves down the coding strand and transcribes the nucleotide sequence to RNA. For this to occur protein complexes capable of phosphorylating RNA pol II have to be recruited to the paused Pol II. This mainly accomplished by positive transcription elongation factor B (P-TEFb) (Ni et al., 2008, Jonkers and Lis, 2015)

but there are some genes whose transcription does not require P-TEFB (Medlin et al., 2005, Rahman et al., 2011). This recruitment of elongation factors leads to the liberation of Pol II from the PIC and initiates its translocation down the coding strand. P-TEFb's recruitment to Pol II also leads to the Pol II CTD being phosphorylated on the serine 2 of the heptapeptide repeat (Zhou et al., 2012, Komarnitsky et al., 2000). It

1.5.3 – Pioneer transcription factors

The restriction of these enhancive DNA sequences is vital to produce an appropriate response to a stimulus, or to prevent expression by an inappropriate cell type. This is accomplished by the function of pioneer transcription factors (such as PU.1) which are able to bind to their cognate sequence motif, even when the DNA is bound to nucleosomes (closed) and unable to be bound by other sequence specific proteins (Adam et al., 2015, Zaret and Carroll, 2011). This interaction is able to cause nucleosomes to "shuffle" to expose a region free of nucleosomes (open) and later, the pioneer factor is able to recruit histone-modifying enzymes to the site to modify the residues of the histone complexes, to further expose DNA, or to signal a site specific function (Chen and Dent, 2014).

Several transcription factors have gained the specialist function to expose regions of chromatin even if the regions lie in normally inaccessible heterochromatin. One of the first described families "pioneer" transcription factors is the Forkhead family (Kaestner et al., 2000). The structure of this protein includes a winged-helix domain which is highly similar in structure to the histone subunit H5. H5 (and its functional relative H1) binds to DNA that is bound to

nucleosomes and facilitates its condensation. In Forkhead proteins this enables the binding of nucleosome bound DNA. Once bound it can recruit chromatin remodelling complexes to expose DNA (Clark et al., 1993).

1.5.4 – Transcriptional Promoters and Enhancers

In order to specifically control protein expression, cells are able to restrict the regions of DNA that are available for transcription factor binding. These promoters and enhancer regions of DNA contain specific sequence motifs bound by specific transcription factors, which facilitate the transcriptional activation or repression of gene expression.

The different activation states of enhancers (whether poised for activation, activated or repressed) can be indicated by the post-translational modifications of the histone complexes bordering the enhancer (key differences between poised and active enhancers are shown in (Figure 1.2). Most active or poised regulatory elements are defined by the presence of mono-methylation (me) or di-methylation (me2) of lysine 4 residue of H3 (H3K4). H3K4me/me2 is often associated with local nucleosome depletion and may act as a foundation step for other histone modifications such as, enabling the acetylation of lysine 27 on histone 3 (H3K27ac) (Creyghton et al., 2010). The H3K4 methylation may also act as a recruitment platform for chromatin remodelling complexes (Nadal-Ribelles et al., 2015). The H3K4me1 can also interact to silence transcription, when combined with other histone marks and is thought to help demarcate the enhancer from other local sequences (Cheng et al., 2014).

The H3K27ac modification is an important link between histone enhancer state and transcriptional activation. This modification is able to recruit members of the bromodomain protein family (Brd). This family of proteins, as well as recognising acetylated lysine residues, act as an intermediary to recruit the P-TEFb complex to that area of chromatin (Jang et al., 2005, Yang et al., 2005). This recruitment of P-TEFb serves to phosphorylate the CTD of Pol II recruited to the enhancer or promoter and initiate transcriptional elongation. Therefore this modification is associated with regions of active transcription. In some poised or repressed enhancers, the H3K27 residue may be tri-methylated (me3) which will then require de-methylation before acetylation can occur, thereby blocking or reducing the recruitment of Brd and P-TEFb. The H3K27me3 mark also acts to recruit Polycomb Repressive Complex 1 (PRC1) to chromatin, which can lead to the compaction of chromatin and the silencing of transcriptional activity at that loci (Grau et al., 2011, van Kruijsbergen et al., 2015).

Enhancers, unlike gene promoters, can affect the expression of genes great distances away from their actual chromosomal location. This problem of distance can be resolved by the looping of chromatin to bring enhancers into proximity to the promoter and TSS of the gene in question. There are many mechanisms by which this can take place, including the use of the chromatin structural protein Cohesin, the insulator protein CTCF and the use of enhancer RNAs (eRNAs) (Natoli et al., 2011, Ghisletti et al., 2010). This enables not only distal enhancers to impact on gene expression, but also allows multiple enhancers and promoters to interact (Fanucchi et al., 2013, Chepelev et al., 2012, Hnisz et al., 2013).

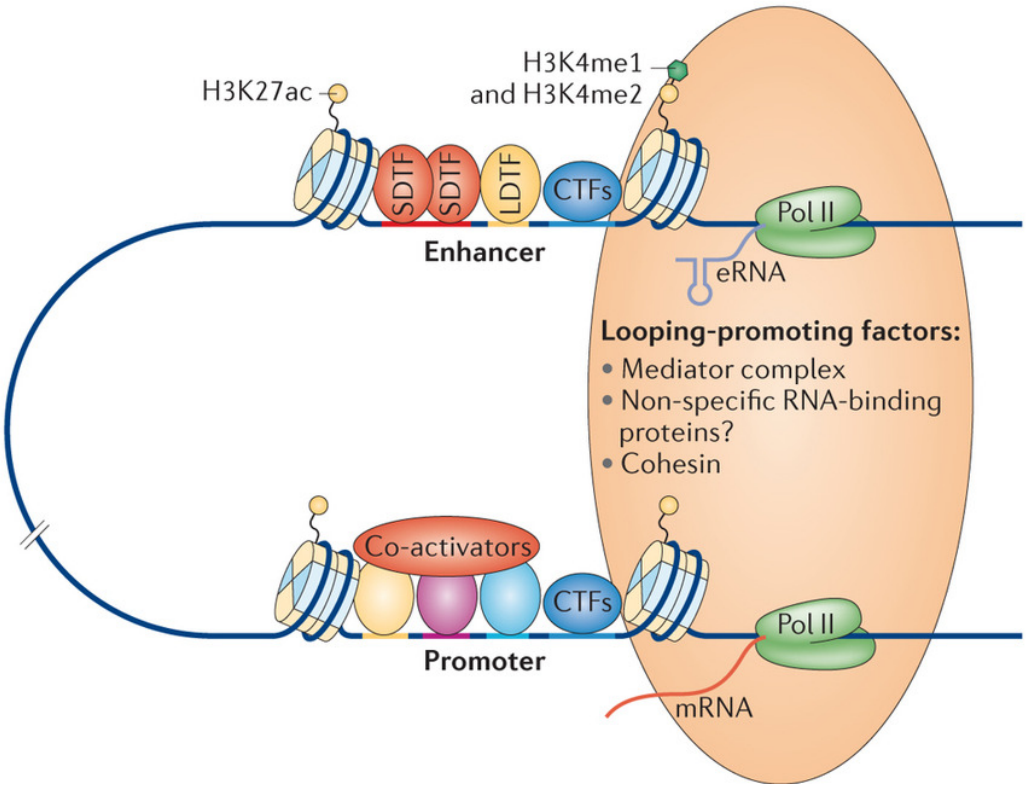

Figure 1.5 – Markers and function of enhancers

Taken from Heinz et al. (2015) (81). Enhancers primed by lineage-determining transcription factors (LDTF) are able to be bound by signal dependent transcription factor (SDTF) and Collaborating transcription factors (CTF), to recruit Pol II and induce the production of eRNAs. Locally bound looping factors (such as Cohesin, CTCF, the mediator complex, or eRNAs) allow the looping of chromatin to bring it into proximity with the promoters of genes. These enhancers typically display H3K4 mono and di-methylation which form the boundary of the enhancer and guide the potential binding of transcription factors. Active enhancers display H3K27ac mark which occurs through the recruitment of p300 and through the bromodomain family of proteins enables the recruitment of P-TEFb and enable transcription.

1.5.5 – Gene regulation in macrophages

There are several key transcription factors that have been described through gene knockout studies as being required for macrophage differentiation and function. The transcription factor PU.1 is expressed at high levels in B cells and monocyte/macrophages). Without this transcription factor macrophages are unable to develop from the yolk sac, fetal liver, or bone marrow (Scott et al., 1997, McKercher et al., 1996, Kierdorf et al., 2013). The expression of this protein is established early during macrophage differentiation and is able to drive the expression of the M-CSF receptor, rendering cells sensitive to this vital macrophage-inducing cytokine (Zhang et al., 1994). In B cells the expression of this PU.1 is much lower in comparison to myeloid cells and the genomic distribution heavily influenced by other transcriptional co-factors (Heinz et al., 2010). PU.1 has also been shown to contribute to induction of inflammatory gene expression in macrophages, and is bound to genomic regions activated by LPS stimulation (Ghisletti et al., 2010).

The transcription factors C/EBPα/β also has a major role in orchestrating macrophage function. Mice lacking these transcription factors lack effective monocyte-macrophage differentiation (Zhang et al., 1996, Pham et al., 2007) as well as having defects in cell metabolism and bactericidal function (Lee et al., 2014). The enforced expression of C/EBPα/β in B cells with low levels of endogenous PU.1 are sufficient to drive these cells towards a macrophage phenotype and cause the loss of B cell surface markers (Xie et al., 2004).

In tissue macrophages the several other transcription factors have been highlighted as having a role in establishing the differential genomic landscape of these cells. Microglia have been shown to utilise SMAD family transcription factors which are activated by TGFβ, while

peritoneal macrophages require retinoic acid (which is present in the peritoneal cavity) in order to establish their phenotype (Gosselin et al., 2014). Other important regulators include: MEF2C (for also for microglia), LXRA (for Kupffer cells), PPARγ (for alveolar macrophages) and RUNX3 (for intestinal macrophages). The transcriptional networks of these cells is of key interest in the field.

1.6 – IL-10

IL-10 is the founding member of the IL-10 family of cytokines, which include: IL-19, IL-20, IL-22, IL-24 and, IL-26 (Sabat, 2010, Zdanov, 2004). These genes display high similarity in gene structure, mRNA and protein sequence, as well as sharing receptor subunits (Williams et al., 2004b). The genes coding for IL-10, 19, 20 and, 24 are also located on the same locus, indicating a common ancestral gene origin, although IL-10 appears as a distinct gene (with significant homology to other identified orthologues) in species that diverged from our own earlier in evolution (such as sharks (Smith et al.). The segregation of IL-19/20/24 into separate genes occurred later in evolution (Wang et al., 2010).

In humans IL-10 is expressed as a 178 amino acid protein, which also includes a 16 amino acid signal peptide, required for its secretion, which is subsequently proteolytically removed. The protein structure itself contains 6 amphipathic helices, similar to the structure of IFNγ and is normally glycosylated (Josephson et al., 2001). Viral homologues of this protein can vary largely in terms of protein sequence, but display high similarity in terms of structure (Zdanov,

2004), though the differences in sequence can lead to differential cell signalling (Ding et al., 2000, Yoon et al., 2012, Liu et al., 1997).

IL-10 was initially described as a soluble inhibitor of T_{H1} cytokine production, secreted by T_{H2} cells upon antigen stimulation (Fiorentino et al., 1989). Since its initial description, IL-10 has been shown to be produced by multiple cells including; T_{regs}, monocytes, mast cells, macrophages and dendritic cells and has shown to be fundamental in the regulation of the adaptive and innate immune system (Murray and Smale, 2012).

1.6.1 – Consequences of IL-10 signalling

1.6.1.1 – On Lymphocytes

<u>T cells</u>

IL-10 induced signalling has profound effects on lymphocytes and affects their role in the immune response. In T cells, IL-10 signalling is able to inhibit the differentiation of naive CD4+ T cells into T_{H1} or T_{H17} cells (Huber et al., 2011, Qu et al., 2012). Existing T_{H1} and T_{H17} cells are also responsive to IL-10 signalling, which causes the inhibition of the inflammatory and immune-stimulatory cytokine production (TNF/ IFNγ for T_{H1} and IL-17/22 for T_{H17}) (Liu et al., 2011, Fiorentino et al., 1989). This has the effect of shifting the immune response from a cell-mediated immune response (mediated by recruited neutrophils and macrophages) to a more humoral immune response (mediated by B cells).

IL-10 signalling is also required by the T_{reg} cell subset for multiple functions. Firstly IL-10 signalling is required to maintain the expression of the transcription factor FOXP3, which is critical for the suppressive function of these cells. Without the ability to respond to IL-10, these cells are also unable to efficiently upregulate their own IL-10 expression and are less effective at restraining immune reactions.

CD8+ T cells also express the receptor for IL-10 and are influenced by IL-10 signalling. Exposure to IL-10 prior to antigen stimulation, inhibits CD8 T cell activation and cytotoxic activity. However after antigen stimulation, IL-10 no longer is able to inhibit cytotoxicity and activation and instead acts to increase IL-2 mediated proliferation of these cells (Ebert, 2000). IL-10 signalling differentially affects memory T cell subsets (delineated by CD4 or CD8 expression), IL-10 inhibits CD4+ memory T cell, function and expansion, but is required for optimal generation of CD8+ T cell memory cells (Foulds et al., 2006, Emmerich et al., 2012, Laidlaw et al., 2015).

<u>B cells</u>

Contrary to its inhibitive effect on T cells, IL-10 has a stimulatory effect on B cells (Itoh and Hirohata, 1995). On exposure to IL-10 B cells have increased MHC class II antigen presentation and survival, which can lead to the initiation of a humoral immune response after encountering a cognate T_{H2} cell (Galbas et al., 2012). In activated B cells, IL-10 is able to enhance the proliferation and survival of germinal centre B cells, as well as enhancing the differentiation of these cells into circulating plasma cells and is also involved in inducing antibody class-switching (Choe and Choi, 1998, Levy and Brouet, 1994, Malisan F, 1996).

1.6.1.2 – On Myeloid cells

Neutrophils

Circulating neutrophils of healthy humans do not express the components of the IL-10 receptor complex on the cell surface and therefore cannot initially be stimulated with IL-10. After a short time in ex vivo culture or, after exposure to an inflammatory stimulus (LPS, TNF), neutrophils upregulate the IL-10 receptor complex and are then susceptible to IL-10-mediated-inhibition of pro-inflammatory cytokine expression (Elbim et al., 2001, Cassatella et al., 2005). IL-10 has been shown to inhibit the phagocytosis and the killing of phagocytosed bacteria in vivo and the neutralisation of IL-10 in models of bacterial infections has been shown to increase survival (Dang et al., 2006).

Dendritic cells

Dendritic cell functions are profoundly altered by IL-10. IL-10 signalling during the differentiation of monocytes to myeloid dendritic cells (mDC) prevents the cells from acquiring classical dendritic cell traits (such as expression of T cell co-stimulatory molecules, IL-12 production and MHC class II presentation) and instead causes them to differentiate into macrophage like cells (De Smedt et al., 1997, Allavena et al., 1998). After monocytes have differentiated to mDCs, they become significantly less sensitive to IL-10s suppressive effects on TNF production and antigen presentation, due to a downregulation of IL10R1 expression and surface presentation (MacDonald et al., 1999, Corinti et al., 2001).

In contrast to its effect on mDCs, IL-10 signalling is able to regulate plasmacytoid dendritic cells (pDC) responses to inflammatory stimuli, inhibiting the secretion of immunogenic cytokines IFNα, IFNγ and, IL-12. IL-10 is also able to induce the apoptosis of pDCs, which is countered by the production of IFNα (which acts as a survival factor for these cells) (Duramad et al., 2003).

Monocytes and macrophages

The response of macrophages to IL-10 is an integral part of controlling the inflammatory response (Pils et al., 2010). Macrophages devoid of an effective IL10R complex or IL-10 develop a hyper-inflammatory phenotype in vitro and in vivo demonstrate a spontaneous gut inflammation in response to the organism's microbiota (Zigmond et al., 2014, Shouval et al., 2014). The addition of IL-10 to differentiating monocytes skews the phenotype of the mature macrophage. Cells exposed to IL-10 have demonstrated increased viability, increased M-CSF binding and receptor expression and, lower antigen-presentation capacity (Hashimoto et al., 1997). This reduced ability to present antigen in response to IL-10 has also been shown *in* vivo in response to infection and this may have a role in the orchestration of the immune response (Nguyen et al., 2012, Chadban et al., 1998, de Waal Malefyt et al., 1991). Macrophages incapable of responding to IL-10 have also been shown to produce greater amounts of inflammatory cytokines and are more susceptible to cytokine induced death from an immune challenge such as LPS (Bosmann et al., 2014, Pils et al., 2010). Macrophages exposed to IL-10 have been previously been described as anti-inflammatory or displaying phenotypic features of an "M2" macrophage (Mia et al., 2014, Lang et al., 2002). This immunosuppressive

phenotype can inhibit important processes such as tissue reconstruction (Eming et al., 2007) as well as inhibiting immune surveillance of tumors (Williams et al., 2016).

1.6.2 – IL-10 signalling

For IL-10 to initiate changes in cell gene expression, it first needs to bind to the IL-10 receptor complex. This extracellular section consists of a tetramer complex containing 2 copies of IL10R1 and IL10R2, of which only IL10R1 binds directly to IL-10. The intracellular section of the complex is associated with Janus kinases (JAKs); JAK1 and Tyk2. JAK1 associates with a JAK binding motif on IL10RA, while Tyk2 interacts with another JAK binding motif on IL10RB. Mouse knockout studies have previously shown that while IL10R1, IL10R2 and, JAK1 are essential for IL-10 signal transduction (Murai et al., 2009); knocking out Tyk2 does inhibit the IL-10 signalling pathway, but may in fact be the target of proteins, which in turn regulate the IL-10 signalling pathway (Pike et al., 2014, Karaghiosoff et al., 2000).

After the binding of IL-10 to the IL10R complex, JAK1 is phosphorylated and in turn phosphorylates two tyrosine residues on IL10R1; Tyr446 and Tyr496 (Lim and Cao, 2006). These phosphorylated tyrosine residues are then able to bind with the SH2 domains of signal transduced activator of transcription 3 (STAT3). This brings STAT3 in close proximity to the active JAK protein and consequently STAT3 is phosphorylated on Y_{705} (Williams et al., 2004b). This causes the dimerization of STAT3 proteins allowing the DNA binding domains to interact with the DNA strand and act as a transcription factor (Hutchins et al., 2012).

IL-10 has also been shown to promote the phosphorylation of the related proteins STAT1 and STAT5 and as well as STAT3 (Finbloom and Winestock, 1995, Wehinger et al., 1996, Zhu et al., 2014). However, the contribution of STAT1 and 5 in IL-10 signalling, are largely uncharacterised. In macrophages, STAT1 phosphorylation by IL-10 is robustly observed after IFNγ pre-stimulation (Herrero et al., 2003) or inhibiting the phosphatase PTPN1B (Pike et al., 2014).

Mice deficient in STAT1 also show a comparable IL-10 response to wild type littermates (Durbin et al., 1996). Indicating that although STAT1 plays a role in IL-10 signalling, the majority of IL-10's effects in macrophages can be attributed to STAT3 (Williams et al., 2004a).

While the JAK/STAT above describes the most well described scheme by which IL-10 alters intracellular signalling, there are other pathways with which IL-10 has been shown to interact. Several studies have shown a role for the PI3K pathway in IL-10 function. In pro-myeloid cells, activation of the IL-10 receptor causes the phosphorylation of insulin receptor substrate 2 (IRS-2) as well as PI3K and Akt (Zhou et al., 2001). In macrophages, inhibitors of PI3K have also been shown to inhibit IL-10 induced or modulated gene expression (Antoniv and Ivashkiv, 2011). However, the mechanism by PI3K could be activated by IL-10 is unknown. These signalling pathways are summarised in Figure 1.6.

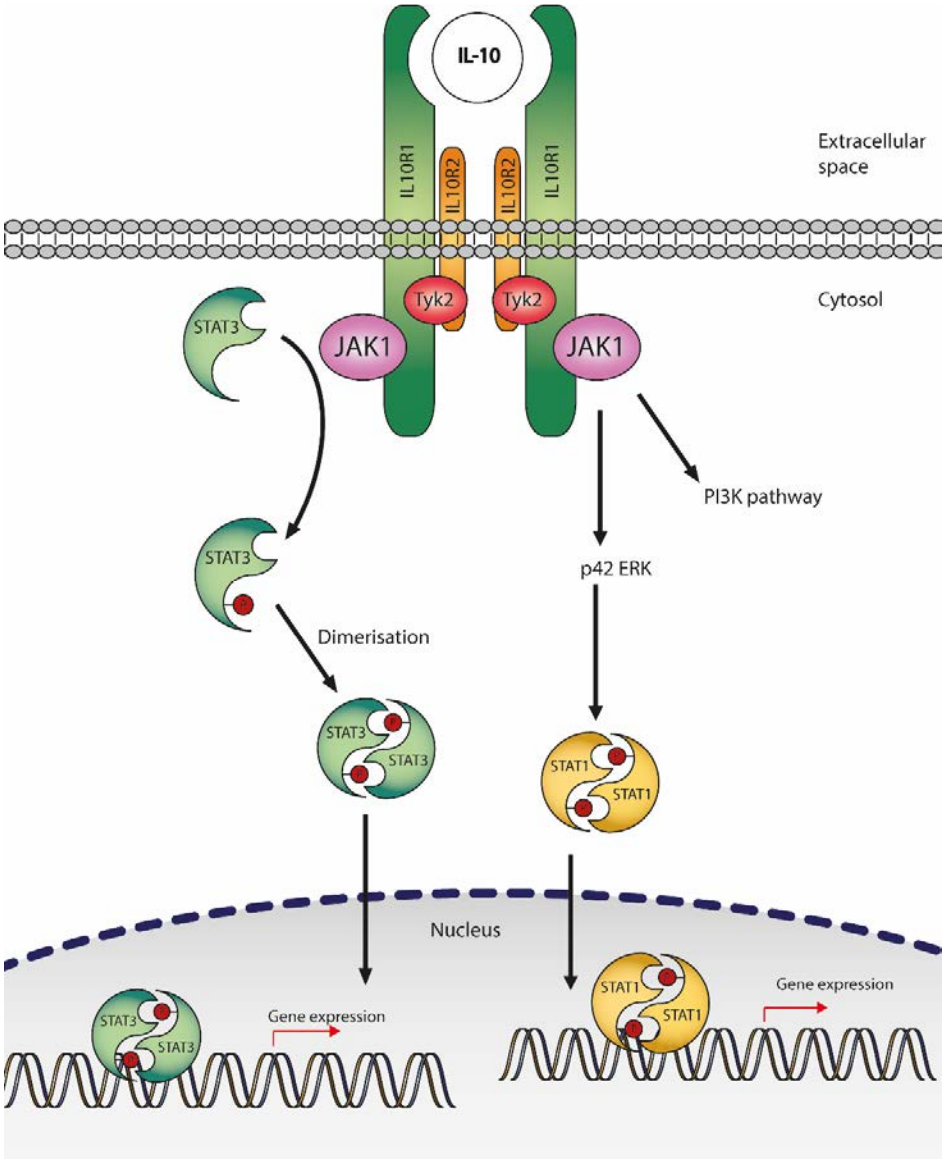

Figure 1.6 – Overview of IL-10 signalling pathway

Schematic of IL-10 activation of JAK1/STAT3 signalling. IL-10 binding to the IL10 receptor which associated kinase JAK1 to phosphorylate one another and tyrosine residues on the cytoplasmic domain of IL10R1. Once phosphorylated these residues act as a binding site for the STAT3, which binds to IL10R1 and is phosphorylated by JAK1. The tyrosine phosphorylation on STAT3 allows the protein to homodimerise and move into the nucleus to facilitate transcription. JAK1 also leads to the phosphorylation of p42 ERK MAPK and the activation of PI3K through unclear mechanisms. The activation of p42 ERK MAPK leads to the activation of STAT1 which can also induce gene expression.

1.6.3 – STAT proteins

In humans there are seven STAT family members: STAT1, 2, 3, 4, 5A, 5B and, 6 (Darnell, 1997). This family of transcription factors share a common set of structural domains: The N-terminal, Coiled-coil, DNA binding, Linker, SH2 and, TAD domains (Mitchell and John, 2005, Levy and Darnell, 2002). The N-terminal, coiled-coil and, linker domains act as surfaces for protein-protein interactions, such as nuclear importins and interactions with cytokine receptors (Zhang et al., 2000, Chen et al., 2003, Ota et al., 2004). The DNA binding domain is an immunoglobulin fold (Ig-fold) type DNA binding structure, which is also used by other DNA binding proteins such as p53 and NF-κB (Chen et al., 1998). The SH2 domain binds to phospho-tyrosine residues on receptor activated STAT proteins and facilitates dimerisation (Darnell, 1997). The TAD domain is able to recruit p300 transcriptional coactivator. This domain is able to be alternatively spliced in many STAT family members and there is a low degree of conservation between orthologues (Paulson et al., 1999).

In order to activate transcription STAT proteins must first dimerise to translocate into the nucleus and to bind to DNA. This is accomplished by the phosphorylation of a conserved tyrosine residue located in the C-terminal TAD domain. Once phosphorylated the tyrosine is then able to be bound by the SH2 domain of another STAT protein forming a dimer (Darnell, 1997). This dimer is then able to bind to nuclear import complexes and be shuttled into the nucleus(Reich, 2013). Once there the STAT dimer is then able to bind to open chromatin containing the STAT consensus motif (selected STAT motifs are shown in Table 1.1).

There is a great similarity in the consensus binding motifs between members of the STAT family (Ehret et al., 2001). Members of the STAT family bind to a palindromic consensus

NAME	MOTIF
STAT1	
STAT3	
STAT4	
STAT5	
STAT6	

Table 1.1 – Table of known STAT family binding motifs

A table of selected STAT family binding motifs derived from ChIP-seq experiments curated by the HOMER program (Rozowsky et al., 2009, Chen et al., 2008, Wei et al., 2010, Liao et al., 2008, Ostuni et al., 2012).

sequence: "TTC$(N)_{1-3}$GAA" where the number of N is dependent on the STAT proteins binding to the sequence. In order to fine tune a response additional co-factors may selectively complex with the STAT dimer, or expose other regions of chromatin for the dimer to bind to (Hutchins et al., 2013).

1.6.3.1 – STAT3

It has been well established that STAT3 is required for the suppressive effects of IL-10, Macrophages transfected with a constitutively active form of STAT3, produce much lower inflammatory cytokines upon LPS stimulation, as well as synthesising more IL-10 inducible genes at the basal state(Williams et al., 2007).

Whole organism deletions of STAT3 are embryonically lethal(Takeda et al., 1997). However, macrophages with STAT3 specifically deleted from the genome, display a high sensitivity to endotoxin shock and have increased levels of circulating inflammatory cytokines(Takeda et al., 1999). A similar effect is observed with macrophages in vitro transfected with a dominant negative version of the protein (mutating the critical tyrosine residue at position 705 to a phenylalanine residue) (Williams et al., 2004a).

As well as the JAK induced Y705 phosphorylation; there are multiple post-translational modifications that can modify STAT3s transcriptional potency and specificity. The best characterised is the phosphorylation of serine 727. This phosphorylation alone does not cause, dimerization or transcriptional activation of STAT3, but instead modifies its transcriptional activity, upregulating a subset of its target genes (Wen and Darnell, 1997, Miyakoshi et al., 2015, Huang et al., 2014, Chung et al., 1997).

Mechanisms of STAT3s suppression of inflammatory gene expression is a contentious point within the field, with multiple transcriptional and post transcriptional mechanisms proposed for this activity. As highlighted in Hutchins et al. (2013 and 2015) this discrepancy could have arisen due to the differences in cell types across these studies.

STAT3's actions are not however solely anti-inflammatory. Many other cytokines including: the IL-6 superfamily of cytokines (Berishaj et al., 2007), the satiety hormone leptin (Bates et al., 2003)) and, the inflammatory cytokine GM-CSF (Gu et al., 2007), also induce tyrosine phosphorylation of STAT3. The major difference between the effects of signalling between these cytokines, is the sustaining of the phosphorylated STAT3 signal. STAT3 is only phosphorylated for a short time after IL-6 stimulation compared to IL-10. This is due to the production of suppressor of cytokine signalling 3 (SOCS3), which binds to "SOCS box" motif in the IL-6 receptor's intracellular domain and directly inhibits the receptor associated JAK proteins (Babon et al., 2003). In the absence of SOCS3 or the "SOCS box", IL-6 phosphorylation is sustained and instead of its pro-inflammatory effect, gains an anti-inflammatory effect similar to IL-10 (Niemand et al., 2003, Yasukawa et al., 2003a).

1.6.4 – IL-10 Mediated Suppression of Inflammation

There is little consensus on the molecular mechanisms by which IL-10 suppresses inflammatory signalling. Smale and Murray (2012) summarise that two major hypotheses exist for IL-10's ability to supress inflammation:

1) There exists a “master regulator” upregulated by IL-10 signalling which exerts the inhibitive effect on inflammation.

2) That IL-10 induces the expression of a number of different negative regulators which act in concert to suppress inflammation in different contexts

There is general agreement however that STAT3’s transcriptional activity is absolutely required for IL-10s inhibitive effects on the expression of inflammatory genes. Cells deficient in STAT3, or expressing a dominant negative version of the protein, produce higher levels of inflammatory cytokines such as TNF and IL6 on LPS stimulation and this production is insensitive to inhibition by IL-10(Williams et al., 2004a, Nakamura et al., 2015). Although there have been some studies that hypothesise the existence of a STAT3-*independent* mechanism for some of IL-10’s inhibitive effects (Chan et al., 2012).

The effect of IL-10 on immune cells is highly variable between cell types even with a common stimulus, indicating that IL-10’s suppression of inflammation may be effected by different mechanisms in different cell types (Hutchins et al., 2012, Hutchins et al., 2015). Another potential confounding factor in identifying IL-10’s effector of inhibition is the difference in exposure time of cells to IL-10 before stimulus. Denys et al. (2002) demonstrated that pre-treatment with IL-10 initiated a differential mechanism of TNF suppression, compared to IL-10 stimulus co-stimulation.

In monocytes and macrophages, IL-10 has been proposed to act through a variety of mechanisms on a multitude of inflammatory genes. These can be stratified into primary response genes (PRGs) and secondary response genes (SRGs) based on the requirement for

nucleosome remodelling or *de novo* protein synthesis (Smale, 2010, Ramirez-Carrozzi et al., 2009, Ramirez-Carrozzi et al., 2006).

PRGs (such as TNF or IL1A) require no new protein synthesis in order for transcription to occur and are often rapidly induced on innate immune signalling. The expression of these genes are critical to the induction of the inflammatory response and some genes (such as TNF) have been shown to be key drivers of inflammatory pathology. The IL-10 mediated inhibition of TNF production has been the focus of much of this research (due its role in inflammatory pathology) and multiple strata of mechanisms have been proposed to explain IL-10's inhibition of its expression, including; inhibition of translation to protein (Chan et al., 2012), reduction in mRNA stability (Schaljo et al., 2009), inhibition of transcription (Murray, 2005, Smallie et al., 2010), modulation of transcription factor function(Wang et al., 1995) and, inhibition of upstream signalling (Curtale et al., 2013, Weaver et al., 2007, Verstrepen et al., 2008).

The inhibition of SRGs by IL-10 (such as IL-6 or IL-12p40) has in contrast received much less attention. Many transcriptional regulators induced by IL-10, have been shown to act on promoters of some of these genes, in order to supress their production by macrophages (El Kasmi et al., 2007, Kuwata et al., 2003, Smith et al., 2011a). It has also been shown that at the IL12p40 loci (Kobayashi et al., 2012), IL-10 is able to inhibit the transcription of these genes, through inhibiting the remodelling of chromatin that is required for their initial expression under inflammatory conditions (Weinmann et al., 2001).

1.6.5 – IL-10 in disease

IL-10s importance in supressing or modulating immune responses is exemplified by the increased susceptibility of individuals with variant sequences in the promoter of IL-10. Single nucleotide polymorphisms (SNPs) in proximal regulatory elements to the IL-10 gene are risk factors for many diseases, including; inflammatory bowel disease (Jostins et al., 2012), Behcet's disease (Remmers et al., 2010), ulcerative colitis (Anderson et al., 2011) and Crohn's disease (Franke et al., 2010). These alterations in the promoter of IL-10 alter its transcriptional induction on stimulation and lead to predisposition to certain diseases (Sakurai et al., 2013).

SNPs in other genes involved in IL-10 signalling are also associated with inflammatory or immune disease incidence and severity. Both the receptor subunits (Moran et al., 2013, Park et al., 2013, Jostins et al., 2012, Franke et al., 2010, Barrett et al., 2009), the kinase JAK1 (Hou et al., 2013, Silva et al., 2010) and the transcription factor STAT3 (Zhang et al., 2014, Jostins et al., 2012, Jakkula et al., 2010). These SNPs are associated with a number of diseases including type I diabetes (Plagnol et al., 2011), Bechet's disease (Remmers et al., 2010), Crohn's disease(Yamazaki et al., 2013) and, ulcerative colitis(Zhang et al., 2014, Moran et al., 2013).

IL-10's key role in limiting and dampening inflammation can be seen in *in vivo* models of disease. Mice lacking the IL-10 gene have increased incidence and severity of many experimental immune challenges, such as arthritis (Cuzzocrea et al., 2001, Tao et al., 2011), sepsis (Latifi et al., 2002) and, asthma (Yang et al., 2000). Mice deficient in IL-10 also spontaneously develop an aggressive form colitis (Sellon et al., 1998), in response to gut microbiota, which is in part a reflection of local macrophage response to IL-10 produced by T_{reg} s (Zigmond et al., 2014, Murai et al., 2009).

Even without disease associated SNPs, IL-10 can still be a driver or aggravator of pathology if expressed inappropriately. Expression of IL-10 in the brain prevents microglial macrophages from being able to clear Alzheimer plaques leading to aggravation of pathology. In mouse models of the disease, blocking the IL-10 pathway or disrupting the gene improves both the clearance of plaques and prevents the formation of new ones (Guillot-Sestier et al., 2015, Chakrabarty et al., 2015).

Many viruses such as Epstein-Bar Virus (EBV) and Cytomegalovirus (CMV) produce orthologues to IL-10 (vIL-10), to suppress the immune response during infection (Liu et al., 1997, Yoon et al., 2012, Kotenko et al., 2000). This capturing of the IL10 gene is believed to have occurred multiple times in history (based on position of the gene within the viral genome and divergence of viral families), with the latest acquisition of the gene occurring after the evolutionary divergence of Old and New world primates (Ouyang et al., 2014, Slobedman et al., 2009, Ding et al., 2000). Interestingly, the mutations acquired in evolution by some vIL-10 forms (such as that produced by CMV), lack the stimulatory properties on B cells but retain the immunosuppressive properties on other cell types (Spencer et al., 2002).

1.6.6 – IL-10 as therapy

Since IL-10s discovery, there have been many attempts to derive clinical benefit from its immune suppressive abilities and its capacity to regulate the pathologies of several mouse models of disease.

Initial phase I clinical trials showed that systemic administration of IL-10 was well tolerated by healthy volunteers with the only side effects being mild flu symptoms in a fraction of volunteers receiving the higher doses of IL-10. In subjects administered with systemic LPS to induce fever, IL-10 only effectively downregulated inflammatory cytokine production, when administered immediately prior or shortly after LPS administration (Pajkrt et al., 1997).

IL-10 however showed disappointing efficacy in phase II trials, in the treatment of Crohn's disease and psoriasis. Patients treated with multiple subcutaneous injections of IL-10 at psoriatic lesions only developed a modest reduction over 6 weeks, which became indistinguishable from the placebo control group at 12 weeks (van Deventer et al., 1997). In patients with Crohn's disease where IL-10 was administered systemically, there was a noticeable reduction in the disease activity score, but it did not result in a reduction in disease pathology or significantly alter the rate of remission, compared to placebo. The lack of efficacy could have been due to the administered IL-10 not reaching the inflamed area of the gut in a sufficient concentration to restrain the disease.

While administration of IL-10 has proved to be an effective treatment in animal models of arthritis (Walmsley et al., 1996), trials in humans have only demonstrated a mild effectiveness (Asadullah et al., 2003). This is likely due to the immuno-stimulatory effects of IL-10 on B cells, which are able to drive the arthritic pathology through autoantibody production.

1.7 – Project Aims

Cytokines and the gene expression changes they initiate are critical for the development, maintenance and resolution of an immune or inflammatory response. Dysfunctions in these stages are often accompanied or preceded by, aberrant expression of cytokines. IL-10 has been shown to be a potent regulator of inflammatory and immune responses but the mechanisms by which IL-10 is able to alter inflammatory gene expression remain unclear. Previous work (Smallie., et al. (2010)) has shown that IL-10 can alter the ability of NF-κB to be recruited to chromatin and therefore reduce its transcriptional activity. I therefore hypothesised that IL-10 is able to alter inflammatory gene expression through the activation or modulation of transcription regulators in macrophages.

Initially I investigated the response of macrophages to LPS, IL-10 or co-stimulation using microarray analysis of gene expression. Analysis of the microarray lead me to investigate whether the effects of IL-10 and LPS in combination as due to an increase rate of gene synthesis using primary transcript QPCR. I also undertook to validate the observed patterns of gene expression in response to LPS and IL-10 at the mRNA and protein level using QPCR, ELISA, Multiplexed cytokine bead assay (Luminex), western blotting and flow cytometry.

In order to interrogate these responses of gene expression to IL-10 signalling I examined the role of the transcription factors STAT3 and NF-κB have been described as having prominent roles in the response to IL-10 and LPS respectively. I undertook to dissect the role of these transcription

factors in the transcriptional response to LPS and IL-10 signalling, utilising siRNA knockdown of gene expression as well as the small molecule inhibitor of NF-κB activity MLN-4924.

After this I investigated in more detail the patterns of gene expression of the gene TNFSF9 (which displayed cooperative regulation with LPS/IL-10) and identified and characterised a putative regulatory element upstream of the TNFSF9 TSS which was demonstrated responsiveness to LPS and IL-10 stimulation.

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 – Cell culture reagents

Dulbecco's modified Eagle's medium (DMEM); Roswell park memorial institute (RPMI) 1640 culture medium.

2.1.2 – Cytokines and stimuli

Cytokine	Concentration	Source
LPS	20 μg/mL	ENZO
IL-10	10 μg/mL	Peprotech
M-CSF	10 μg/mL	Peprotech

Table 2.1 – Recombinant cytokines used in this study

2.1.3 – Signalling inhibitors

Inhibitor	Source
LY294002	ENZO
MLN-4924	Peprotech
Ruxolitinib	Selleckchem
Wortmanin	Sigma Aldrich
SB203580	Sigma Aldrich
BAY 11-7082	Sigma Aldrich
JNK	Simon Arthur (Glasgow)
Cycloheximide	Sigma Aldrich

Table 2.2 – Signalling inhibitors and concentrations used in this study

2.1.4 – Adenoviruses

The NF-κB and STAT3 reporter adenoviral constructs were gifted by Dr Tim Smallie and Dr Lynn Williams of the Kennedy Institute for rheumatology. The NF-κB luciferase construct contains a firefly luciferase open reading frame (ORF) downstream of a TATA-like promoter (derived from Herpes simplex virus thymidine kinase promoter) and 4 concatenated artificial NF-κB binding motifs (single binding motif sequence is as follows: "GGAATTTCC") synthesised based on the consensus sequence of p65:p50 dimer (Campbell et al., 2004, Sanlioglu et al., 2001).

The STAT3 luciferase reporter construct promoter consist of 4 concatenated repeats of a modified oligonucleotide probe previously shown to bind to STAT3 (Horvath et al., 1995). The sequence of a single the STAT binding unit of this reporter "GGTTCCCGTAAATGCATCA" (STAT binding site is underlined). This promoter was previous cloned upstream of a Tata-like promoter (derived from Herpes simplex virus thymidine kinase promoter) and a firefly luciferase open reading frame (Besser et al., 1999) which was then subsequently cloned into a adenoviral vector (Williams et al., 2007, Staples et al., 2007).

2.1.5 – Plasmid Vectors

Vector name	Application	Antibiotic resistance	Other screening	Source
TOPO 2.1	Cloning	Ampicillin/Kanamycin	Blue/white screenable	Invitrogen
pGL4.26	Luciferase assay	Ampicillin	None	Promega
rL-TK	Luciferase	Ampicillin	None	Promega

Table 2.3 – Plasmid vectors used in this study

2.1.6 – Bacterial culture reagents

Lennox L broth	20 g/L	(Invitrogen)
Lennox L agar	38 g/L	(Invitrogen)
Ampicillin	100 mg/mL	(Sigma Aldrich)
Kanamycin	50 mg/mL	(Sigma Aldrich)

2.1.7 – General buffers and solutions

Phosphate Buffered Saline (PBS)	137 mM NaCl
	3 mM KCl
	8 mM Na_2HPO_4
	1.5 mM KH_2PO_4
	pH 7.3

FACS buffer	1% Heat inactivated fetal calf serum
	0.05% Sodium Azide
	PBS
Cytoplasmic Lysis Buffer	50 mM Hepes-KOH(pH 7.5)
	140 mM NaCl,
	1 mM EDTA
	10% (v/v) Glycerol
	0.5% (v/v) NP40
	0.25% (v/v) Triton X-100
Nuclear Wash Buffer	10 mM Tris-HCL pH 8
	200 mM NaCl
	1 mM EDTA
	0.5mM EGTA

Nuclear Lysis Buffer	10 mM Tris-HCL pH8
	100 mM NaCl,
	1 mM EDTA
	0.5 mM EGTA
	0.1% (v/v) Na-Deoxycholate
	0.5% (v/v) N-Lauroylsarcosine
RIPA Buffer	50 mM Hepes-KOH pH 7.6
	500 mM LiCl
	1 mM EDTA
	1% NP40 (IGEPAL)
	10% Na-Deoxycholate

2.1.8 – Luciferase assay buffers

Luciferase Lysis Buffer	0.65% (v/v) NP40 (IGEPAL)
	10 mM Tris-HCl pH8
	1 mM EDTA
	150mM NaCl

Luciferase Assay Buffer	1% (v/v) Triton-X-100
	25 mM Tris-phosphate pH 7.8
	8 mM $MgCl_2$
	1 mM EDTA
	15% (v/v) Glycerol

2.1.9 – Western blotting buffers

Protein lysis buffer	50mM Tris HCL
	0.25M NaCl
	3mM EDTA
	3mM EGTA
	0.5% (v/v) NP-40
	10% (v/v) Glycerol
Western Sample buffer (5x)	375mM Tris HCL pH 8
	10% (v/v) SDS
	50% (v/v) Glycerol
	12.5% (v/v) β-mercaptoethanol
	0.1% (m/v) Bromophenol blue

Running buffer	25 mM Tris
	192 mM Glycine
	0.1% SDS
	pH 8.3
Wash Buffer	1 x Phosphate Buffered Saline
	0.1% Tween 200
Blocking buffer	1 x Phosphate Buffered Saline
	5% (w/v) Milk Powder
	0.1% Tween 200

2.1.10 – ELISA solutions

ELISA Wash Buffer	1 x Phosphate Buffered Saline
	0.01 % Tween 200
ELISA Blocking Buffer	1 x Phosphate Buffered Saline
	2% (w/v) BSA

2.1.11 – ChIP solutions

Dynabeads Blocking buffer	0.5% (w/v) BSA
	1 x Phosphate Buffered Saline
Salt wash buffer	1M Tris EDTA
	50mM NaCl
Bead elution buffer	1M Tris EDTA
	2% (w/v) SDS

2.1.12 – Primers

Listed here are the oligonucleotide primers used in this study, separated by experiment type. All primers were purchased from MWG Eurofins.

Gene Symbol	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Target
CCL8	TGTCCCAAGGAAGCTGTGAT	TGGAATCCCTGACCCATCTCT	Human
CD274	CCTACTGGCATTTGCTGAACG	AGTGCAGCCAGGTCTAATTGT	Human
CH25H	CTTTCCGTGGAGGACCACTC	GTGAGAGTGATGCAGGTCGT	Human
CXCL10	CCTGCAAGCCAATTTTGTCCA	TGTGGTCCATCCTTGGAAGC	Human
DUSP1	ACAACCACAAGGCAGACATCA	CAGTGGACAAACACCCTTCCT	Human
FFAR2	GCTACCTGGGAGTGGCTTTC	CATAACCCAGGCCACCAGAG	Human
GADD45B	ACGAGTCGGCCAAGTTGATG	CGCACGATGTTGATGTCGTT	Human
GAPDH	GTCAGCCGCATCTTCTTTTGC	AATCCGTTGACTCCGACCTTCC	Human
IFNB1	TGGCACAACAGGTAGTAGGC	AGTGGAGAAGCACAACAGGAG	Human
IL1A	CTCACGGCTGCTGCATTACA	CAGCAGCACTGGTTGGTCTT	Human
IL1B	CAACAGGCTGCTCTGGGATT	CTGGCGAGCTCAGGTACTTC	Human
NFKBIA	GAAGTGATCCGCCAGGTGAA	CTCACAGGCAAGGTGTAGGG	Human
NFKBID	CCGAGACAGGCTGGATTGTG	CATGTTGACAAAGGTCCGCAG	Human
NFKBIZ	CTCAACCTGAGCTACTTCTACGG	CTTGAAAGGGGCCTCTCTGCT	Human
NR4A2	TGAAGAGAGACGCGGAGAAC	CAGCCGAGTTACAGGCGTTT	Human
TNF	CCCCAGGGACCTCTCTCTAAT	TCTCTCAGCTCCACGCCATT	Human
TNFAIP3	CCCTTGGAAGCACCATGTTTG	GGTTGGGATGCTGACACTCC	Human
TNFSF9	ACAGTCTTGGGACTCTTCCG	ACCCAGGCTGGACGTTATTC	Human
TNIP3	AATTTTGTATACTTGCTCTCACCC	TCTTGTTGATGGTTCAGCACA	Human
Tnfsf9	CCTGCTCAGACCCCATAAAA	GCATACAGAGACTGGGAGCTG	Mouse
Gapdh	CATCATCTCCGCCCCTTCTG	CATCACGCCACAGCTTTCC	Mouse

Table 2.4 – Primers used for the detection of mature mRNA transcripts by QPCR

GENE SYMBOL	FORWARD PRIMER SEQUENCE (5'-3')	REVERSE PRIMER SEQUENCE (5'-3')
CD274	ACTTCTCCATGCTGTTCCCA	TCCTTTCATTTGGAGGATGTGC
IL1A	CACGCCTCTGGAATCAATGG	ACTGCCCAAGATGAAGACCA
IL1B	GGGAACTGGGCAGACTCAAA	TCCTGATCATGTGACCTGCT
NFKBIA	TTGCGCTCATAACGTCAGAC	TGGGCTATGGAGAATGGAGTC
TNF	GCAGTCAGATCATCTTCTCG	AGGTACAGGCCCTCTGATGGCAC
TNFAIP3	GCCCACACCAGACTGATACT	CACAAGGCAGACATCAGCTC
TNFSF9	CGGGAGTGTAGAACAGGTGT	GAGGGTCCCGAGCTTTCG
TNIP3	CAGGATGCCTTGAATATCAAGTGTTC	TTCTCCACAGGATCATGCCACTTC

Table 2.5 – Primers used to detect mRNA primary transcripts by QPCR

GENE SYMBOL	FORWARD PRIMER SEQUENCE (5'-3')	REVERSE PRIMER SEQUENCE (5'-3')
IL6	CGAAGCTGCAGGCACAGAACC	CAACAACAATCTGAGGTGCCCATG
NFKBIA	TTGCGCTCATAACGTCAGAC	TGGGCTATGGAGAATGGAGTC
TNF	GCAGTCAGATCATCTTCTCG	AGGTACAGGCCCTCTGATGGCAC
TNFAIP3	GCCCACACCAGACTGATACT	CACAAGGCAGACATCAGCTC
TNFSF9	CGGGAGTGTAGAACAGGTGT	GAGGGTCCCGAGCTTTCG

Table 2.6 – Primers used to detect enrichment of proteins at downstream gene regions by ChIP

GENE SYMBOL	FORWARD PRIMER SEQUENCE (5'-3')	REVERSE PRIMER SEQUENCE (5'-3')
TNFSF9	TTCTCGAGAGTTTTCACCGCTTCTTGGC	TTAGATCTCAGTGGAACCCTGAGTGTCA

Table 2.7 – Primers used to amplify human genomic regions including restriction sites

2.1.13 – Antibodies used in this study

Target	Species Reactivity	Organism	Clone	Source
RELA	Human	Rabbit	C-20	Santa Cruz
Tubulin	Human	Mouse	DM1A	Sigma Aldrich
STAT3	Human	Mouse	#9132	Cell signalling
Phospho-Y_{705} STAT3	Human	Mouse	D3A7	Cell signalling
Laminin A/C	Human	Mouse	612163	BD Bioscience
TNFAIP3	Human	Rabbit	D13H3	Cell signalling
TNIP3	Human	Rabbit	SAB3500087	Sigma Aldrich

Table 2.8 – Antibodies used for Western blotting

Target	Species Reactivity	Organism	Clone	Source
RNA-polymerase II	Human	Rabbit	H-224x	Santa Cruz
Phospho-serine2 RNA-polymerase II	Human	Mouse	Ab84988	Abcam
IgG isotype	Human	Mouse	Ab46540	Abcam

Table 2.9 – Antibodies used in ChIP

Target	Species Reactivity	Organism	Clone	Source
TNFSF9-PE	Human	Rabbit	5F4	Biolegend
CD274-Alexafluor450	Human	Mouse	M1H1	eBiocience
IgG-κ-PE	Human	Mouse	P3.6.2.8.1	Biolegend
IgG-κ-Alexaflur450	Human	Mouse	MOPC-21	Biolegend

Table 2.10 – Antibodies used in flow cytometry

Target	Species Reactivity	Organism	Clone /Batch	Source
TNF	Human	Rabbit	551220	BD Pharminogen
TNF	Human	Mouse	554511	BD Pharminogen
Streptavidin-HRP	Human	Mouse	PY998	R&D

Table 2.11 – Antibodies used in TNFα ELISA

2.2 – Methods

2.2.1 – Cellular methods

2.2.1.1 – Isolation of monocytes from leukocyte cone

Leukocyte cones, which are residues of cells leftover from plateletpheresis with heparin, were purchased from the National Blood Service, in accordance with the University of Birmingham and National Blood Service ethics.

The blood cells were recovered from the cone by removing the tubing at either end of the cone and flushed through with 20 ml of Hanks BSS. The suspended cells were then diluted up to a volume of 40 mL and layered on an equal volume of Lympholyte H and centrifugated at 400 x g for 20 mins with low acceleration and no braking.

The density gradient formed in the tube during centrifugation prevents peripheral blood mononuclear cells (PBMCs) from moving from the interface between the blood solution and the Lympholyte H, while allowing lighter material (including erythrocytes) to sediment at the bottom of the tube.

After the centrifugation the PBMCs were harvested from the interface and washed 3 times in serum free RPMI 1640. Cells were then counted and diluted to a concentration of 20 x 10^6 cells/ mL in 10% heat inactivated fetal calf serum (hiFCS) and layered onto a 0.88 x PBS 46% percoll solution. This was then centrifugated for 30 mins at 400 x g with low acceleration no brakes. This monocyte-enriched fraction was collected and washed before being resuspended to a concentration of 2 x 10^6 cells/ mL in RPMI 1640. This 10 mL of this solution was then added to a 10 cm^2 cell culture dish and left for 1 hour in the incubator to allow the monocytes to adhere. After this time the supernatants containing contaminating cells were removed and the adherent cell layer washed with RPMI to further reduce the amount of contaminating T cells. The adherent cells were then cultured in 5% HI-FCS RPMI with 50 ng/mL M-CSF over 5 days to differentiate the adherent monocytes into macrophages.

2.2.1.2 – siRNA transfection of primary human macrophages

Macrophages were harvested after 2 days of differentiation in M-CSF supplemented RPMI. These cells were then re-plated into 12 well tissue culture plates at a density of 10^6 cells per well and left overnight to adhere in 5% HI-FCS RPMI. The next day the cell culture supernatants was siphoned off the cell layer and replaced with serum free RPMI without phenol red.

The transfection reagent was formed from Dharmafect I, OPTIMEM media and an appropriate concentration of STAT3 targeted siRNA or scrambled oligonucleotide control. These 3 reagents were mixed together by pipetting and left to stand for 20 minutes at room temperature prior to application to the cells.

After 20 minutes the transfection reagent was then added to the wells of the cells, drop-wise around the plate to allow a consistent concentration of transfection reagent throughout the well. The plate was then left in the incubator for 2 hours before being replaced by 5% RPMI (phenol red free) with 50 ng/ ml M-CSF. These cells were then left to mature for a further 3 days before harvesting to allow knockdown of STAT3 protein.

2.2.1.3 – Adenoviral transfection of primary human macrophages

Primary human macrophages were harvested using mechanical dissociation and plated at a density of 1 x 10^5 cells per well of 96 well plates and rested overnight in 5% HI-FCS RPMI media. The next day cells were infected with 100 MOI of adenovirus in serum free RPMI for 2 hours. After this the virus containing media was siphoned off and replaced with 5% HI-FCS RPMI and rested overnight prior to stimulation the next day.

2.2.1.4 – Cell culture

All cell cultures were maintained at 37^{o}C and 5% CO_2 atmosphere. Primary human macrophages were harvested using cell dissociation buffer.

2.2.1.5 – Cryo-preservation of Cell lines

Cryo-preservation of RAW 264.7 cells was accomplished by suspending cells in a 10% DMSO 90% HI-FCS solution. These cells were then frozen initially at -80^{o}C before being transferred for liquid nitrogen.

Revival of frozen stocks was accomplished by thawing the cryopreserved cells at 37^{o}C before washing the cells in DMEM to remove the DMSO, before seeding at high density overnight, to allow viable cells to adhere to the tissue culture flask, before harvesting and reseeding at an appropriate density the next day.

2.2.1.6 – Transfection of RAW cells with plasmid DNA

RAW 264.7 cells were seeded at a density of 25 x 10^4 cells per well of a 24 well tissue culture in 0.5 mL of 10% HI-FCS DMEM (with 1% Pen/Strep) plate left overnight to allow the RAW cells to adhere. The next day for each well to be transfected: 0.5 µg of reporter plasmid DNA was mixed 50ng of control rL-TK luciferase plasmid in OptiMEM media to a volume of 25 µL and mixed with 1.8 µL of FuGENE®HD reagent, this was then pipetted 15 times and left for 10 minutes at room temperature. After 25 µL of the transfection mixture was added to each well of the plate and left for 18 hours to allow for the transfection to occur. After 18 hours the cell culture supernatants with the transfection reagent were removed and replaced with fresh 10% HI-FCS DMEM (with 1% Pen/Strep) and stimulated.

2.2.2 – Molecular biology

2.2.2.1 – Polymerase Chain Reaction (PCR)

Polymerase chain reaction PCR was used to amplify a target region 276bp of human genomic DNA upstream of the gene TNFSF9. For this reaction 1 ng of Human DNA (Sigma Aldrich) was used for the template, primers designed to target the desired region and containing restriction digest sites

(described in Table 2.7) and GoTaq® G2 hot start master mix (Promega). The reaction mix was prepared to a final volume of 10µL (detailed below). The lid of the PCR machine was heated to 95°C to prevent condensation in the lid of the reaction tube. The PCR was accomplished with 30 cycles of the following program:

1) Initial Denaturation at 95°C for 30 seconds
2) Annealing of primers to template temperature at 59.2°C 30 seconds
3) Polymerase extension for 30 seconds at 72°C

After this a final elongation step was p for 5 minutes before being cooled to 4°C.

REAGENT	VOLUME
DNA Template (100 ng/µL)	1 µL
GoTaq® G2 hot start master mix (2x)	5 µL
Forward Primer (100 pM)	1 µL
Reverse Primer (100 pM)	1 µL
H_2O	(remaining volume)
Final reaction volume	50 µL

2.2.2.2 – Topo® 2.1 cloning

To incorporate the amplified PCR fragment into a bacterial plasmid for large scale replication the TOPO® 2.1 cloning system was used. This system exploits the property of *Taq* polymerases to polymerise a single adenosine base to the 3' end of a PCR product. These 3' adenosines "overhang" is then able to hybridise to a single thymidine "overhang" on a linearised vector, allowing the PCR to fit into the vector.

The protein Topoisomerase I is able to bind DNA duplexes after a specific binding motif (CCCTT) and cleave the phosphodiester bond of the DNA backbone before ligating the 5' hydroxyl group to a 3' phosphate group.

1 µL of a 50 µL PCR reaction was added to 1µL of master mix, which contained the topoisomerase I and vector and diluted salt solution to a volume of 5 µL. This mixture was then left at room temperature for 15 minutes to allow the incorporation of the PCR insert to the TOPO II vector. After 15 minutes the mix was transferred to TOPO oneshot compotent bacteria and transformed according to section 2.2.2.6.

2.2.2.3 – Restriction digestion of plasmid

For sub-cloning the TNFSF9 ECR from the TOPO II vector to a luciferase containing plasmid (PGL4.26), *XhoI* and *BglII* restriction enzymes were purchased from New England Biolabs. A restriction digest reaction was set up with the following amounts:

REAGENT	AMOUNT
DNA	3 µg
NEB 10x restriction buffer (3.1)	5 µL
Enzyme	1 µL of each enzyme
H_2O	(remaining volume)
Final reaction volume	50 µL

The restriction digest was conducted at 37^{o}C over 2 hours.

2.2.2.4 – DNA digest purification

To resolve the products of a restriction digest reactions by fragment size, completed restriction digests were mixed with 10 µL 6x DNA loading buffer. The digest was then loaded onto an 0.7% (w/v) agarose TE gel, with SYBR safe DNA dye (Invitrogen), alongside a DNA size ladder. The fragments were then electrophoresed across the gel, for 90 minutes at 120 V. After this time, the bands were visualised using a bright box and filter and the band corresponding in size to the desired fragment was excised from the gel and purified using the QIAGEN Gel purification kit.

2.2.2.5 – Ligation

To ligate a digested plasmid and insert, 100ng of digest plasmid was mixed with a 3x molar ratio of insert. This was then mixed with 1µL of ligase buffer and 1µL of T4 ligase enzyme and made up to a volume of 10µL. This reaction mix was left to occur overnight at 4^{o}C, before being used to transform chemically competent bacteria the next day.

2.2.2.6 – Transformation of Chemically competent bacteria

Frozen 20 µL aliquots of Silver competent *E. coli* (Thermo Scientific) were thawed on ice and 1-4 µL of ligation product or 1ng of purified plasmid was added and gently mixed with the bacteria. The DNA:bacteria mix was then incubated on ice for 30 minutes before being placed in a 42^{o}C water bath for 30 seconds and returned to ice for 2 minutes. Cells were then incubated with 500 µL of SOC media for 30 minutes at 37^{o}C to allow for antibiotic resistance genes to be expressed. Pre-warmed LB plates with selective antibiotic (and for TOPO II plasmids 1.6ng of X-gal and 40µL

of 100mM IPTG spread on the surface of the plate) were inoculated with 200 µL of transformed bacteria and incubated overnight at 37^{o}C. Discrete colonies were then selected for larger liquid cultures the next day. In the case of TOPO II plasmids, only white or light blue colonies were selected for larger culture and insert confirmation.

2.2.2.7 – Isolation of RNA

Total RNA was extracted from MDM and RAW 267.4 cells using Qiagen RNeasy kit, typically 1 x 10^6 cells were used for QPCR and 2 x 10^6 cells were used for microarray analysis data points. After stimulating the cells, supernatants were removed and the adherent layer was washed with sterile PBS. The cells were then lysed in the supplied RLT buffer, supplemented with 0.1% β-mercaptoethanol. These lysates were then homogenised using QIAShredder spin columns. Homogenised lysates were then mixed with an equal volume of 70% ethanol and added to an RNA binding column and impurities were washed out with the supplied buffer.

While bound to the column the RNA was treated with an on-column DNase (QIAGEN), prior to the elution step. RNA was then eluted in RNase free water and stored at -80^{o}C. RNA was quantified and quality was assessed by nanodrop.

2.2.2.8 – Preparation of samples for Microarray and analysis

Sample RNA purified from cells was analysed by Nanodrop spectrophotometer for quantification and identification of solvent contamination. Solvent contamination was indicated by high absorbance at wavelengths of 280nm and 230nm when compared to the peak of absorbance for

nucleic acids (260nm). Samples with low or indistinguishable solvent contamination with a concentration of >100 ng/µL were taken for further microarray analysis.

The initial LPS/IL-10 microarray shown in chapter 3 was performed with a Affy HuGene 1_0 st v1 chip. The MLN4924 microarray in chapter 4 was performed on an Agilent SurePrint G3 GE 8x60kv2. cDNA labelling, hybridisation and image capture were performed by Dr Tim Smallie (for the Affymetrix arrays) and Oxford Gene Technology (for the Agilent array).

In microarray analysis of gene expression; RNA harvested from a cell or a population of cells is purified and reverse transcribed into cDNA. This cDNA is then chemically coupled to fluorescent dyes, which enables the visualisation of the cDNA under specific wavelengths of light. The fluorsenct sample cDNA is then placed on a microarray ChIP which contains many oligonucleotide probes of various sequences and lengths between 25nt-60nt. The cDNA is then given time to hybridise and then washed to remove cDNA not specifically or weakly bound to a probe. The cDNA is then visualised using a laser at a wavelength specific to the fluorophore and the amount of cDNA bound to a probe is quantified by the intensity of the fluorescence at that position.

2.2.2.9 – Generation of cDNA from total RNA

For the generation of cDNA from total RNA, the iScript reverse transcriptate group of reagents were used. In a standard reaction 2.5µL of total RNA (typical total quantity of 100ng) was mixed with 1 µL iSript reaction mix (5x), 1.25µL of RNase free water and, 0.25µL iScript reverse

transcriptase. The reaction mix contains a mixture of random hexamer oligonucleotides and poly-thymidine primers and will therefore amplify all thypes of RNA not only mature mRNA.

After the reverse transcriptase reaction was set up, the mixture was placed in a PCR thermocycler and heated to 25^{O}C for 5 minutes, before being heated to 42^{o}C for 30 minutes. After this the reverse transcriptase was inactivated by heating to 85^{o}C for 5 minutes.

2.2.2.9 – QPCR

QPCR is a method to quantify the abundance of an mRNA by measuring fluorescence of incorporated fluorogenic probe or intercalating dye. The SYBR green dye emits fluorescence when intercalated with dsDNA, with the level of fluorescence directly proportional to the amount to dsDNA.

During PCR amplification, dsDNA copies of the amplicon are produced which SYBR green can intercalate with. As more amplicons are produced each cycle, the level of fluorescence increases.

A standard QPCR reaction used in this study is shown in Table 2.12.

REAGENT	VOLUME
cDNA	2.4 µL
SYBR green Master mix (2x)	4 µL
Forward Primer (100 pM)	0.4 µL
Reverse Primer (100 pM)	0.4 µL
H_2O	0.8 µL
Final reaction volume	8 µL

Table 2.12 – Reaction mix for a standard QPCR reaction

The threshold cycle (C_t) is the number of PCR cycles required for a SYBR green reaction to produce fluorescence signal above a background level (an example is shown in). A relative level of mRNA abundance can be then inferred by comparing the C_t of a gene of interest to the C_t of a housekeeping gene (in this study GAPDH is used), which is not affected by conditions of the experiment.

$$\Delta C_t = C_t \text{ (target gene)} - C_t \text{ (housekeeping gene)}$$

$$\Delta\Delta C_t = \Delta C_t \text{ (condition 1)} - \Delta C_t \text{ (condition 2)}$$

$$\text{Fold change} = 2^{-(\Delta\Delta Ct)}$$

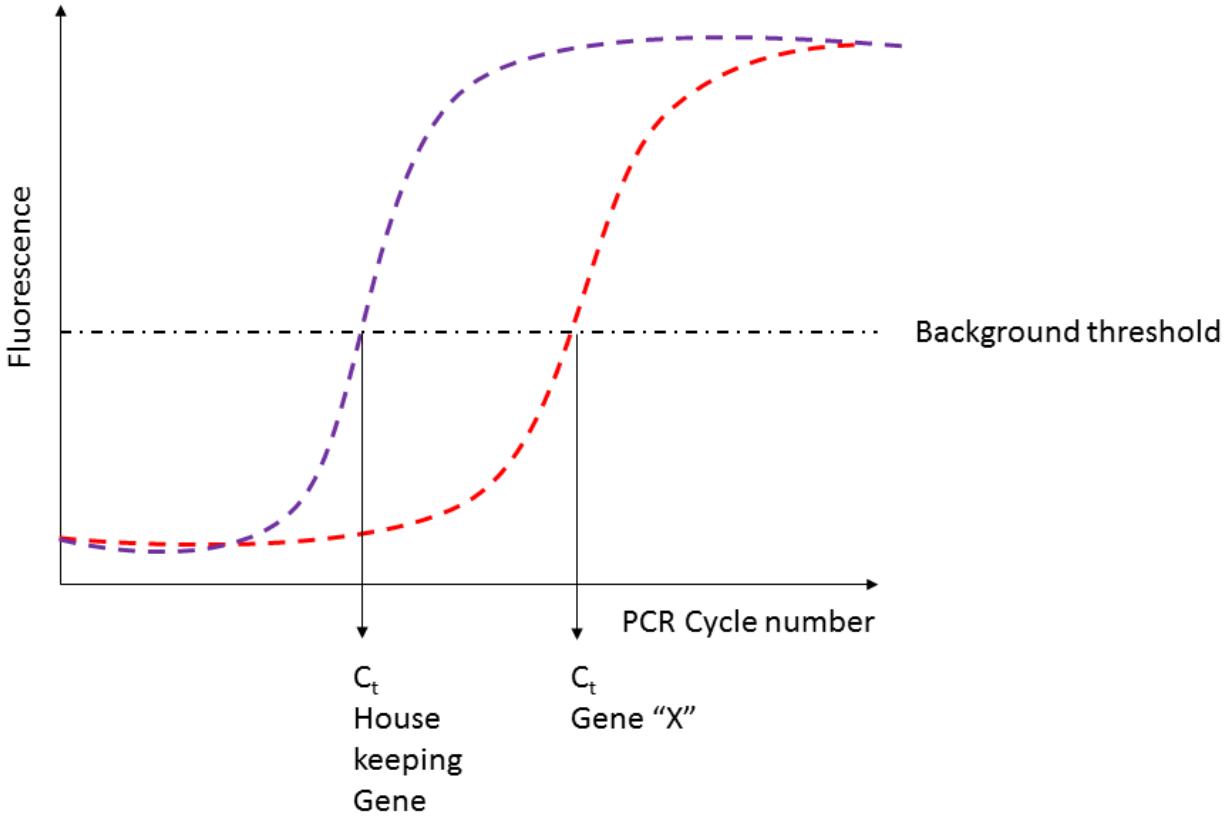

Figure 2.1 – Calculating threshold cycle for QPCR

A typical QPCR experiment, with fluorescence of a reaction on the Y axis and cycle number on the X. Each reaction analyses the abundance of 1 target gene or 1 housekeeping gene. As the number of PCR cycles increases the amount of amplicon increases and therefore increases in fluorescence. After fitting a curve to the data points acquired the cycle at which the sample fluorescence reaches a threshold above background (C_t)

This method of quantifying gene expression assumes that the efficiency of the PCR reaction is close to 100%. Prior to use in my experiments, the efficiency of the PCR reaction for each primer pair was assessed using a standard curve method. In this method, template cDNA was serially diluted 1:5 with molecular biology grade water for use as a template for a QPCR reaction. The C_t for each dilution was then plotted against the dilution factor and the gradient of this graph was used to calculate efficiency using the formula below. Primers were only used for QPCR if the efficiency was greater than 80%.

$$\text{Efficiency (\%)} = (-1+10^{(-1/\text{gradient})})*100$$

2.2.2.10 – Enzyme linked immunosorbent assay (ELISA)

The concentration of secreted TNF-α was measured by sandwich ELISA. Between each step the wells were washed with ELISA wash buffer three times. All antibodies and the HRP:Streptavidin conjugate were diluted in 0.5% BSA PBS. 96 well ELISA plates were coated with 100 µL of TNF capture antibody overnight at 4^{o}C. The wells were then washed and blocked with 100 µL of ELISA Blocking Buffer, for 2 hours at room temperature. After washing; 100ul of cell culture supernatants (some supernatants were diluted in 0.5% BSA to adjust the concentration of secreted TNF-α into the range of the standard curve) were added to the wells alongside serially diluted recombinant human TNF standards (10 ng/mL – 13 pg/mL) and a negative control of PBS 0.5% BSA in triplicate, for two hours at room temperature. The plate was washed and then 100ul of biotin-conjugated secondary anti-TNF antibody for 1 hour at room temperature. After the next set of washes 100ul of HRP:Streptavidin conjugate was added to the wells for 1 hour at room

temperature. After this final set of washes the samples were developed using 100ul of TMB peroxidase substrate system (KPL Inc). The reaction was then stopped using 50 µL of 2M H_2SO_4 and read at 450nm on an ELISA plate reader and analysed using GEN5 software.

2.2.2.11 – Flow cytometry

1 x 10^6 MDM cells were stimulated for a selected time and were harvested by scraping in 200 µL of cell dissociation buffer. The harvested cells were then pipetted gently to remove aggregates of cells. The cell suspension was then divided in half for separate staining mixtures and centrifugated at 2000 rpm for 3 minutes at 4^{o}C. One aliquot of stimulated cells was then stained with 0.5µg of anti TNFSF9-APC antibody and 0.5µg of anti CD274-Alexfluor450 in 50µL of FACS buffer, while the other aliquot was stained with the same amount of isotype control antibody. The cells were then left for 30 minutes at 4^{o}C in the dark to allow the antibodies to bind to the cell surface antigen. Cells were then fixed with FixPerm solution (Biolegend) for 15 minutes in the dark at 4^{o}C and washed 3 times prior to being suspended in 100µL of FACS buffer. Fixed cells were then examined by using a Cyan flow cytometer (Beckman Coulter) and the Summit software package. The strategy for gating on single cells for analysis based on laser scattering is detailed in Figure 2.2.

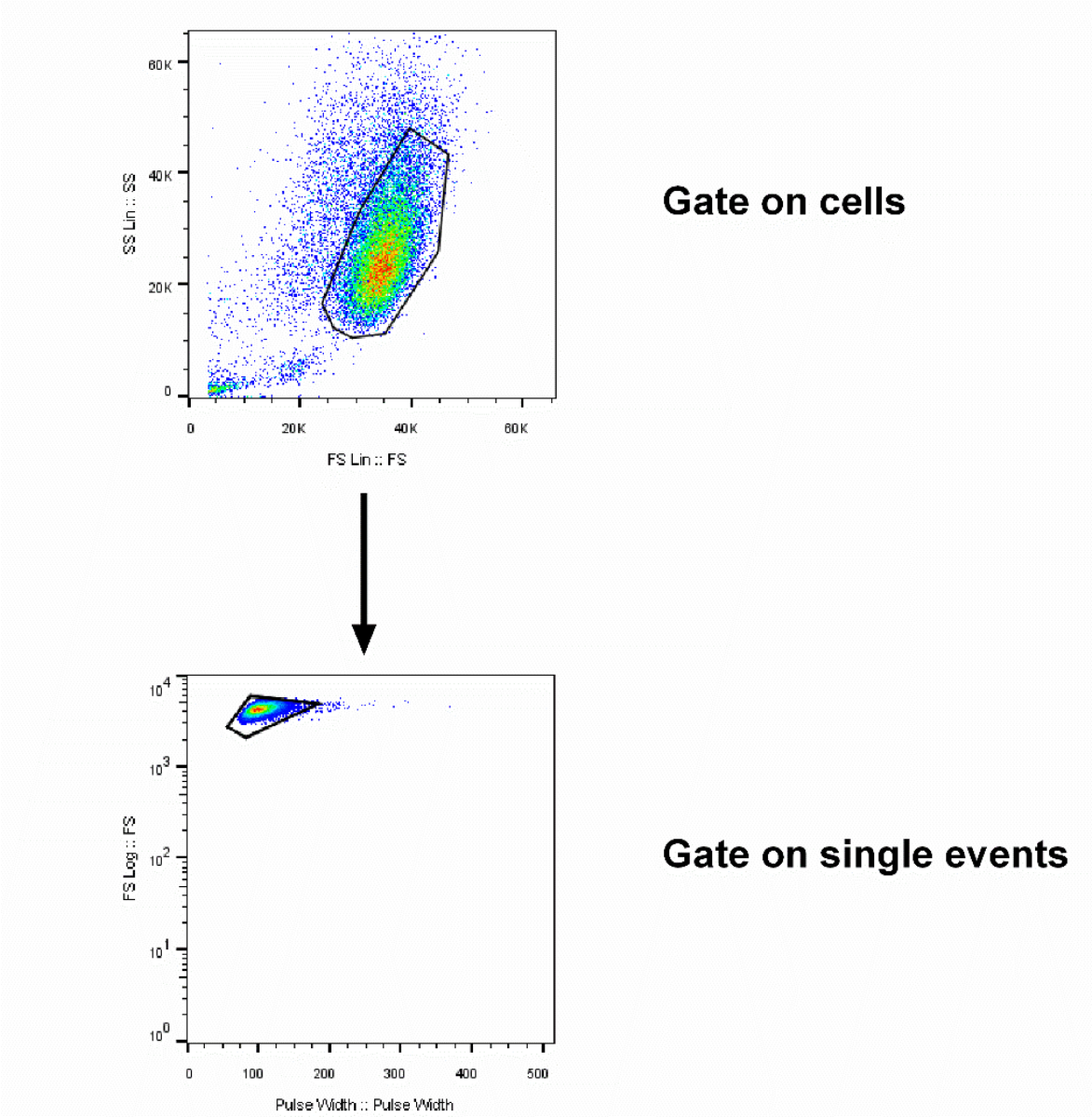

Figure 2.2 – Flow cytometric gating strategy

Single cell events were gated on by first selecting the population of events of forward scatter (FS) between 20K and 50K and a side scatter (SS) of between 15K and 45K, to exclude cellular debris from further analysis. This population was then gated further on the pulse width and FS to exclude doublet or multiple event acquisitions being further analysed. For each sample roughly 20000 events for each sample were collected in this second gate for further analysis.

2.2.2.12 – Luminex

Multiplexed cytokine bead assay for: CCL8 and CXCL10 were purchased from R&D and used in accordance with the manufacturer's protocol.

2.2.2.13 – Western blotting

2.2.2.13.1 – Sample preparation

$2x10^6$ MDM cells were seeded in each well of a 6 well tissue culture plate. After stimulation cell culture supernatants were removed and the cells washed twice in PBS. After washing, cells were lysed in 80µL of cold protein lysis buffer, supplemented with protease and phosphatase inhibitors. These lysates were then centrifuged in QIAshredder spin columns to shear genomic DNA, thereby decreasing viscosity of the samples. At this point samples were quantified for protein expression using a Bradford assay (BioRad) according to manufacturer's instructions.

2.2.2.13.2 – Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS is an amphipathic detergent which is able to associate with denatured protein and coat it in a charge. Allowing the proteins to be electrophoresis across a gel.

Lysates were mixed with an appropriate volume of sample loading buffer (section) and boiled for 10 minutes at 97^{o}C to ensure denaturation of the proteins. After this samples were centrifuged at 13,000 rpm for 1 minute to pellet insoluble cell debris and were loaded onto a precast poly acrylamide gel (Biorad) and run at 100 V for 90 minutes.

2.2.2.13.3 – Protein transfer

Polyvinylidene difluoride (PVDF) membranes (Bio-Rad) were washed briefly in methanol and placed under the gels. The gel/PVDF sandwich was then placed between layers of buffer soaked tissue (provided with the PVDF membrane) and placed in the Trans-blot turbo system-cassette. The cassette was then loaded into Trans-blot Turbo and proteins transferred to the membrane over 7 minutes at a constant of 25V and 2.5 A.

2.2.2.13.4 – Immunoblotting

PVDF membranes were blocked with blocking buffer (section 2.1.8) for 1 hour at room temperature. This coated areas on the membrane where protein had not been transferred from the SDS-PAGE gel. The membrane was then washed 3 times in western blotting wash buffer (section 2.1.8) for 5 minutes each time. The membrane was then incubated with primary antibody suspended in blocking buffer, overnight at 4^{o}C. Again the membrane was washed in western blotting wash buffer 3 times, before being incubated with a secondary HRP-conjugated antibody for 1 hour at room temperature. The membrane was then washed again and incubated with the chemiluminescent substrate for HRP ECL reagent (Thermo scientific) for 1 minute, before being visualised on a chemidoc.

2.2.2.14 – Nuclear Cytoplasmic subcellular fractionation

After stimulation cells were lifted from tissue culture plates using cell scraper into 1 mL of PBS and pelleted at 800g for 1 minute. The cell pellets were then gently resuspended in cold

Cytoplasmic Lysis buffer and left on ice for 10 minutes. The cell suspension was then subjected to centrifugation at 800g for 1 minute again and the supernatant which comprised the cytoplasmic fraction was siphoned off and stored. The pellet was then resuspended in nuclear wash buffer and left for 10 minutes at room temperature, after which the nuclear suspension was centrifugated at 800g for 1 minute to pellet the nuclei. The supernatant was then removed and the pelleted nuclei were lysed in nuclear lysis buffer.

2.2.2.15 – Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation enables the quantification of DNA:protein interactions, by chemically joining the DNA and protein molecules in close proximity (cross-linking). These complexes can then be precipitated with antibodies specific to a DNA binding protein of interest.

2.2.2.15.1 – Isolation of fixed nuclei

10x107 of mature MDM were seeded onto 10 cm^2 tissue culture dishes and left to adhere overnight and stimulated the following day. After stimulation 1 mL of cell culture supernatant was removed for analysis by ELISA and cells were fixed with 1% (final concentration) formaldehyde (Sigma) in the remaining cell culture medium for 10 minutes. To quench the molecule cross-linking capacity of the formaldehyde 125mM Tris pH 7.5 (final concentration) was added to the cell culture medium, which after briefly mixing to ensure a complete quenching of formaldehyde was discarded. The cells were then washed 3 times in ice cold PBS, to remove the remaining Tris and formaldehyde. Fixed cells were then harvested by scraping in ice cold PBS

supplemented with protease and phosphatase inhibitors (Roche) and transferred into 1.5 ml lobind tubes (Eppendorf). Cells were then pelleted by centrifugation at 400g for 1 minute at 4^{o}C and the supernatant discarded.

In order to remove the cytoplasmic fraction the cell pellets were gently resuspended in ice cold Cytoplasmic Lysis buffer supplemented with protease and phosphatase inhibitors (Roche) and left on ice for 10 minutes. The cell suspension was then centrifugated at 800g for 1 minute again and the supernatant (the cytoplasmic fraction) was removed. The pellet was then resuspended in nuclear wash buffer and left for 10 minutes at room temperature, after which the nuclear suspension was centrifugated at 800g for 1 minute to pellet the nuclei. The supernatant was then removed and the pelleted nuclei were lysed in nuclear lysis buffer.

2.2.2.15.2 – Chromatin fragmentation

In order to break down the cross-linked chromatin into fragments that allows efficient QPCR reactions (~500bp fragments), nuclear fractions were sonicated at 20% amplitude on a vibracell sonicator (Sonics) for 8 x 12 second pulses. Between each round of sonication the fractions were incubated on ice for 1 minute to prevent the sample from overheating.

2.2.2.15.3 – Immunopreciptation

Chemically fixed protein:DNA complexes were isolated by immunoprecipation with Dynabeads Protein G (Invitrogen). 1 day prior to the nuclei isolation and sonication steps, Dynabeads were washed with ice cold Dynabeads blocking buffer three times. The beads were then suspended in

20µg/mL ChIP antibody or isotype control (Table 2.9) and left overnight rotating at 4°C. The following day the coated beads were washed 3 more times with dynabead blocking buffer, before being resuspended in 500µL of Nuclear lysis buffer and 100µL of sheared sample chromatin and left rotating at 4°C overnight.

Samples were then centrifugated at 16000g for 1 minute and 200µL of the supernatant was retained for use as an input DNA control. The beads were then washed 5 times with ice cold RIPA buffer, supplemented with protease and phosphatase inhibitors followed by one wash with salt wash buffer .Beads were then centrifugated at 400g for 3 minutes and resuspended in 200µL of bead elution buffer and incubated at 65°C for 15 minutes, resuspending the beads every 2 minutes. The beads were then centrifugated again at 400g for 3 minutes and the supernatants were extracted and incubated at 65°C overnight in lo-bind tubes (Eppendorf) to reverse the chemical cross-linking of the protein:DNA complexes.

2.2.2.15.4 – DNA purification

After reversing the cross-links DNA was purified using QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol and eluted in 200µL of molecular biology grade water. The immoprecipitation was then assessed by QPCR (2.2.2.9).

2.2.2.15 – Luciferase assay

Cells transfected with plasmid DNA or adenoviral plasmids containing luciferase were stimulated and cell culture supernatants removed. The cells were then lysed in 100µL of luciferase assay lysis buffer (section). 50µL of this was then removed and added to 120µL of luciferase assay buffer

(section) and 30µL of Luciferin (Promega) and luciferase activity assessed using a plate luminometer. After 40 minutes of repeated 1 second per well acquisition, Stop-and-Glo (Promega) reagent was added to the wells. The addition of this reagent, quenches the firefly luciferase activity but also provides a substrate for the control *Renilla* luciferase plasmid under control of a 760bp human thymidine kinase promoter (rL-TK). The *Renilla* luciferase activity was acquired for 1 second per well exposures for a further 40 mins. The *Renilla* luciferase activity was then used to normalise differences in transfections between the different stimulations and inhibitor treatments.

2.3 – Bioinformatic and statistical methods

2.3.1 – Microarray analysis

Microarray image files were prepared by Oxford Gene Technology LTD (OGT) and analysed by Partek Genomic Suite®. Feature extraction file for each array were quantile normalised to account for inter array variation, prior to the statistical analysis using a multivariate ANOVA. Duplicate probes for genes and transcripts were filtered to a single probe which demonstrated the highest level of expression.

2.3.2 – Microarray merging

2.3.2.1 – Yugene

Comparison between microarray datasets were done using the Yugene method (Cao et al., 2014). In this methods, all probes were ranked in terms of “expression” (fluorescence intensity when

probes bind to complementary sequences in fluorescently labelled cDNA) and assigned a value based on the fraction of probes whose expression is less than the probe of interest (low expression values tending toward 0 and higher expressing values tending to 1).

The log_2 expression data from the microarray samples was taken from Partek Genomic Suite® and transformed by the Yugene function of the Yugene R package.

2.3.2.2 – Z-score

Log_2 expression scores of all the probes were transformed using a Z-score transformation, which standardises two sets of values based on the mean of the sets and therefore allows comparisons between sets of data that vary greatly in magnitude. The z scoring was performed by the *scale* function in R, with the equation shown below where: x = expression a probe, $\boldsymbol{X}$ = mean expression of all probes in that data set and n = number of probes in the dataset.

$$Z\ score = \frac{(x - \boldsymbol{X})}{\sqrt{\sum \left(\frac{x - \boldsymbol{X}}{n - 1}\right)}}$$

2.3.3 – Principal Component analysis (PCA)

The PCA method attempts to highlight the largest differences in multivariate datasets. To accomplish this the data for each sample is mean centred and then co

PCA analysis was accomplished using the PCA function of the "MixOmics" R package, with mean centred data, but without scaling (Le Cao et al., 2009, González, 2011).

2.3.4 – Pearson correlation

Pearson correlation statistics were calculated on the 1000 most variable genes (defined by the highest standard deviation between samples) on log2 expression values. The Pearson correlation matrix was computed using the "cor" function of R and a heatmap of these data was drawn by the "ComplexHeatmap" package.

2.3.5 – Gene ontogeny analysis

Microarray probe lists for each hierarchical cluster were transformed into the respective ENSEMBL gene ID and added as a search query to InnateDB (www.InnateDB.com) (Breuer et al., 2013, Lynn et al., 2008, Lynn et al., 2010).

2.3.6 – Transcription Factor motif enrichment

Microarray probe lists for each hierarchical cluster were transformed into the respective ENSEMBL gene ID and assessed for transcription factor motif enrichment using HOMER. The program assessed the enrichment of transcription factor binding motifs between -2000bp and +500 bp from the transcription start site (TSS) of the gene.

2.3.7 – Multiple alignment of syntenic DNA sequences

Sequences syntenic to regions of human refseq DNA were acquired through the *liftover* tool in the UCSC genome browser. These sequences were then aligned using the Clustal Omega.

(Goujon et al., 2010, Li et al., 2015, McWilliam et al., 2013, Sievers et al., 2011)

2.3.8 – 3rd party data access-and credits

PU.1, H3K27ac ChIP-seq tracks from human MDM were retrieved from (Pham et al., 2012) and H3K4me3, H3K4me1 ChIP-seq tracks and Dnase-seq data produced by the blueprint consortium (Saeed et al., 2014) was accessed through the UCSC browser.

This study makes use of data generated by the Blueprint Consortium. A full list of the investigators who contributed to the generation of the data is available from www.blueprint-epigenome.eu. Funding for the project was provided by the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 282510 – BLUEPRINT.

Chapter 3 – IL-10 modulation of LPS-induced transcription

3.1 – Introduction

Dissecting the mechanisms macrophage responses to LPS have been most often studied using macrophages derived of bone marrow of inbred mouse strains, using either M-CSF (which generates homogenous macrophage populations) or GM-CSF (which produces mixed populations of macrophage and dendritic cells (Helft et al., 2015). This approach allows the use of genetically modified (e.g. knock out) mouse strains to interrogate the roles of specific factors in the response, as well as reducing inter-individual variation. However, there are abundant differences in gene expression between mouse and human macrophages. Extrapolation from mouse studies to the behaviour of human macrophages in health or disease can therefore be difficult.

Macrophages constitute an extremely heterogeneous population of cells, in terms of origin, of function and, patterns of gene expression. It is therefore naive to think that there exists a single archetypal macrophage population, which exists as a model for all subpopulations. Tissue resident macrophages from different anatomical sites display clear differences from one another and from bone marrow derived macrophages (Lavin et al., 2015b, van de Laar et al., 2016). However, certain core responses of macrophages appear to be shared. For practical and ethical reasons, large numbers of tissue resident macrophages are extremely difficult to obtain from healthy humans. Macrophages derived from patients with chronic inflammatory disease or cancer must be treated with caution because of the possible confounding effects of disease and treatment.

In this thesis I wished to investigate the effect of IL-10 on primary human macrophages, because of the relevance of these effects to inflammation, resolution, health and, disease. I chose to use

macrophages derived from healthy donor monocytes using M-CSF, because these could easily be generated in large numbers and as relatively homogenous populations. Monocyte-derived macrophages (MDM) differentiated with M-CSF were expected to recapitulate at least some of the IL-10 responses of tissue-resident macrophages. Given the variety of methods for macrophage isolation, it is important to report the method of isolation and differentiation clearly, and to avoid excessive extrapolation of the results (Murray et al., 2014).

3.2– Results

3.2.1 – IL-10 selectively modulates LPS-induced gene expression

The ability of IL-10 to inhibit LPS-induced gene expression has been the focus of many studies, generating a plethora of hypotheses for a mechanism for this action. In order to establish a mechanism for IL-10's action it was first necessary to characterise the effects of LPS and IL-10 induced signalling on the macrophage transcriptional program.

To investigate whether IL-10 is able to modulate the LPS-induced transcriptome, monocyte derived-macrophages (MDM) from 4 independent donors stimulated with LPS, IL-10 or in combination for one or four hours were analysed by the Affymetrix HuGene-1_0-ST V1 microarray platform (performed by Dr Tim Smallie). This platform contains 33296 oligonucleotide probes which have been annotated based on sequence to bind to 24832 unique mRNA transcripts corresponding to 24329 genes.

Due to the number of samples in the experiment, it was necessary to perform the chip hybridisation and image capture in a number of batches. In order to account for batch variation, the data from the microarray were pre-processed using quantile normalisation. In this method the relative intensity between the majority of microarray probes is assumed not to change over an experiment and therefore the distributions between samples are similar and can be normalised to one another. Figure 3.1 highlights the variation in probe intensity before and after quantile normalisation in the experiment. Although there was some variation in the distribution between samples (Figure 3.1A) after quantile normalisation the samples became comparable in distribution (Figure 3.1B).

Once the data had been processed to enable comparison the intensities of the microarray probes were extracted. The samples were assessed for the similarity between donors and timepoints to ensure robust statistical comparisons later on in the analysis. To accomplish this, I utilised a Pearson correlation which analyses the strength of a linear correlation between two samples. All samples were compared in a pairwise fashion and the resulting Pearson values (structured as a matrix) were then hierarchically clustered (using an average linkage algorithm) to restructure the matrix, placing more similar samples adjacent to one another (Figure 3.2).

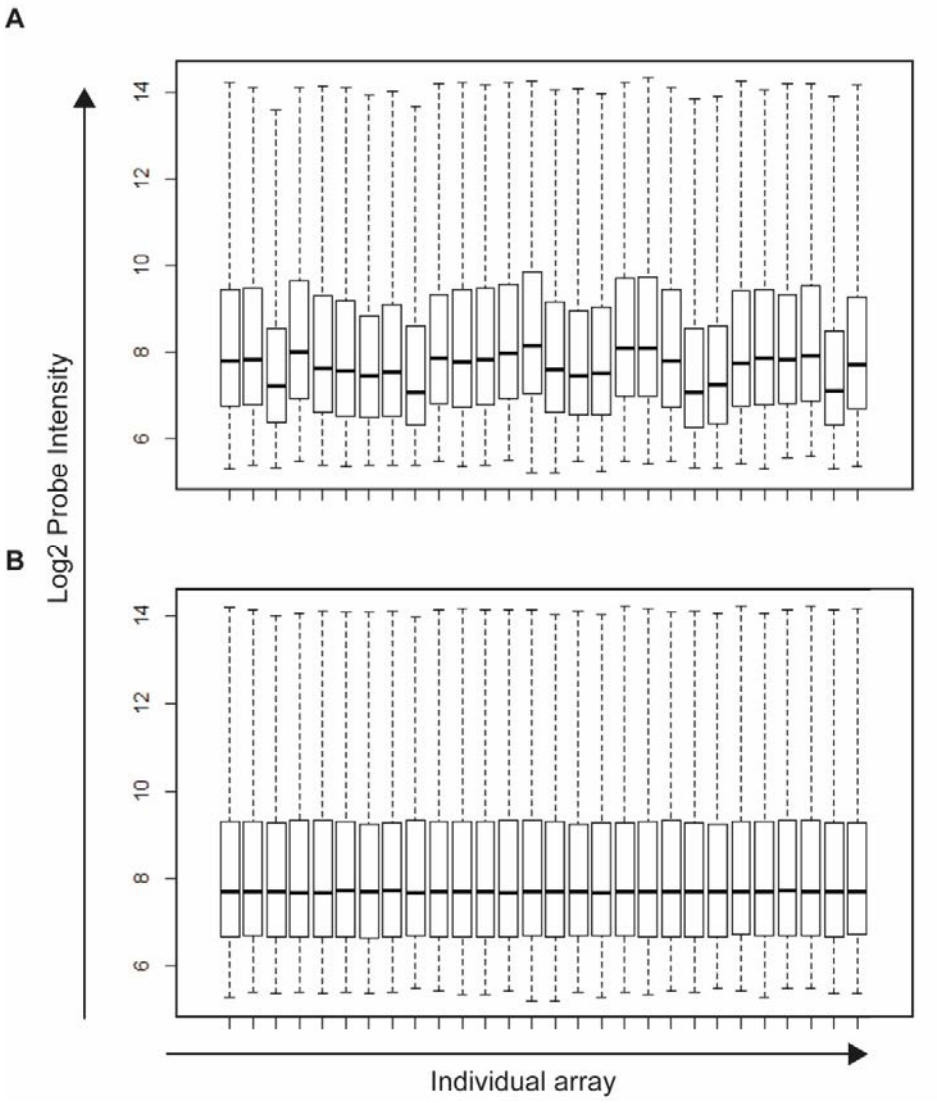

Figure 3.1 – Distribution of microarray probes across multiple chips prior and post quantile normalisation

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 1 or 4 hours with 10 ng/mL LPS. Cells were then harvested for RNA and microarray analysis performed on the samples (Samples prepared by Dr Tim Smallie). The probe intensities on each microarray chip were extracted and the range of intensities are shown above, prior to quantile normalisation (A) and after quantile normalisation (B).

not separate. After 4 hours all samples clustered according to treatment. LPS alone and LPS and IL-10 stimulation were highly similar to one another but still segregated according to treatment rather than the donor, whereas the IL-10 treated samples were quite closely correlated with untreated samples. This correlation analysis did not identify any potential outliers and indicated that the data would allow for robust statistical analysis of the dataset.

In order to visualise the differences between the stimulated transcriptomes of the microarray, I performed a principal component analysis (PCA) on the whole microarray using the $\log_2$ expression values generated by Partek Genomics Suite and the R package "MixOmics" principal component analysis. A principal component analysis is a method of visualising variation between samples with large number of measurements per sample and reducing the variation seen into principal components (PC) that account for some of the differences in the data. Figure 3.3 shows the proportion of the dataset variation explained by each principal component. In these data it can be seen that the first three PCs explain over half of the variance of the dataset (0.45, 0.13 and 0.06 for PC1, 2 and 3 respectively) with remaining components (4-28) each explaining less than 0.05 of the data each.

Knowing that the first three principal components explained the majority of the variation in the data, I examined the distribution of the data in the first three principal components, as shown in Figure 3.4. The most noticeable feature about the data is the clustering of the four donors according to treatment, indicating that the major changes to the transcriptome on stimulation are consistent between individuals.

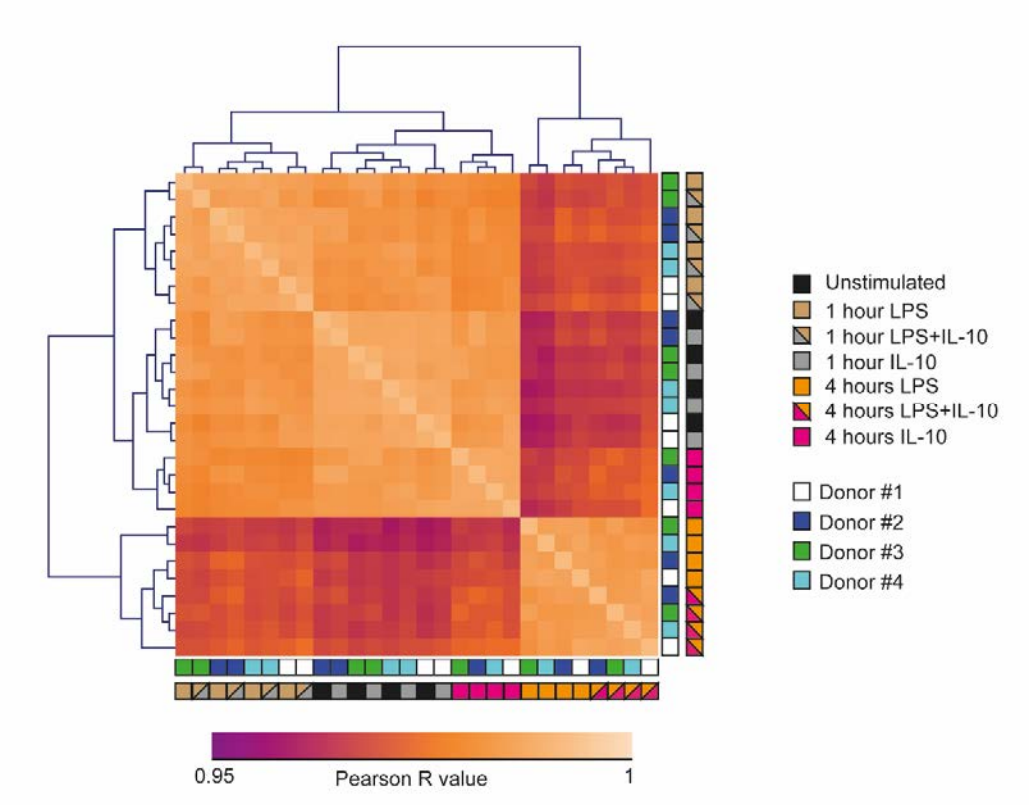

Figure 3.2 – Pearson correlation of the LPS/IL-10 microarray

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 1 or 4 hours with 10 ng/mL LPS. Cells were then harvested for RNA and microarray analysis performed on the samples (Samples prepared by Dr Tim Smallie). Individual samples across the experiment were compared using a Pearson correlation and the resulting correlation coefficient matrix was hierarchically clustered using an average linkage algorithm.

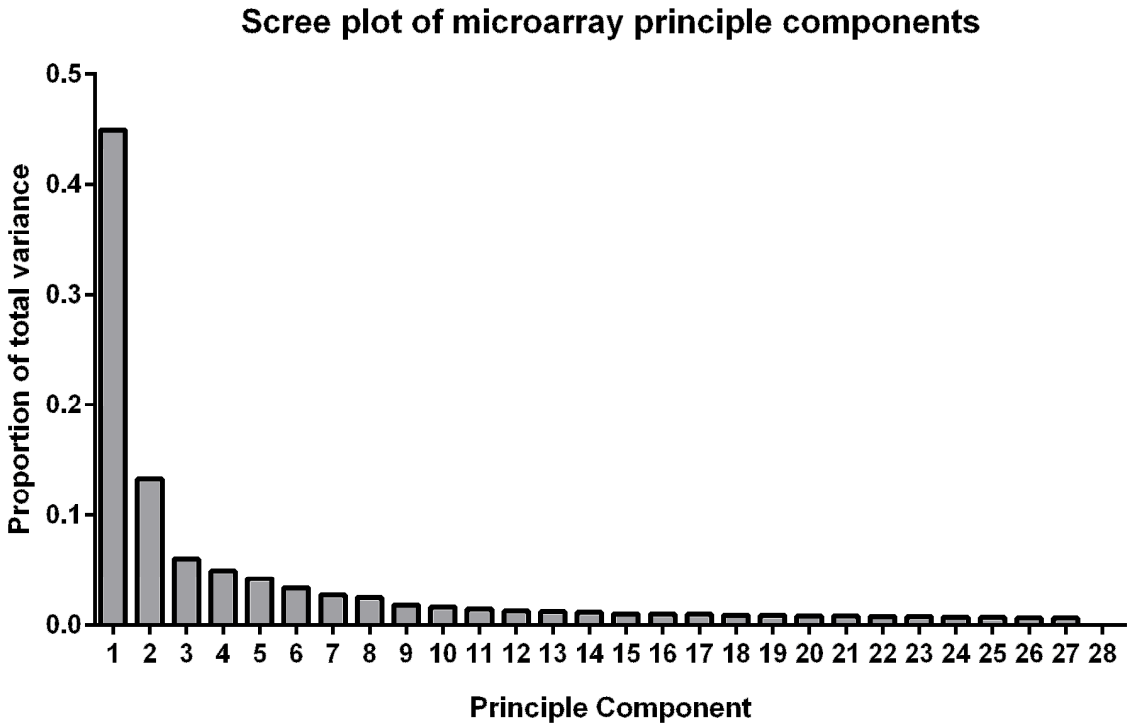

Figure 3.3 – Scree plot of principal components contribution to the variation in the IL-10 microarray

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 1 or 4 hours with 10 ng/mL LPS, 10 ng/IL-10 or in. Cells were then harvested for RNA and microarray analysis performed on the samples (Samples prepared by Dr Tim Smallie). Principal component analysis was then performed data and the eigenvalues of each component were plotted as a fraction of the variance of the whole dataset.

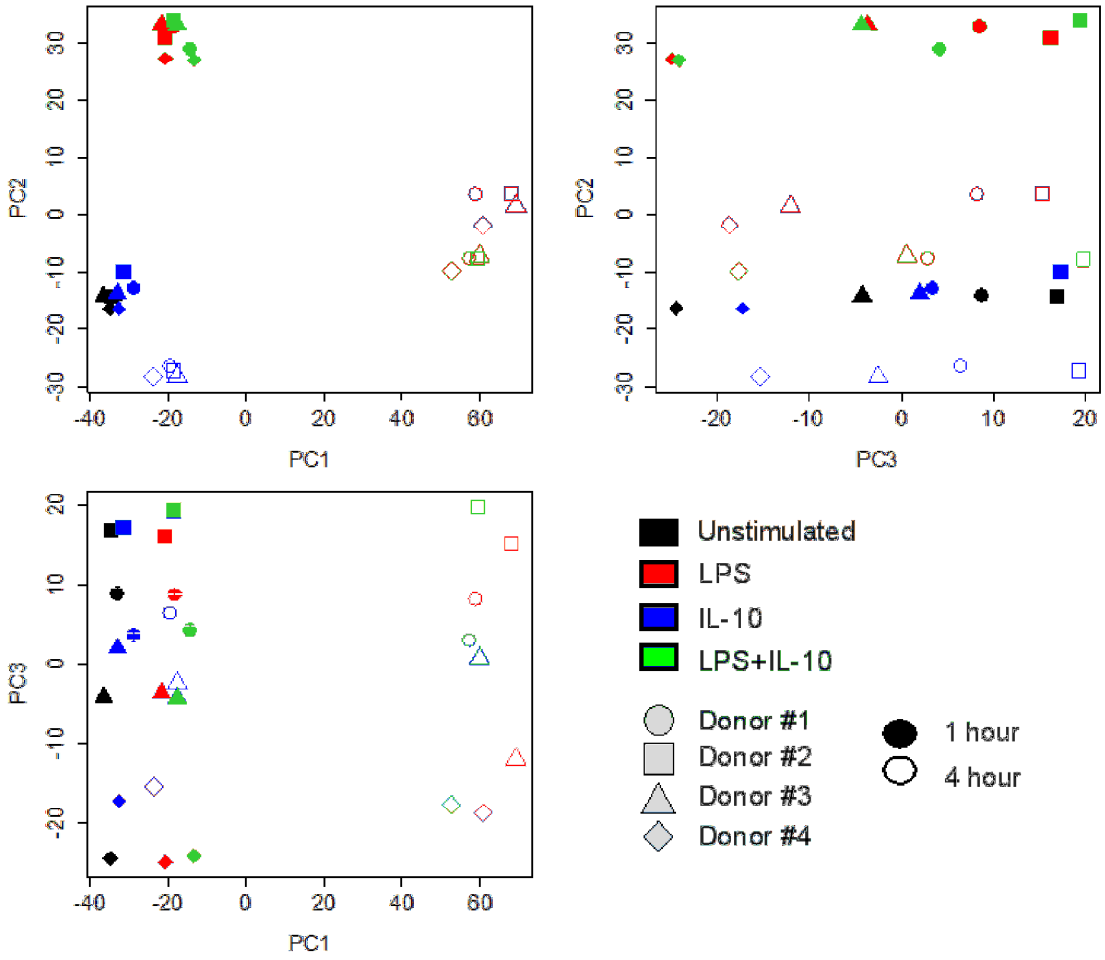

Figure 3.4 – PCA of LPS/IL-10 stimulated macrophage transcriptomes

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 1 or 4 hours with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination. Cells were then harvested for RNA and microarray analysis performed on the samples (Done by Dr Tim Smallie). The raw data from the chips was quantile normalised and the variation in the dataset visualized by PCA (MixOmics package).

The first 2 PCs (corresponding to the two largest sources of variation) separated the one and four hour LPS-stimulated cells. While I expected the two timepoints to separate in the PCA, the fact that they separated in two separate principal components, indicates that the LPS modulated program of gene expression, significantly changes between the two timepoints.

Another interesting feature of the PCA is the much smaller degree of change in the transcriptome, when cells were stimulated with IL-10. In the first two PCs there was very little difference between unstimulated cells and cells treated with IL-10 for one hour. At four hours there was a more pronounced change in both PC1 and PC2 between unstimulated and IL-10 stimulated cells. However, this shift was small in comparison to the changes caused by LPS stimulation. Cells treated with LPS or LPS and IL-10 in combination separated to some extent at one hour and more clearly at four hours. In each case the effect was orthoganol to the effect of LPS. At one hour, LPS stimulation caused a shift mostly in PC2, whereas the addition of IL-10 separated samples (albeit weakly) in PC1. At four hours the inverse was true; LPS stimulation separated samples in PC1 while the addition of IL-10 separated them in PC2. The orthoganol shifts caused by LPS and IL-10 may reflect broadly opposing effects on macrophage gene expression.

The third PC did not describe differences according to time of stimulation or stimulus used, but instead separated the donors indicating that despite the identical conditions producing broadly similar effects (shown by PC1 and 2), on a genome wide scale the variation between individuals was still able to produce a significant effect. However, the level of this variation was much smaller than the effect of the stimuli (PC1; 45% variation, PC2; 13% variation, PC3; 6% variation).

Both the Pearson correlation analysis and principal component analysis indicated that most changes of gene expression in response to LPS and IL-10 were robust and reproducible between individuals, although some inter-individual variation was present. The effect of IL-10 on the macrophage transcriptome were small in comparison with the effects of LPS. Either in the presence or absence of LPS, effects of IL-10 on the macrophage transcriptome were greater at four hours than at one hour.

I then examined how LPS and IL-10 stimulation affected expression of individual genes over time. The microarray was analysed by multivariate ANOVA, which took into account: sample stimulation, time, donor and, batch. Genes were filtered on significant changes in expression from unstimulated samples (>2 fold difference, ANOVA p-value<0.05) and I examined the number of shared modulated genes between the two time points. Figure 3.5 and 3.6 show that the macrophage transcriptome was more profoundly altered at the four hour timepoint compared to one hour time point. After one hour of LPS stimulation 214 genes were induced by LPS and 80 genes were significantly repressed. After four hours 403 genes were induced by LPS and 833 genes were repressed.

IL-10 treatment produced a much smaller change in gene expression compared to LPS. IL-10 alone induced only 31 genes after one hour and 139 at four hours. It also repressed the expression of only 4 genes at one hour and 49 after four hours of stimulation. These results are not surprising, given the high Pearson correlations between unstimulated and IL-10 stimulated samples (Figure 3.2).

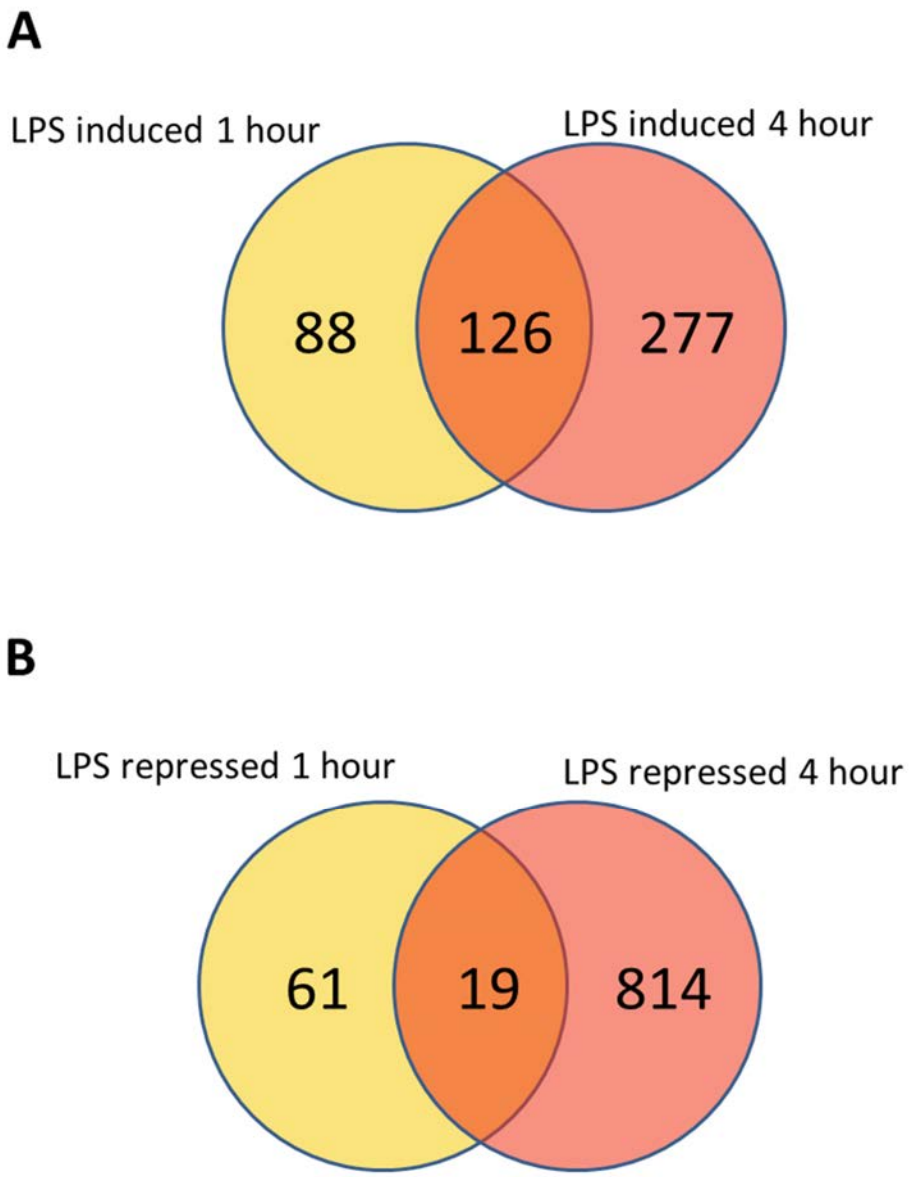

Figure 3.5 – Comparison of LPS induced genes at 1 and 4 hours

Microarray data was filtered on genes that changed expression on LPS stimulation (>2 fold change, ANOVA p-value <0.05). Venn diagrams showing the number of probes A) shared by those induced by LPS, B) repressed by LPS stimulation.

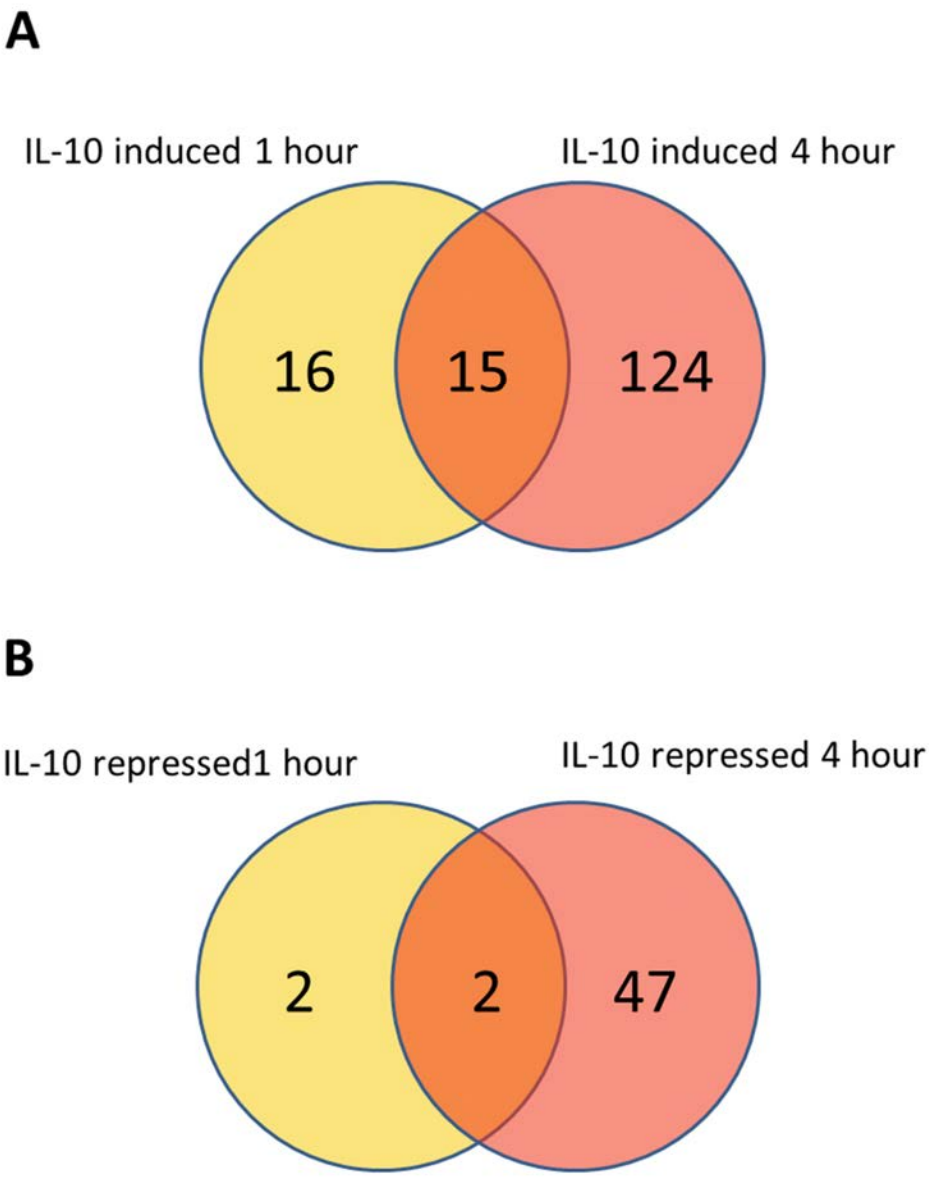

Figure 3.6 – Comparison of IL-10 induced genes at 1 and 4 hours

Microarray data was filtered on genes that changed expression on IL-10 stimulation (>2 fold change, ANOVA p-value <0.05). Venn diagrams showing the number of probes A) shared by those induced by IL-10, B) repressed by IL-10 stimulation.

I then assessed whether the alterations in gene expression on stimulation were either due to the modulation of strongly (or weakly) expressed genes or whether the changes in gene expression were confined to genes intermediately expressed. To do this I correlated the Log_2 of the gene expression for all unique genes from all conditions and plotted them against the unstimulated values (Figure 3.7). From this I observed that the distribution of induced (and repressed genes) was spread across a range of levels in unstimulated cells.

Both Pearson correlation and the principal component analyses indicated a somewhat restricted rather than general effect of IL-10 on the macrophage transcriptional response to LP. However, IL-10 is very well known to inhibit the LPS-induced expression of many genes. Therefore, I undertook a hierarchical clustering approach to examine in more detail the effects of IL-10 on the transcriptional response to LPS. I extracted the significantly LPS induced genes at 1 and 4 hours (upregulated >2 fold, p value <0.05) and hierarchically clustered according to the gene expression behaviour in response to LPS and IL-10. This analysis (Figure 3.8 and) identified three distinct patterns of gene expression: transcripts that were inhibited by IL-10 (labelled as “A”), transcripts whose expression was not affected by IL-10 (labelled “B”) and transcripts whose expression was cooperatively regulated by LPS and IL-10 (labelled “C”).

At the one hour timepoint 42% of the LPS induced genes were inhibited by IL-10 while an equal number of genes were unaffected by IL-10 signalling, with the remaining 19% being cooperatively regulated by LPS and IL-10. At four hours the pattern shifted and almost 3/4 of LPS induced gens were unaffected by IL-10 signalling. The proportion of IL-10 inhibited probes dropped to 20% and the cooperatively regulated cluster dropped to just over 8% of the total LPS-induced probes.

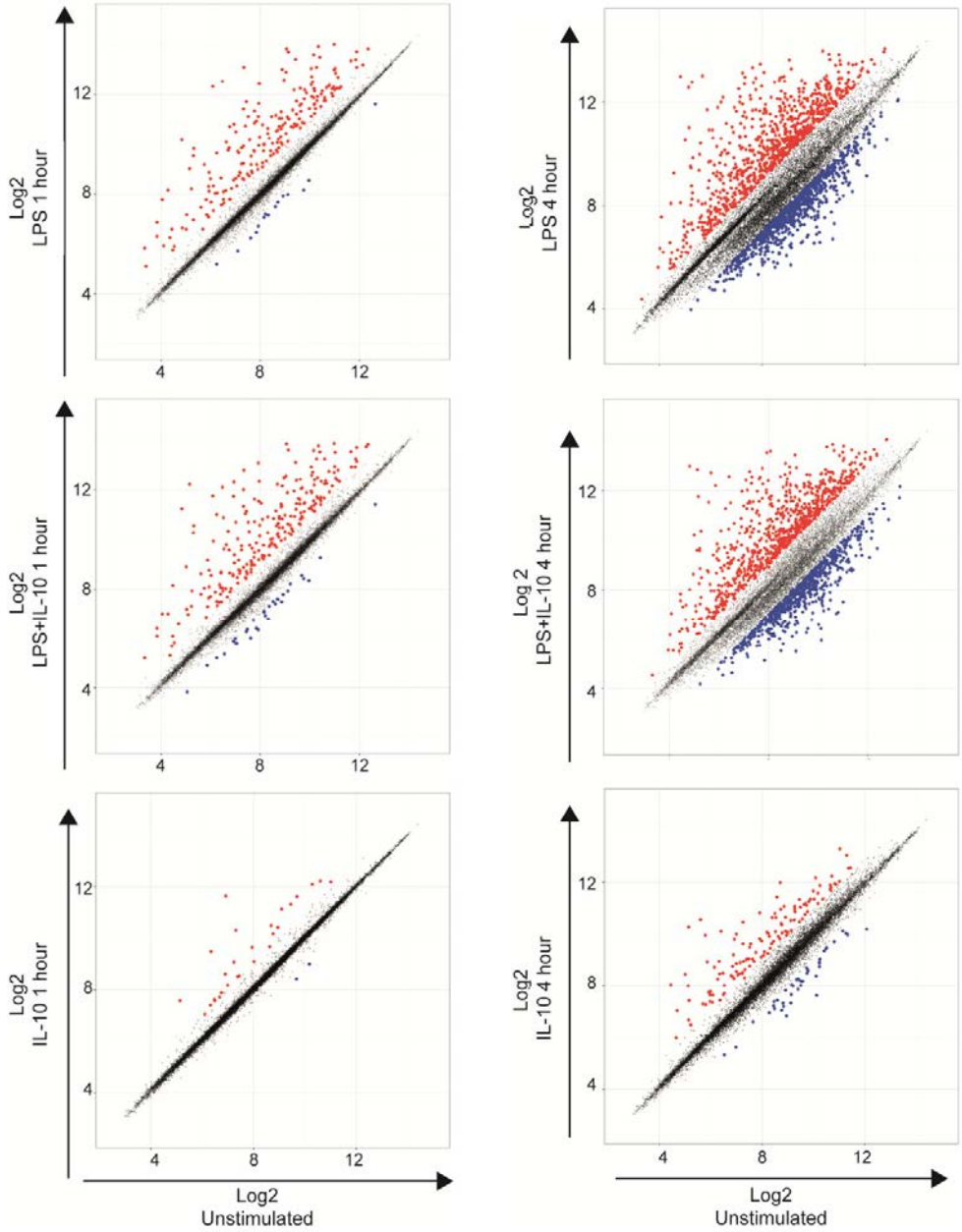

Figure 3.7 – Correlation plots showing changes in gene expression between stimulated and unstimulated macrophages

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 1 or 4 hours with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination. Cells were then harvested for RNA and microarray analysis performed on the samples (Done by Dr Tim Smallie). Genes whose expression was altered greater than 2 fold compared to unstimulated and whose change was statistically significant (ANOVA p value<0.05) are highlighted in red (for those upregulated) and blue (for those downregulated).

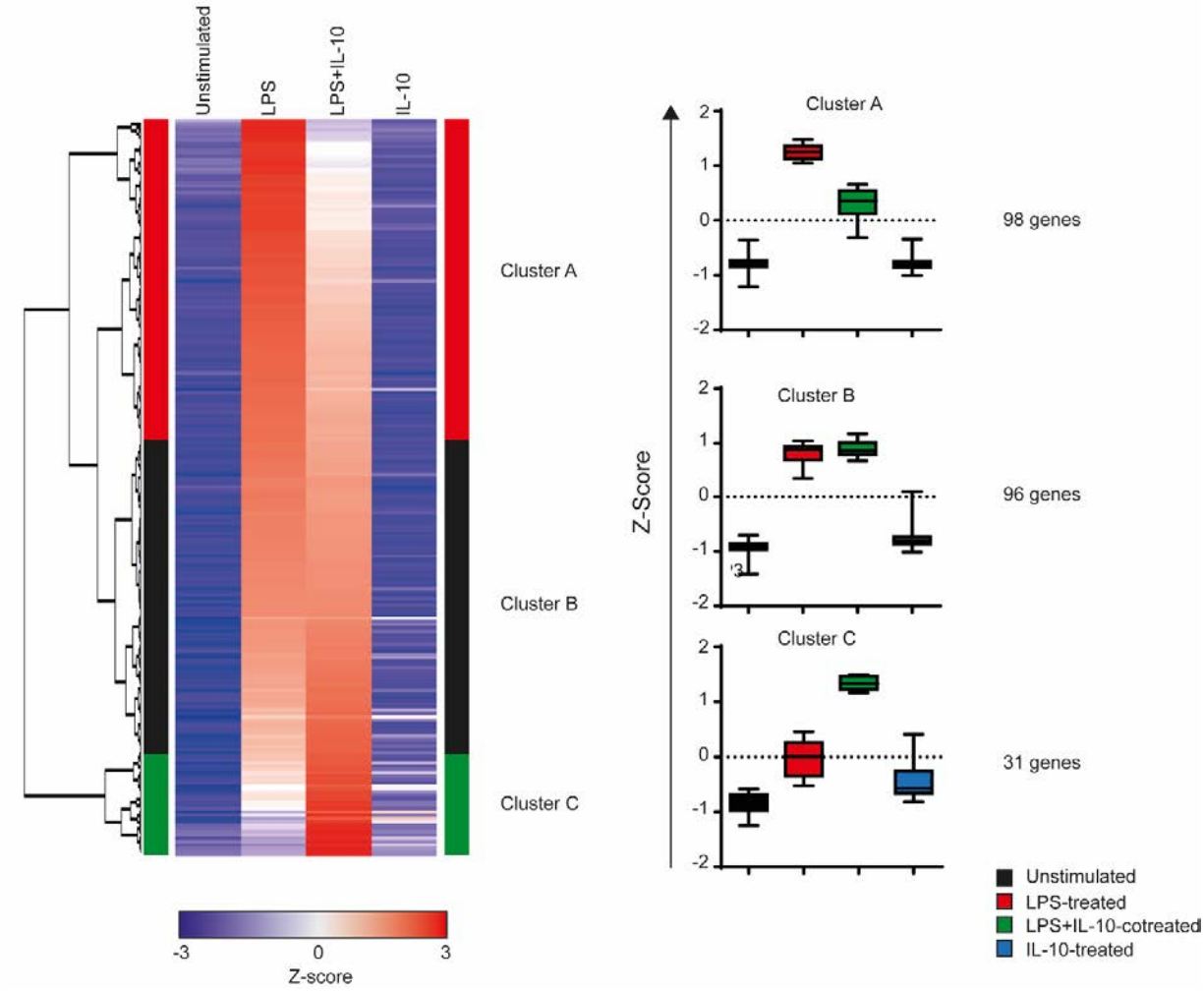

Figure 3.8 – Hierarchical clustering of LPS/IL-10 1 hour transcriptome.

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 1 hour with LPS, IL-10 or in combination. Cells were then harvested for RNA and microarray analysis performed on the samples (Oxford Gene Technology). The raw data from the chips was quantile normalised and duplicate probes removed. LPS-induced genes (>2 fold above unstimulated, ANOVA p value<0.05) were clustered using average linkage hierarchical clustering. Z-scores of genes in each cluster shown in the box and whisker plots.

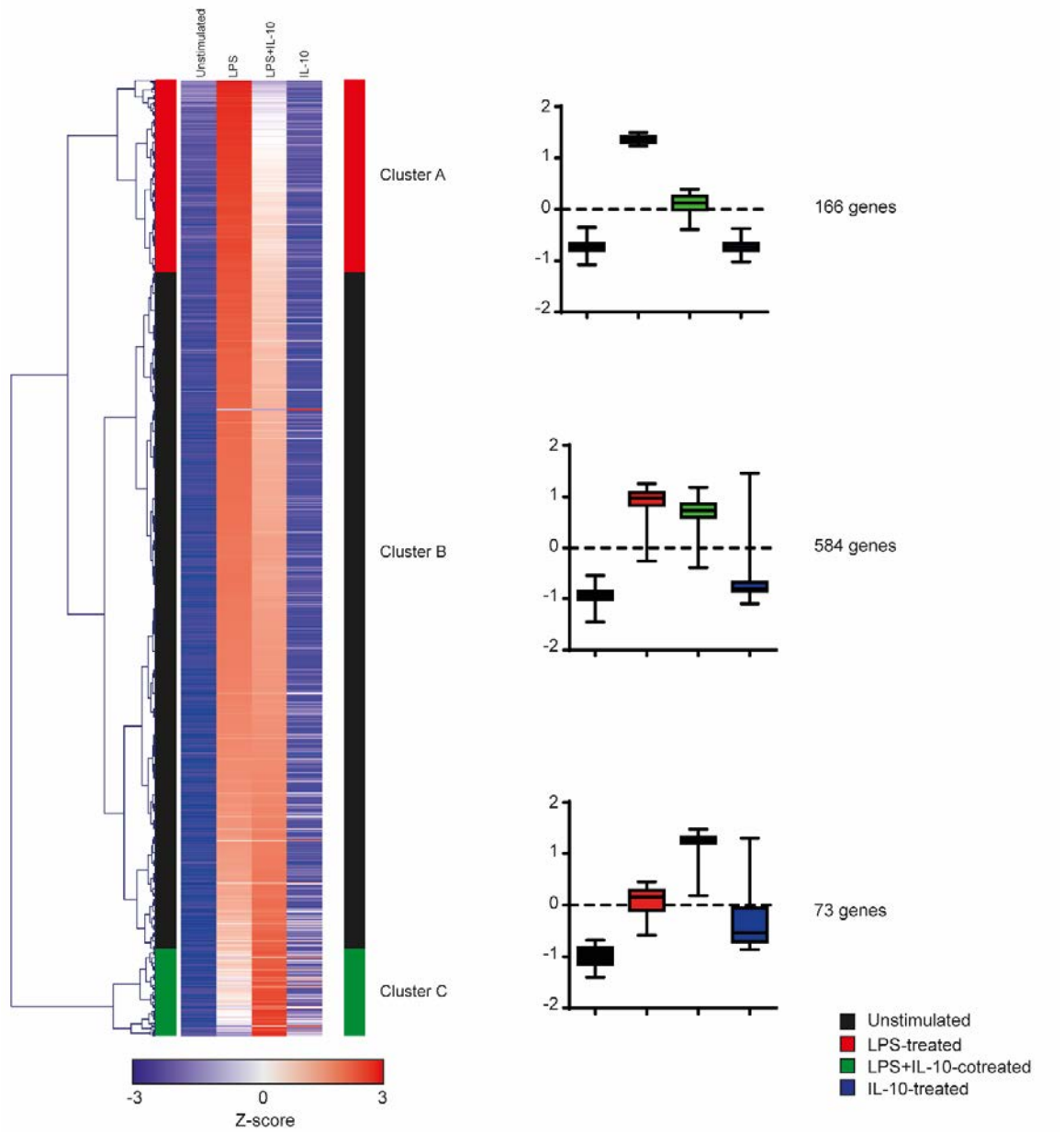

Figure 3.9 – Hierarchical clustering of LPS/IL-10 4 hour transcriptome.

$2x10^6$ primary human macrophages from 4 independent donors were stimulated for 4 hour with LPS, IL-10 or in combination. Cells were then harvested for RNA and microarray analysis performed on the samples (Oxford Gene Technology). The raw data from the chips was quantile normalised and duplicate probes removed. LPS induced genes (>2 fold above unstimulated, ANOVA p value<0.05) were clustered using average linkage hierarchical clustering. Z-scores of genes in each cluster shown in the box and whisker plots.

These results were surprising, given PCA and Pearson correlation of the same data. However, given that the PCA and Pearson correlation do not filter on LPS induction, it is possible that the differences in this analysis were masked by largely unchanged expression of many genes at the global level.

In order to understand this shift in IL-10 responsiveness to IL-10 I examined genes induced at one and four hours after LPS stimulation and assessed the change in proportion of IL-10 responsive clusters between these time points (Figures 3.10, 3.11 and 3.12). There was a clear redistribution of probes from IL-10 inhibited and cooperatively induced at one hour, to IL-10 insensitive at four hours (shown by the blue chords connecting the two sides). About half of the genes inhibited or cooperatively regulated by IL-10 at one hour became unaffected by IL-10 at four hours, while the majority of 1 IL-10 unaffected genes, remained so at four hours. Interestingly, 6 genes that were cooperatively regulated by IL-10 at one hour were inhibited by IL-10 at four hours, while no genes inhibited at one hour became cooperatively regulated at four hours. Some example genes are shown in Figure 3.12 To illustrate changes in the effect of IL-10

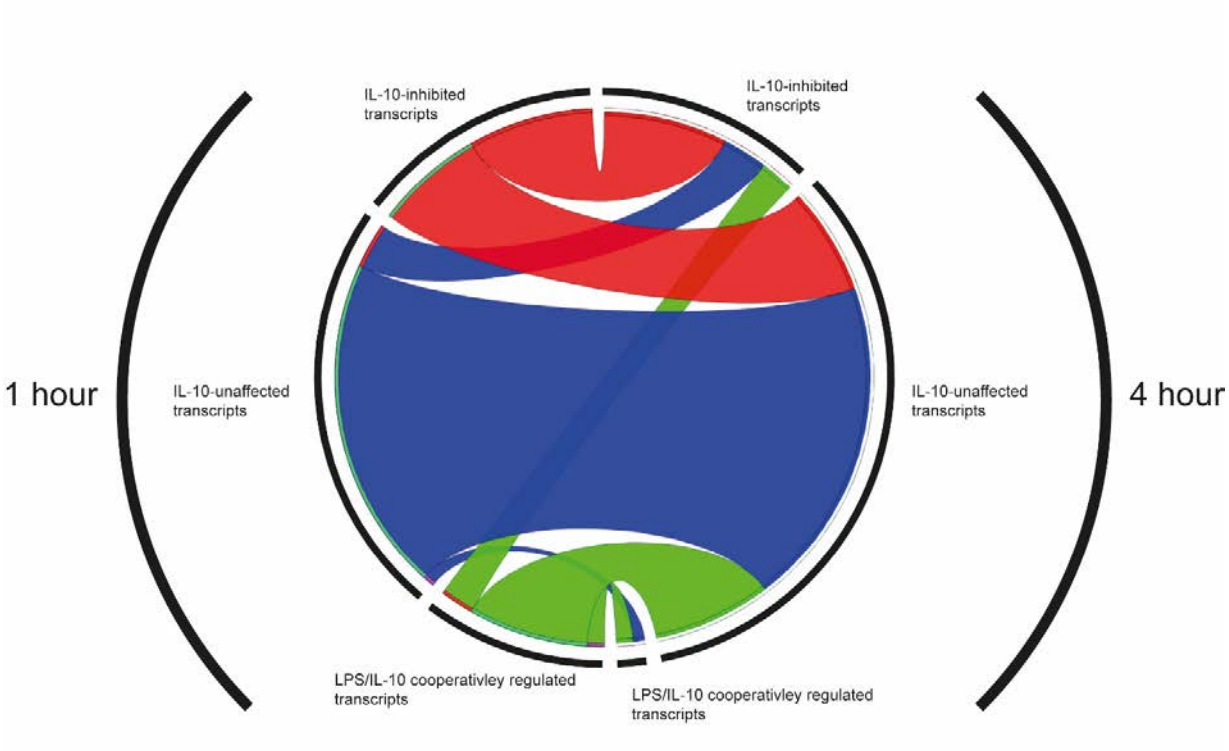

Figure 3.10 – Chord diagram of IL-10 clusters of probes induced by LPS at 1 and 4 hours

Microarray expression data of genes induced by LPS hours (>2 fold induction, ANOVA p value <0.05) at both 1 and 4 hours were hierarchically clustered according to their response to IL-10. The change in cluster assignment between the two time points is shown by thickness of the chords joining the segments.

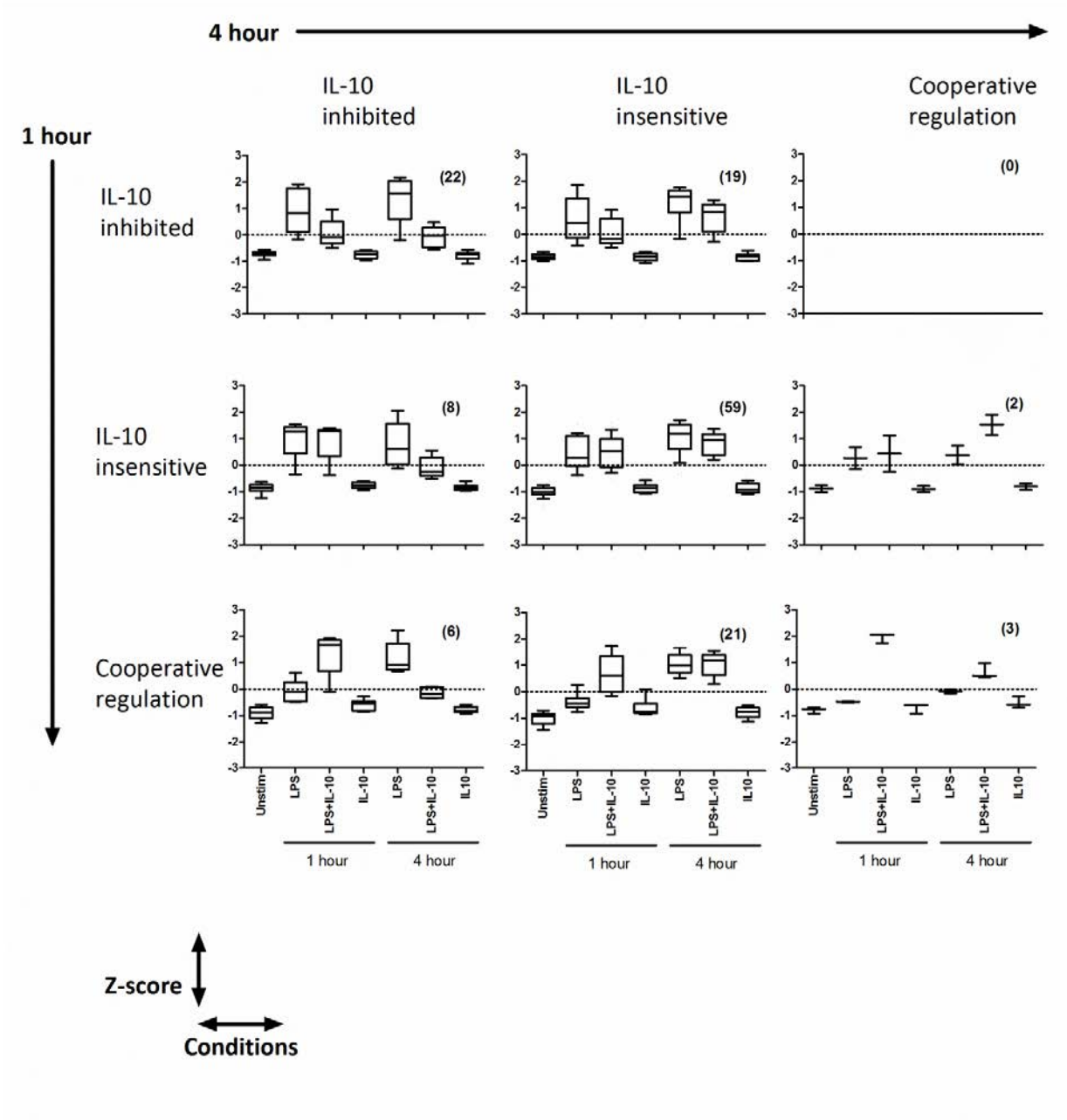

Figure 3.11 – Z-score distribution of 1/4 hours LPS-induced gene expression.

Microarray expression data of genes induced by LPS hours (>2 fold induction, ANOVA p value <0.05) at both 1 and 4 hours were hierarchically clustered according to their response to IL-10. genes were then sorted according to their 1 hour and 4 hour clusters and normalised by Z-score.

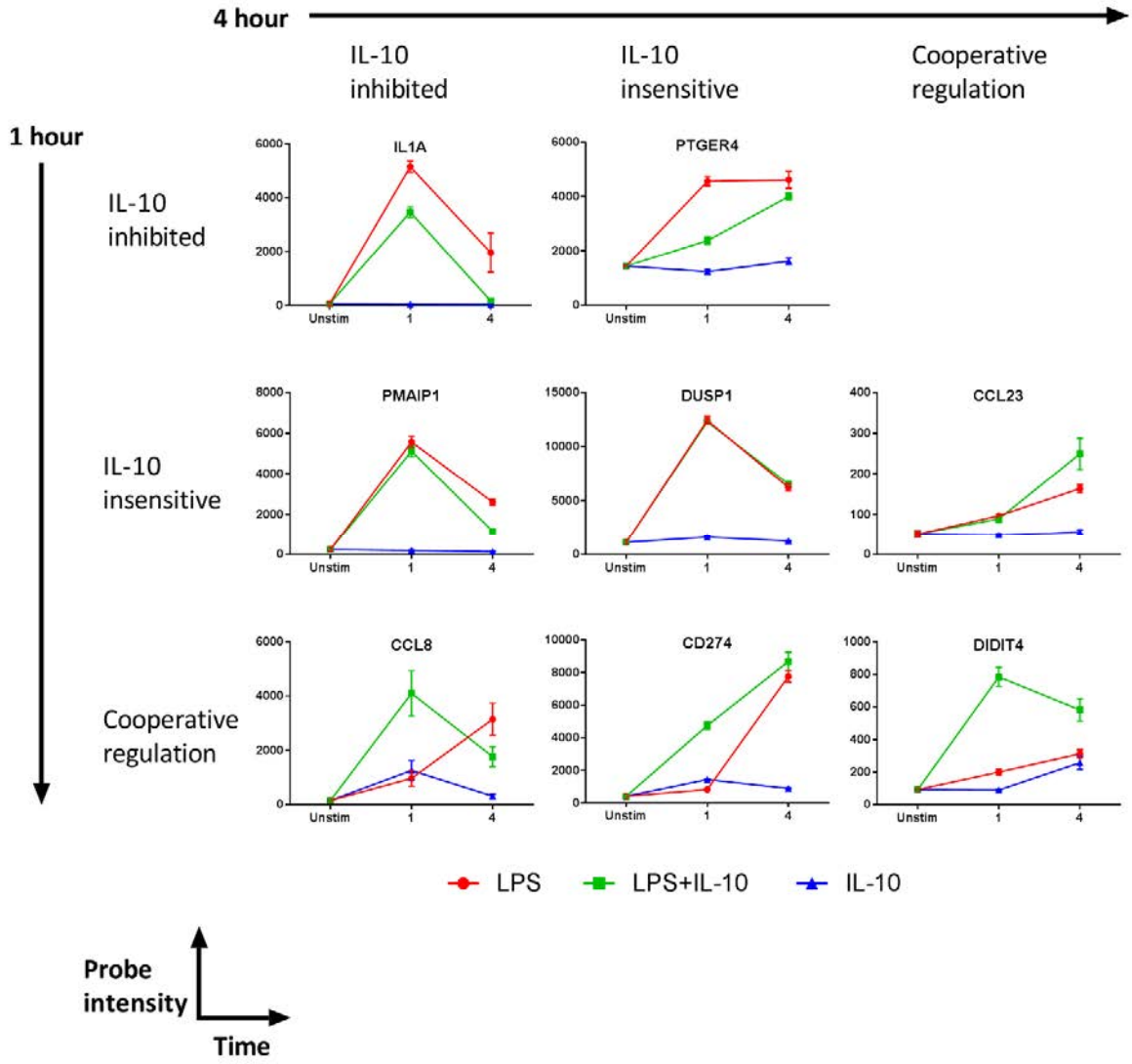

Figure 3.12 – Representative gene expression profiles of 1 and 4 hours LPS-induced genes.

Microarray expression data of genes induced by LPS hours (>2 fold induction, ANOVA p value <0.05) at both 1 and 4 hours were hierarchically clustered according to their response to IL-10. genes were then sorted according to their 1 hour and 4 hour clusters and normalised by Z-score.

3.2.2 – Validation of microarray data

To evaluate whether the observations in the microarray were repeatable, I selected 18 genes that were induced by LPS at one hour and displayed different responses to the addition of IL-10. The genes selected were: *CCL8*, *CD274*, *CH25H*, *CXCL10*, *DUSP1*, *FFAR2*, *GADD45B*, *IFNB1*, *IL1A*, *IL1B*, *NFKBIA*, *NFKBID*, *NFKBIZ*, *NR4A2*, *TNF*, *TNFAIP3*, *TNFSF9* and, *TNIP3* (highlighted in Figure 3.13).

In order to assess the reproducibility of the QPCR assays in this experimental setup, 4 donors were assessed for the expression of the genes (mentioned above) compared to the housekeeping gene *GAPDH* (data shown in Figure 3.13). Many of the genes assayed had less than 1% the expression of *GAPDH* in unstimulated cells, with the lowest expression (*NFKBIZ*) reaching the threshold of fluorescence late into the QPCR reaction. On LPS stimulation, these genes consistently rose to a detectable level of expression. Since in unstimulated cells the transcripts were weakly expressed and difficult to detect, fold inductions compared to unstimulated conditions would be inappropriate to use to compare between samples. I therefore normalised the data sets to the LPS stimulated sample to allow more consistent comparisons between the data. I then assayed the levels of expression the genes by QPCR, on MDM from 4 independent donors stimulated in identical manner to those used in the microarray (10 ng/mL LPS, 10 ng/mL or in combination for one hour).

The QPCR values were generated, by normalising to the housekeeping gene *GAPDH* and the LPS-stimulated value (due to very low levels of transcripts in the unstimulated samples). These values were then analysed by Pearson correlation to the microarray values (also normalised to the LPS sample) to assess the similarity of the two methods (side by side comparison shown in Figure 3.14

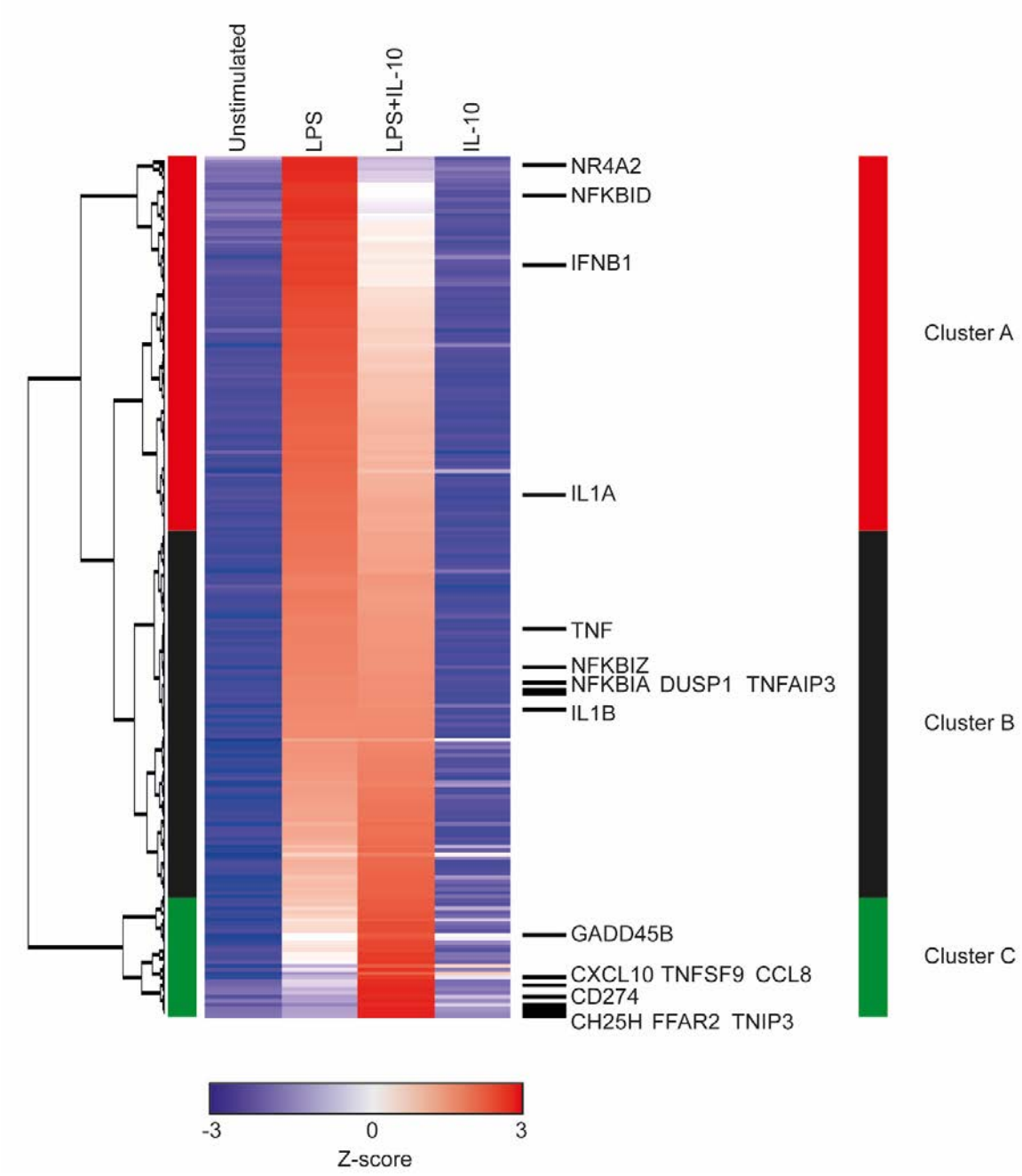

Figure 3.13– Selection of genes for microarray validation

LPS inducible genes were selected from each of the clusters on the basis of differential response to IL-10 and on a known role in the immune response, inflammation or, cell signalling. The position of each of the selected genes in the hierarchical clustering is indicated.

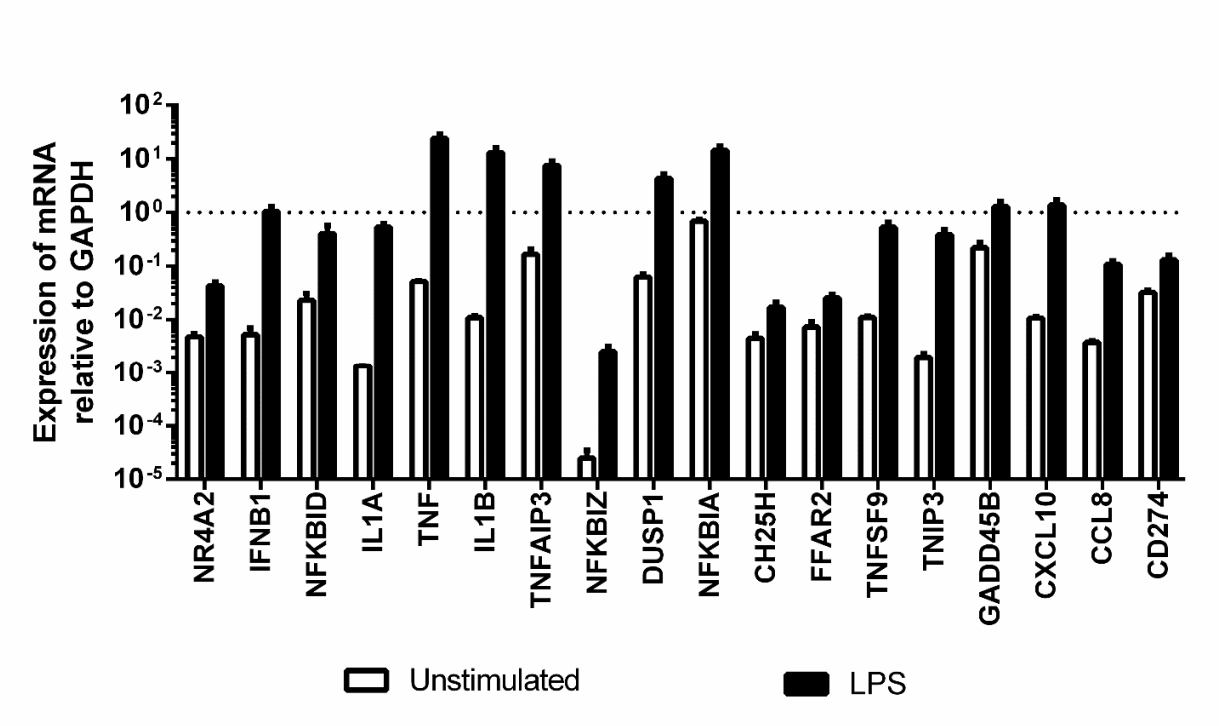

Figure 3.14 – Induction of gene expression on LPS stimulation

Primary human macrophages of 4 independent donors were stimulated with LPS for one hour or left untreated and analysed for the expression of 18 LPS-induced genes and the housekeeping gene *GAPDH*. The expression of each gene was normalised with respect to *GAPDH*.

and a table of the Pearson correlations between the microarray and QPCR values is shown in Table 3.1).

Most of the selected genes had high Pearson correlation coefficients between the microarray and QPCR values, with the highest correlation coefficient being *NR4A2* with 0.991. The lowest correlation coefficient was that of *CH25H* which had a correlation coefficient of 0.729. The level of sensitivity between the two assays, was consistent for most of the genes assayed. However, the LPS/IL-10 cooperatively regulated genes *TNFSF9*, *CCL8* and, *CXCL8* showed a more exaggerated LPS/IL-10 cooperative regulation when measured by QPCR. Overall the genes selected demonstrated a high level of concordance with the microarray data set.

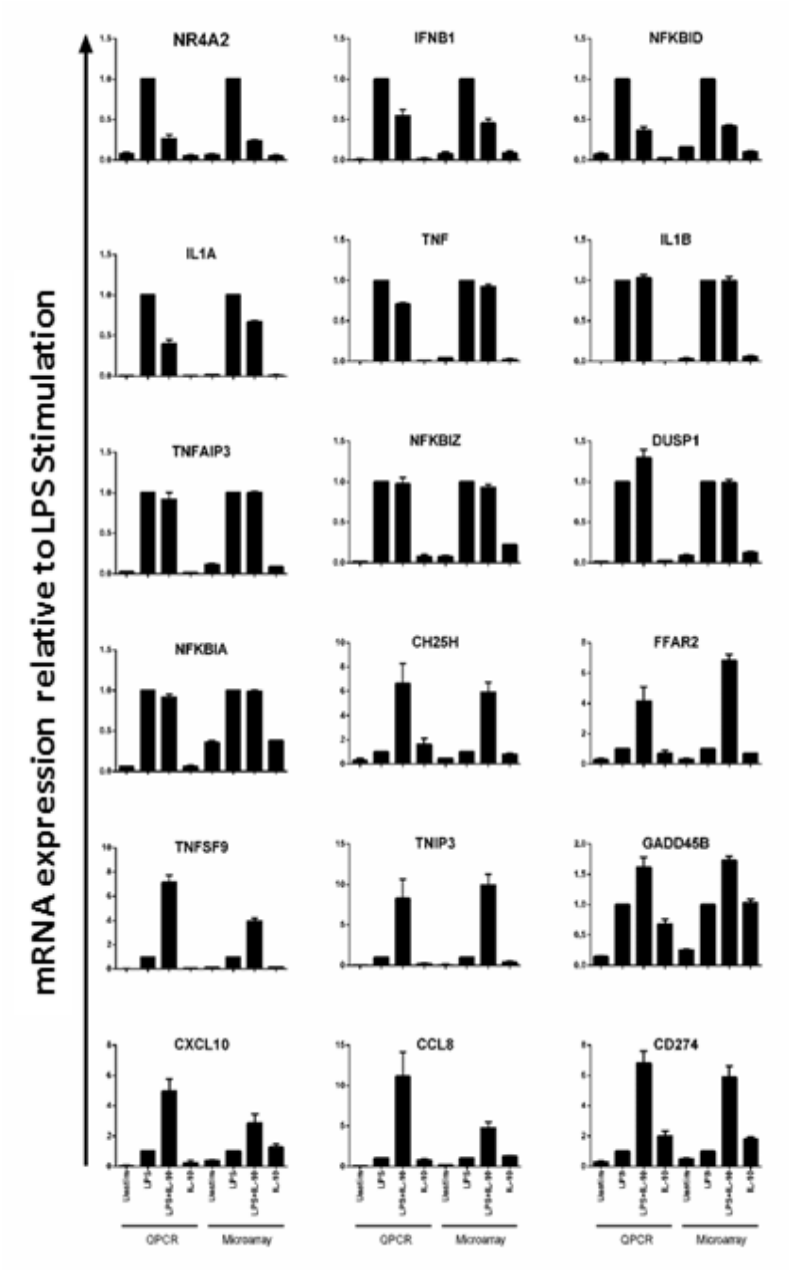

Figure 3.15 – Comparison of microarray and QPCR data

Primary human macrophages of 4 independent donors were stimulated with LPS, IL-10 or in combination for one hour and analysed for the expression of 18 LPS-induced genes and the housekeeping gene *GAPDH*. The expression of each gene was normalised with respect to *GAPDH* and the LPS-stimulated sample. This was then compared to the LPS normalised expression of samples analysed by microarray.

Gene Name	Pearson correlation between QPCR and microarray values
NR4A2	0.991
IFNB1	0.956
NFKBID	0.992
IL1A	0.958
TNF	0.983
IL1B	0.993
TNFAIP3	0.987
NFKBIZ	0.975
DUSP1	0.962
NFKBIA	0.996
CH25H	0.729
FFAR2	0.840
TNFSF9	0.972
TNIP3	0.914
GADD45B	0.910
CXCL10	0.876
CCL8	0.927
CD274	0.853

Table 3.1 – Pearson correlation statistics between the QPCR and microarray data

Primary human macrophages of 4 independent donors were stimulated with LPS, IL-10 or in combination for one hour and analysed for the expression of 18 LPS-induced genes and the housekeeping gene *GAPDH*. The expression of each gene was normalised with respect to *GAPDH* and the LPS-stimulated sample. This was then compared to the LPS normalised expression of samples analysed by microarray. Correlation between the two analyses was assessed by Pearson correlation.

3.2.3 – IL-10 inhibited genes display differential ontology to those cooperatively regulated by LPS/IL-10

Gene lists from the microarray clusters (IL-10 inhibited, insensitive and cooperatively regulated) were assessed for gene ontology enrichment using InnateDB. This web-based program used an over-representation analysis, comparing the enrichment of GO terms associated with genes in a list to the GO terms in a randomly selected background list of genes. The p-value produced from this enrichment analysis was then adjusted with a Benjamini and Hochberg correction to take into account multiple testing. The enriched GO terms were then filtered on adjusted p-values lower than 0.05 and lists between clusters were compared.

Of the terms significantly enriched in each of the one hour clusters (Figure 3.15) 4 terms appeared in multiple clusters. These terms include: “Cytokine-cytokine receptor signalling”, “TNF signalling” and “Toll-like receptor signalling” (full list see Appendix Table 8.1-8.8) All of these terms can be attributed to the up regulation of gene expression on LPS I initially filtered on. Interestingly, there were a number of enriched GO terms unique to each cluster (6, 28 and 2 for the IL-10 inhibited, insensitive and cooperative clusters respectively) indicating a divergence in function between the three groups. The GO terms enriched in the IL-10 inhibited cluster include “Epithelial signalling in H. pylori infection” and “transcriptional misregulation in cancer”. The IL-10 insensitive cluster includes other GO terms related to infection such as: “Leishmaniasis”, “Malaria” and “African trypanosomiasis”. However, there were other GO terms of interest in this including: “Adipocytokine signalling pathway” and “FoxO” signalling pathway” indicating that the genes

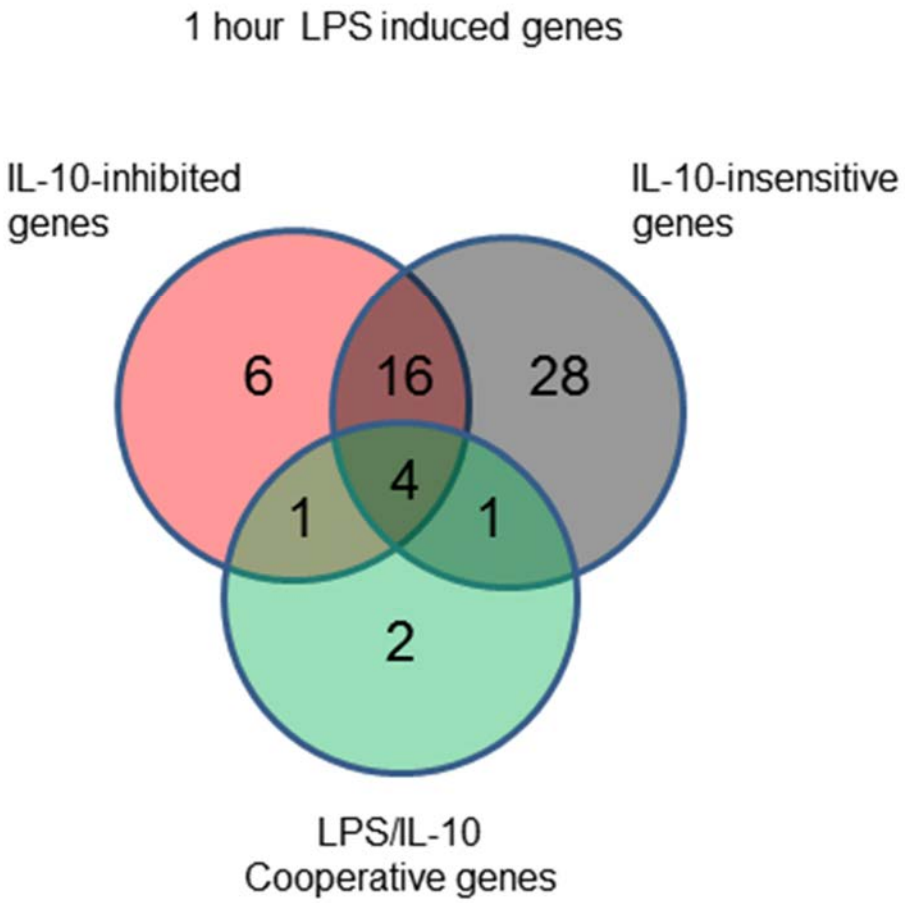

Figure 3.16 – Venn diagram of enriched gene ontology terms in the 1 hour clusters

ENSEMBL IDs of genes induced by LPS (>2 fold increase, ANOVA $p<0.05$) at 1 hour and separated into 3 groups by hierarchal clustering. Each cluster was then assessed for gene ontology (GO) term enrichment through over representation analysis (performed by InnateDB) and filtered for a multiple-comparison corrected p-value <0.05. Full tables of these GO terms are located in the appendix (Tables 8.1-4).

spared IL-10's inhibitory effect may alter the cell signalling pathways possible in a macrophage after exposure to LPS. Between the IL-10 inhibited and unaffected genes, there was considerable overlap in enriched GO terms, indicating that there may be some degree of functional o ontological overlap. The two terms uniquely enriched in the LPS/IL-10 cooperative cluster were: "Hepatitis C" and "Primary bile acid biosynthesis".

The four hour enriched GO terms (Figure 3.17) were more distinct in comparison, with only one GO term shared between the clusters ("Cytokine-cytokine receptor interaction") and a much lower enrichment of shared GO terms between two clusters. The inhibited IL-10 genes are enriched for "Notch signalling pathway" elements as well as those involved in "adherens junction" and "salmonella infection".

The IL-10 unaffected cluster has a notably higher number of enriched GO terms which is likely due to its much larger size compared to the other clusters. Similar to the one hour timepoint this includes ontology terms such as "Influenza A" and "Measles" indicating these genes were also upregulated in disease. However, there are also a number of GO terms which include cell signalling components including: "NF-κB signalling pathway", "Apoptosis" and, "Prolactin signalling pathway".

The LPS/IL-10 cooperative cluster has 3 significantly enriched GO terms: "PPAR signalling pathway", "PI3K-Akt signalling pathway" and, "Fatty acid biosynthesis". This indicates that cooperatively regulated genes may be inducing specific signalling pathway components or regulators and treated macrophages may have different responses to stimulus after being treated with LPS and IL-10.

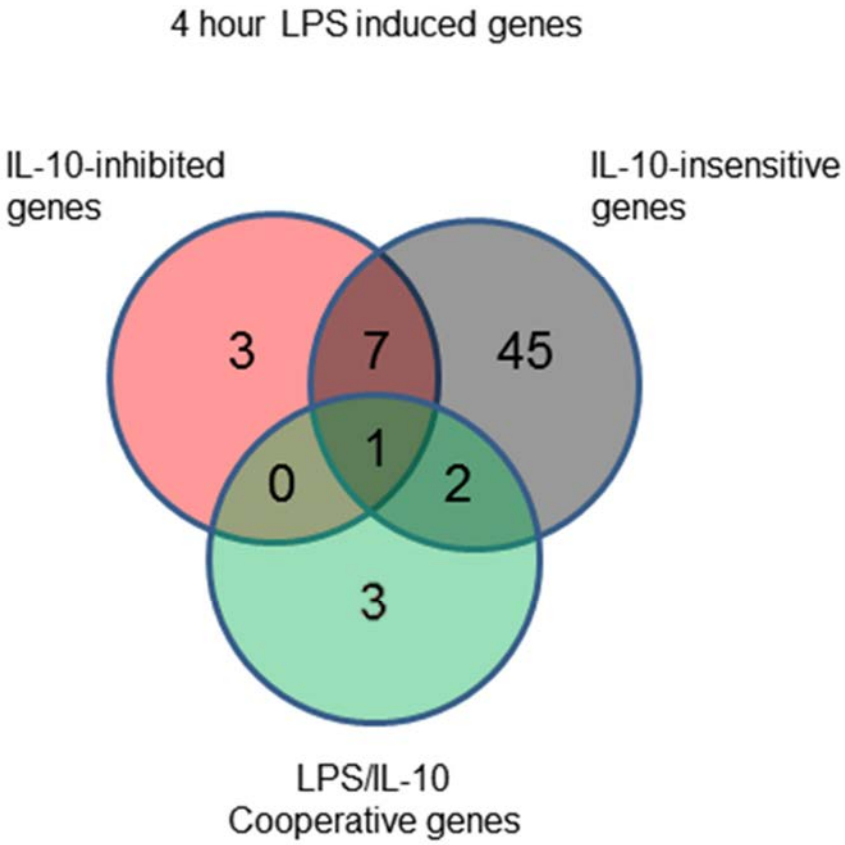

Figure 3.17 – Venn diagram of enriched gene ontology terms in the 4 hour clusters

ENSEMBL IDs of genes induced by LPS (>2 fold increase, ANOVA $p<0.05$) at 4 hour and separated into 3 groups by hierarchal clustering. Each cluster was then assessed for gene ontology (GO) term enrichment through over representation analysis (performed by InnateDB) and filtered for a multiple-comparison corrected p-value <0.05. Full tables of these GO terms are located in the appendix (Tables 8.5-8)

3.2.4 – IL-10 dependent modulation of gene expression occurs at the primary transcript level

After observing the effect of IL-10 on LPS-induced gene expression, I wanted to examine whether these patterns of gene expression represented changes in gene transcription or altered stability of mRNA transcripts. To accomplish this I used primary transcript (PT) QPCR to detect unspliced mRNA transcripts (Lipson and Baserga, 1989). PT-QPCR is used to quantify the abundance of the primary transcript of a gene. A primary transcript (PT) is the first product of gene transcription, which contains both the introns and exons of a gene and lacks 3' polyadenylation, seen in mature mRNA (see Figure 3.18)(Lodish H, 2000).

Due to the inclusion of introns in the PT, it can be distinguished from mature mRNA in QPCR, by using a primer that anneals within an intron or exon/intron boundary. However, this technique is susceptible to chromatin contamination which can also act as a template for the reaction. To reduce the chromatin contamination of the lysates, the RNA was DNase treated during isolation. In addition, control cDNA reactions were performed, in the absence of reverse transcriptase to assess the extent of chromatin contamination of the samples. These reactions did not have detectable or had extremely low levels of amplicon detection demonstrating the specificity of the PT reaction.

Macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120, 240 or 480 minutes. Cells were then lysed and RNA from the cells was extracted. The harvested RNA was treated with DNase to reduce chromatin contamination. The RNA was then

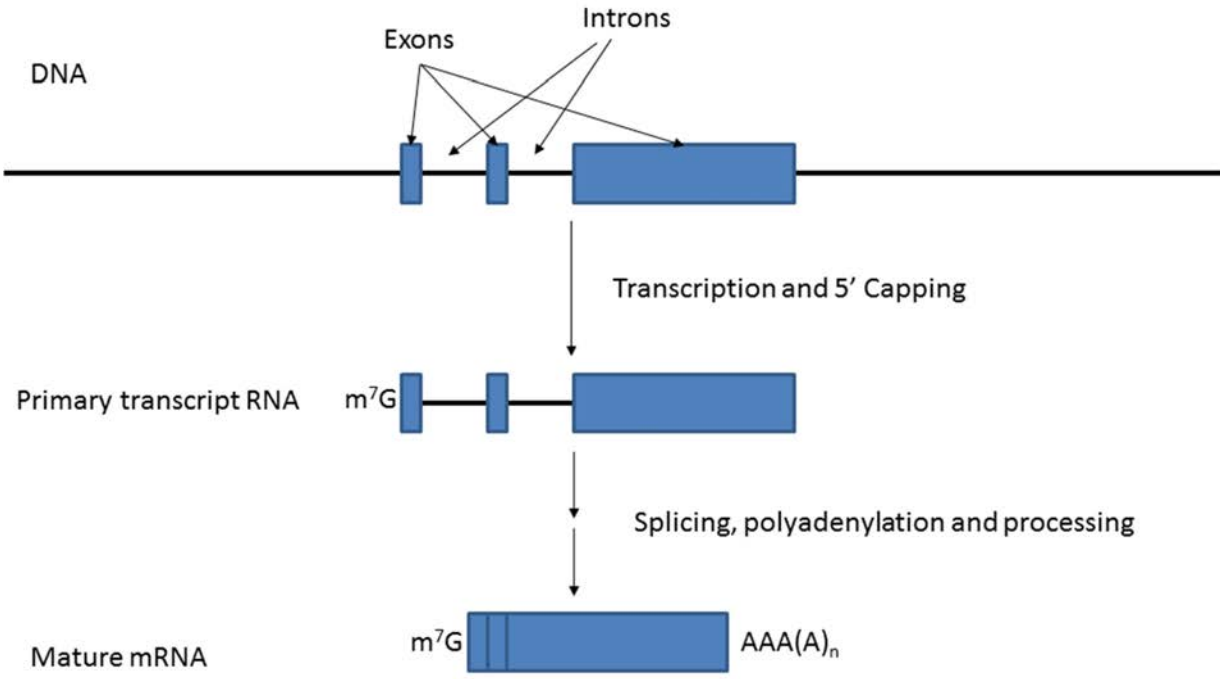

Figure 3.18 – mRNA production and maturation schema

Figure describing the production of mRNA. First RNA Pol II is recruited and released from the transcription start site and translocates from 5' to the 3' end of a gene. During this translocation it replicates the template strand of DNA into a reverse complementary RNA which is capped with a 7-methyl-Guanidine base, co-transcriptionally. After RNA Pol II reaches the end of the RNA is released (the primary transcript RNA). After release the RNA is then spliced, polyadenylated and processed into mature mRNA.

reverse transcribed and assessed for primary and mature transcript production by SYBR green QPCR.

Examining the expression of two LPS-induced genes inhibited by IL-10 (TNF and IL1A), there was a clear peak of LPS-induced transcription at 60 minutes at both the level of primary and mature mRNA transcript levels (Figure 3.19). For TNF mature mRNA this level of expression steadily drops from this point till 480 minutes where it has just under a quarter of its maximal expression. IL1A retains its high expression after 120 minutes and then begins to lose its expression level, which returns to almost unstimulated levels by 480 minutes. At the PT level TNF behaves much the same as at the mature transcript level, with only minor differences in the level of expression at each timepoint. With IL1A, there is a differential pattern of induction at the PT compared to the mature transcript level. The PT expression is induced on LPS stimulation which then peaks at 60 minutes. After this the level of expression rapidly falls to 20% of its expression by 120 minutes. The PT then remains at this level at 240 minutes before disappearing at 480 minutes.

In the case of IL1A PT, IL-10 directly inhibits primary transcript production at 30 and 60 minutes, before the LPS-induced transcription rapidly disappears at 120 minutes. In contrast to *TNF* this indicates that IL-10s inhibition of *IL1A* is entirely due to inhibition at the level of transcription. At the mature transcript level, both *TNF* and *IL1A* were inhibited by IL-10 at the 60, 120 and 240 minute timepoints. While there appeared to be an IL-10 mediated inhibition at 30 and 480 minutes, the decrease was not statistically significant.

After noting the effects of IL-10 on the primary transcript of inhibited genes, I then selected 3 genes (*NFKBIA*, *IL1B* and *TNFAIP3*) that were induced by LPS but not affected by IL-10 in the

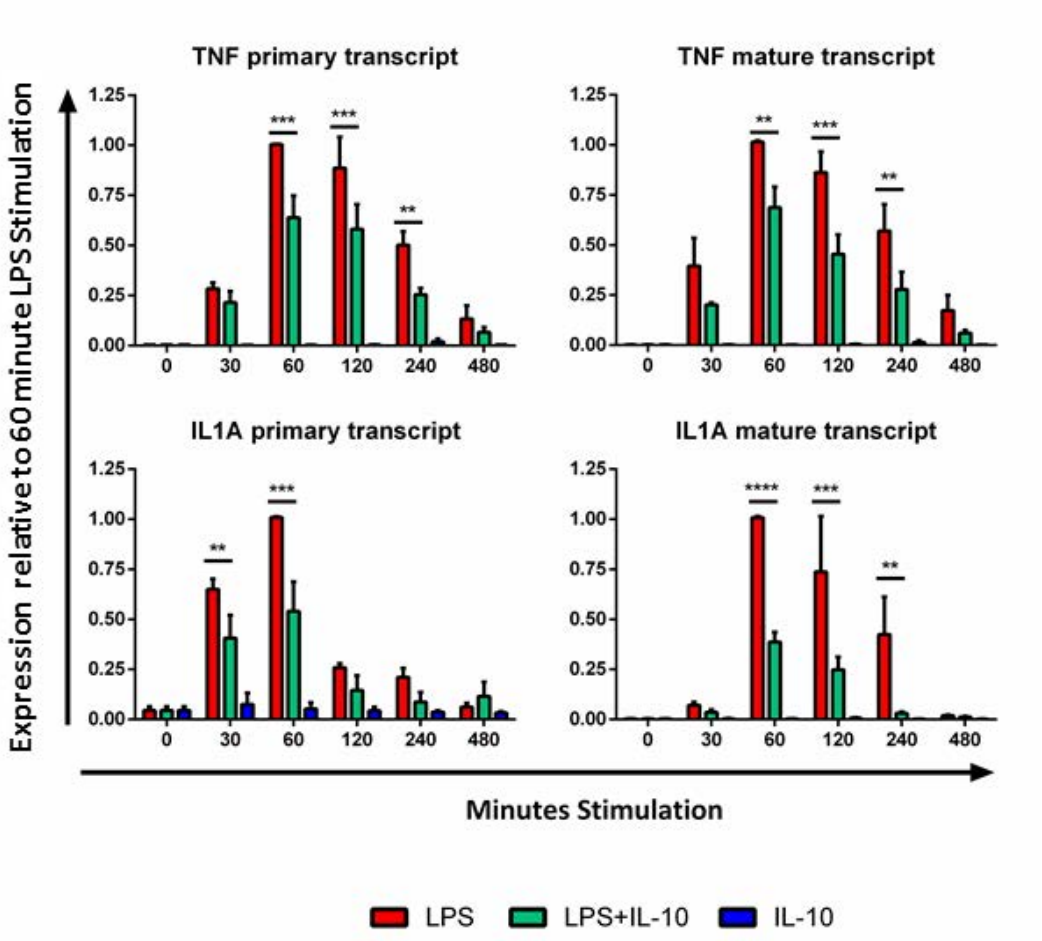

Figure 3.19 – Primary and mature transcript mRNA of IL-10 inhibited

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120, 240 or 480 minutes. The cells were then harvested for RNA and assessed for primary transcript and mature transcript mRNA levels of *TNF*, *IL1A* and *GAPDH* SYBR green QPCR and normalised to *GAPDH* between samples and the 60 minute LPS stimulation for each gene. Statistical comparisons between the LPS and LPS+IL-10 were conducted using a matched 2-way ANOVA, with a Bonferroni-correction for multiple comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Data represents 3 independent donors and error bars represent SEM.

specific effect. All three of the genes examined showed high levels of expression at all timepoints post LPS stimulation, with *NFKBIA* and *TNFAIP3* mature transcript levels peaking at 60 minutes and *IL1B* peaking at 120 minutes (Figure 3.20). On stimulation with LPS and IL-10 there no significant difference between the LPS stimulation at any timepoint for *NFKBIA* and *TNFAIP3* mature transcript, while *IL1B* demonstrated a statistically significant inhibition of gene expression at 240 minutes, which then returned to no significant difference by 480 minutes. At the PT level all three genes microarray, to examine whether the repression of transcription rate was a genome wide or gene were rapidly induced by LPS and expression levels peaked at 30 minutes. This level of LPS induction then slowly reduced to half the maximal expression (for *NFKBIA* and *TNFAIP3*) or little over a quarter (for *IL1B*). The addition of IL-10 did not affect the expression of *NFKBIA* significantly at any timepoint. *TNFAIP3* PT was inhibited by IL-10 at

240 mins although, this inhibition did not persist to the 480 minute timepoint. Interestingly, IL-10 seemed to act cooperatively with LPS at the *IL1B* PT, significantly inducing the expression at 60 minutes. This cooperation also appears at 30 minutes but is not statistically significant. Given the difference between the PT and MT of *IL1B* there is a hint that the *IL1B* transcripts are actually more unstable on IL-10 stimulation compared to LPS alone.

I then selected 3 genes that were cooperatively regulated by LPS and IL-10 at one hour: *TNFSF9*, *TNIP3* and *CD274* (Figure 3.21). *TNFSF9* MT displayed statistically significant LPS/IL-10 cooperation at 60 and 120 minutes. At the level of PT there was a significant cooperative effect at 30 and 60 minutes, which steadily decreased over the course of 8 hours. In the samples treated with LPS alone *TNFSF9* PT was induced in a biphasic manner, peaking at 30 and 120 minutes, with

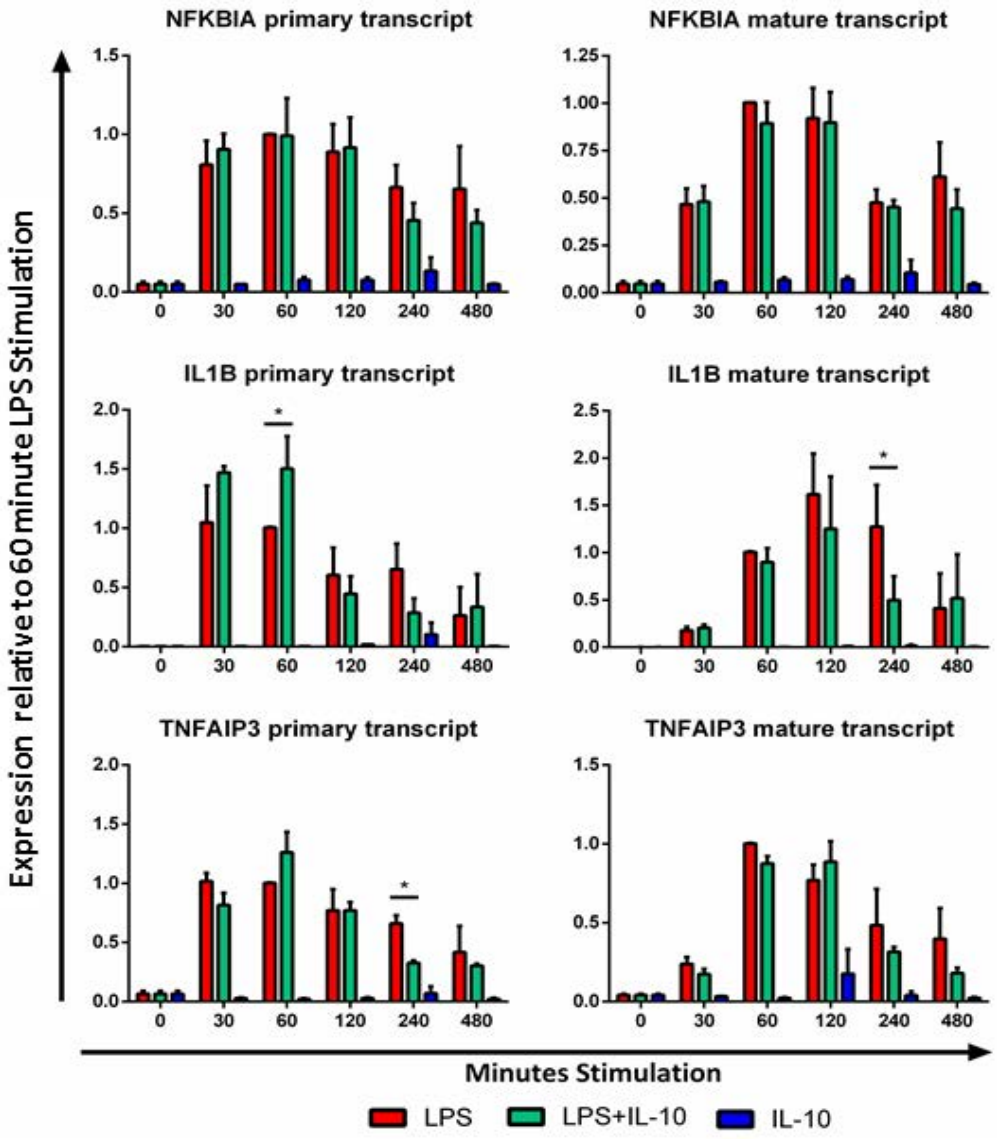

Figure 3.20 – Primary and mature transcript mRNA of IL-10 insensitive genes

10^6 primary human macrophages from 3 independent donors, were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120, 240 or 480 minutes. The cells were then harvested for RNA and assessed for primary transcript and mature transcript mRNA levels of *IL1B*, *TNFAIP3*, *NFKBIA* and *GAPDH* SYBR green QPCR and normalised to *GAPDH* between samples and the 60 minute LPS stimulation for each gene. Statistical comparisons between the LPS and LPS+IL-10 were conducted using a matched 2-way ANOVA, with a Bonferroni-correction for multiple comparisons. * $p<0.05$.

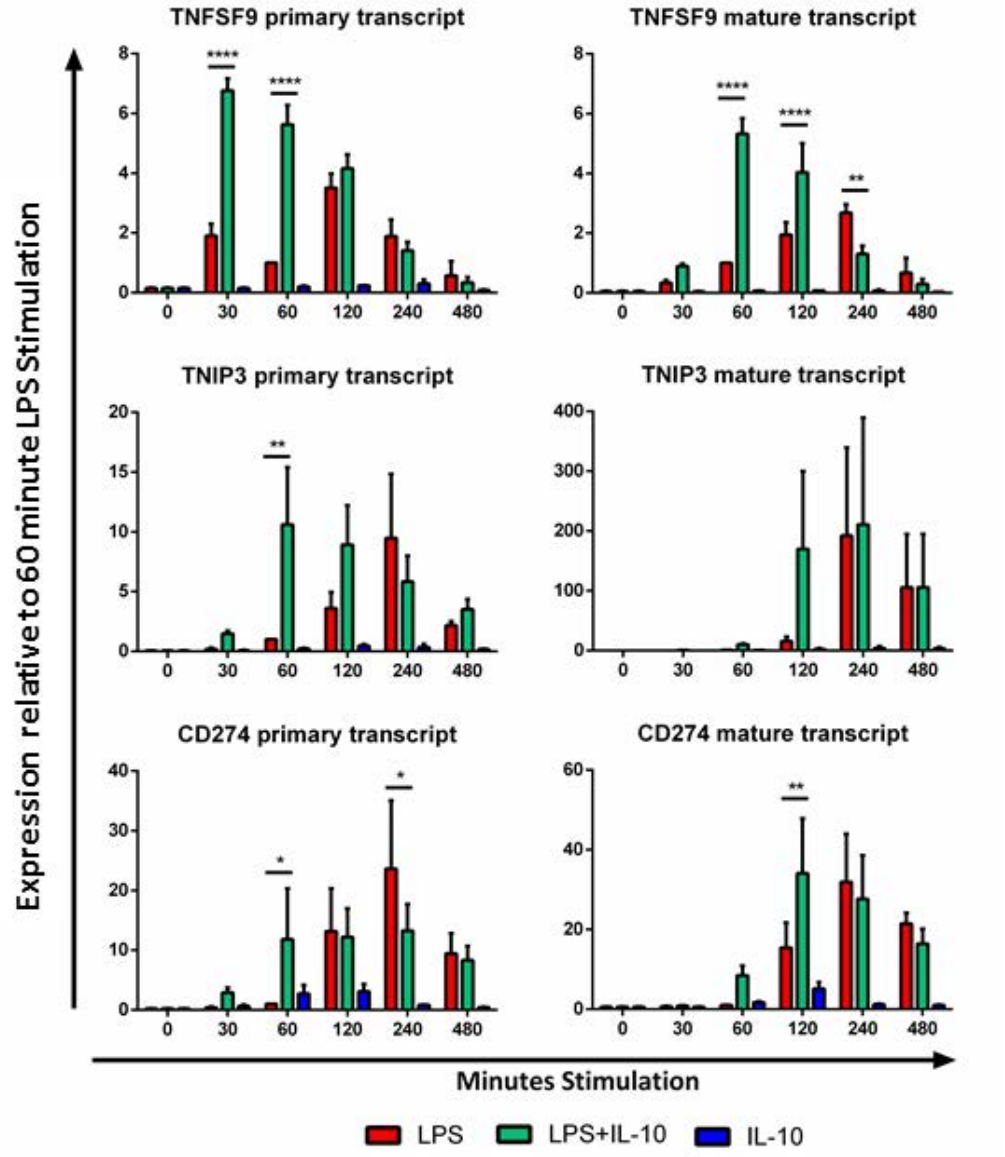

Figure 3.21 – Primary and mature transcript mRNA of LPS/IL-10 cooperatively regulated genes

10^6 primary human macrophages from 3 independent donors, were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120, 240 or 480 minutes. The cells were then harvested for RNA and assessed for primary transcript and mature transcript mRNA levels of *TNFSF9*, *TNIP3*, *CD274* and *GAPDH* SYBR green QPCR and normalised to *GAPDH* between samples and the 60 minute LPS-stimulated for each gene. GAPDH between samples and the 60 minute LPS stimulation for each gene. Statistical comparisons between the LPS and LPS+IL-10 were conducted using a matched 2-way ANOVA, with a

Bonferroni-correction for multiple comparisons. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Error bars represent SEM.

a reduction in expression at 60 minutes. With the MT LPS induction of the transcript slowly increased over four hours, before dropping to the same level as LPS+IL-10 by 8 hours.The levels of *TNIP3* and *CD274* induction were quite different between the donors analysed and therefore many timepoints lack statistical significance. However the PT of both *TNIP3* and *CD274* were significantly upregulated by LPS/IL-10 above LPS alone treatment at 60 minutes. In the case of *CD274* PT the induction by LPS alone significantly overtook the LPS/IL-10 treated samples at 240 minutes, before falling to the same level at 480 minutes.

The induction of PT and MT by LPS at both *TNIP3* and *CD274* peaked at 240 minutes, slowly increasing after stimulation, before dropping to levels equal to LPS+IL-10 at 480 minutes.

3.2.5 – IL-10's effect on LPS-induced gene expression can be observed at the protein level

In order to determine whether IL-10 mediated regulation of mRNA abundance was reproduced at the protein level, the following genes were analysed for protein production: *TNF, CCL8, CXCL10, TNFAIP3, TNFSF9, CD274, TNIP3*. Secreted levels of TNF, CCL8 and, CXCL10 protein were analysed by Luminex and ELISA, Intracellular TNFAIP3 and TNIP3 protein levels were assessed by western blotting. The cell surface expression of CD274 and TNFSF9 were assessed by flow cytometry.

CD274, TNFSF9, TNIP3, CCL8 and CXCL10 are genes cooperatively regulated by LPS/IL-10 at the early timepoint, though all 4 appear to change pattern of expression later on (microarray values for these genes are included in section 8.3). TNFAIP3 is at all timepoints insensitive to IL-10 and TNF is well known to be inhibited by IL-10. Biosynthesis of IL1α and β is subject to an additional

inflammasome-mediated stage, which operates under a complex regulatory system. I therefore elected not to investigate the production of these proteins (Howard et al., 1991, Groß et al., 2012, Rathinam et al., 2012). Similarly, I did not assess the production of NFKBIA, as it is heavily modified and degraded by LPS signalling and therefore would be difficult to assess its *de novo* production.

The protein TNFAIP3 is clearly induced by LPS and LPS+IL-10 by 60 minutes and peaked at 120 minutes, reducing to close to unstimulated levels by 8 hours (Figure 3.22). LPS+IL-10 does appear to subtly inhibit the expression at 8 hours compared to LPS alone. In the IL-10 alone samples, TNFAIP3 was not induced at any timepoint compared to unstimulated cells. The cooperation of LPS and IL-10 in the expression of TNIP3 is visible at the protein level at 120 minutes. By 240 minutes this cooperative induction is reduced and by 480 minutes the LPS and LPS+IL-10 stimulated samples contain roughly equal amounts of TNIP3 expression. IL-10 alone stimulated samples do not induce TNIP3 at any of the timepoints tested.

The secretion of the cytokines CXCL10 and TNF, all were significantly inhibited by IL-10 at 4 and 8 hours (Figure 3.23). TNF additionally displayed inhibition at 2 hours significantly. At the remaining timepoints, IL-10 appears to inhibit the cytokine production if LPS induces detectable cytokine production. These observations (while consistent) are not statistically significant.

Examining expression of the inhibitory cell surface receptor CD274 (Figure 3.24), there is a clear induction on LPS and LPS+IL-10 stimulation between 4 and 8 hours. At 8 hours there is a statistically significant increase in mean fluoresce intensity (MFI) (a representation of mean surface expression per cell). This increase is not statistically significant in the median fluorescence intensity, or the percentage of CD274+ cells.

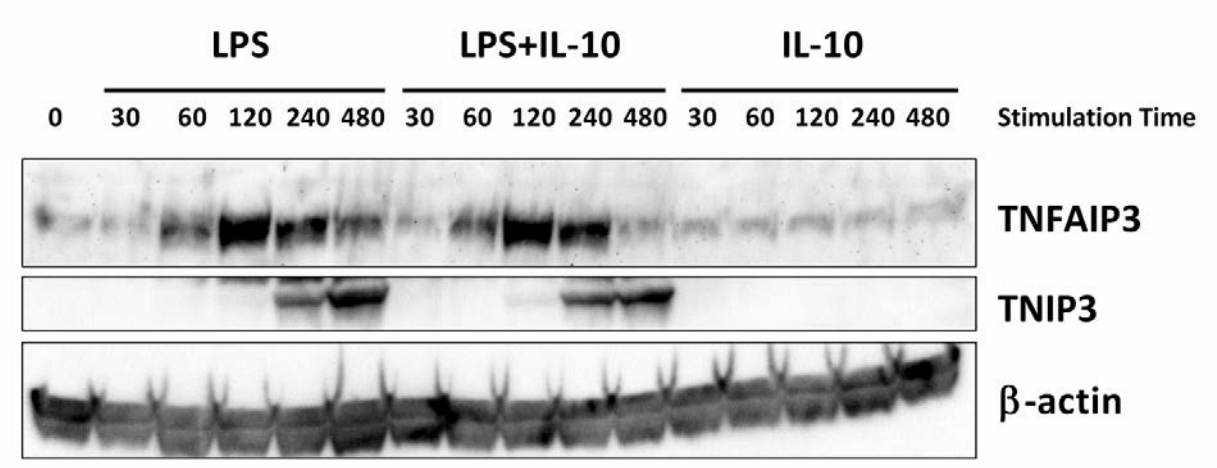

Figure 3.22 – Western blot of TNFAIP3 and β-actin after LPS/IL-10 stimulation

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120, 240 or 480 minutes. The cells were then lysed and assessed for TNFAIP3, TNIP3, β-actin proteins expression by western blot. Representative of two independent experiments

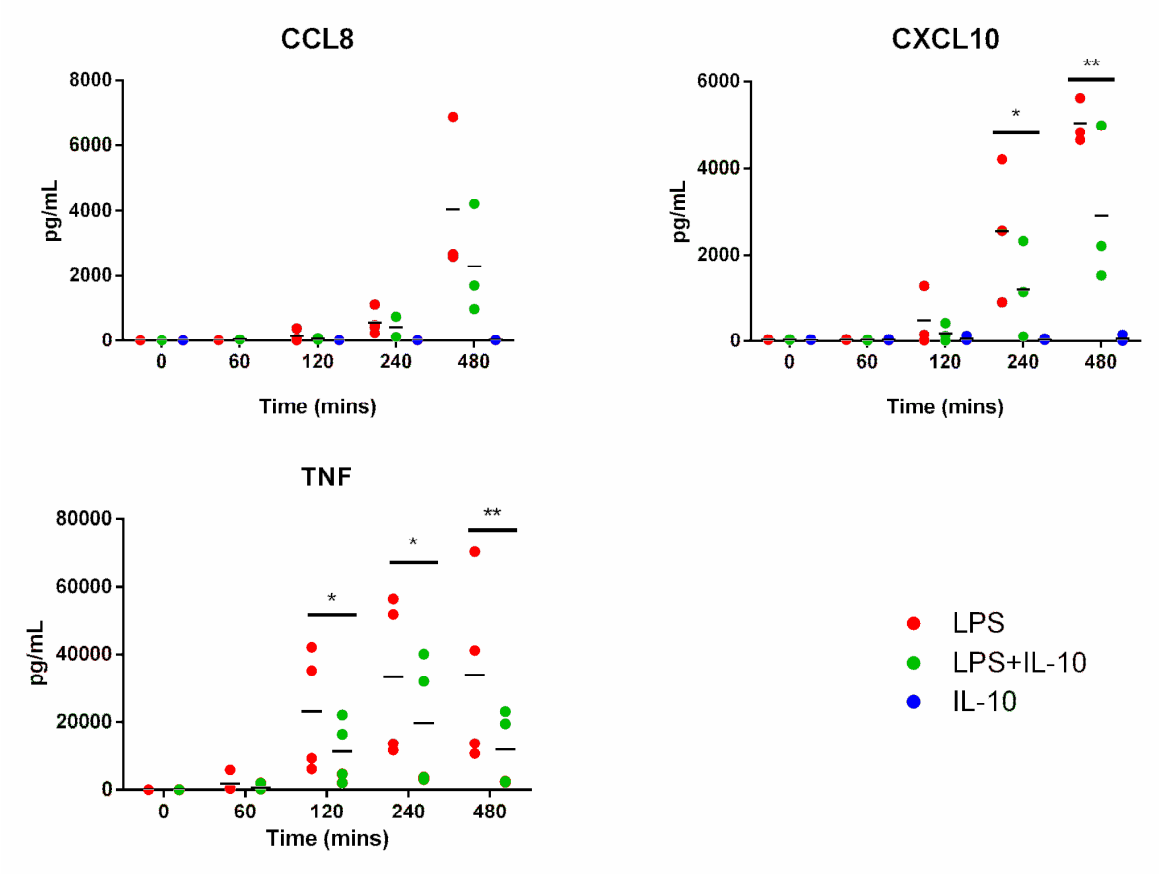

Figure 3.23 – Secretion of cytokines CCL8, CXCL10, TNFα after LPS/IL-10 stimulation

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for: 60, 120, 240 or 480 minutes. Cell culture supernatants were then analysed by multiplex cytokine assay (CCL8, CXCL10) and ELISA (TNF) for cytokine secretion. Statistical comparisons between the LPS and LPS+IL-10 were conducted using a matched 2 way ANOVA, with a Bonferroni-correction for multiple comparisons (* $p<0.05$, ** $p<0.01$).

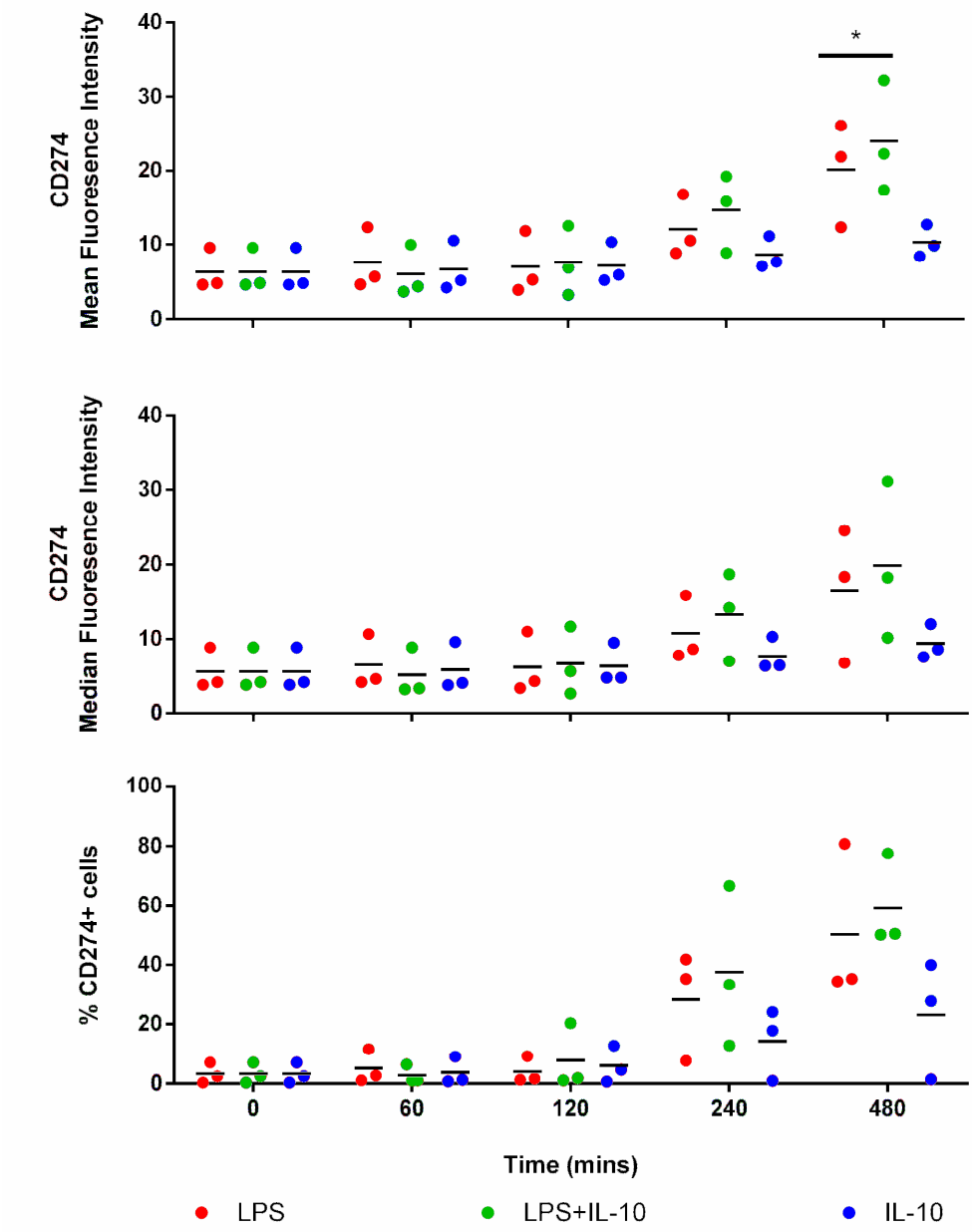

Figure 3.24 – Flow cytometry analysis of CD274 protein expression after LPS/IL-10 stimulation

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for: 60, 120, 240 or 480 minutes. Cells were then harvested and stained for CD274 or with an isotype control. Cells were then assessed for antibody binding for flow cytometry gating on single cell events. Statistical significance between the conditions was computed using a matched 2-way ANOVA with Bonferroni correction for multiple comparisons ($p<0.05$ = *).

TNFSF9 on the other hand, is highly expressed and presented on the surface on co-stimulated cells as early as 60 minutes (Figure 3.25). This expression peaks (with both MFI and percentage of TNFSF9+ cells) at 2 hours before falling back to almost basal expression at 8 hours. LPS alone treated cells only begin to show detectable TNFSF9 on the surface at four hours and by eight hours this expression has diminished. In both the case of CD274 and TNFSF9, IL-10 alone did not induce expression of these proteins. (Representative histogram plots are located in the appendix section 8.4).

Overall, the correlation of gene and protein expression was mixed. Several genes (TNF, TNFAFIP3, TNFSF9 and at the earlier timepoints, TNIP3) displayed a good correlation between gene expression and later protein expression. However other genes (CD274 and CXCL10) did not seem to behave similarly to their initial gene expression. Their expression at later timepoints (CD274 became unaffected by IL-10 and CXCL10 inhibited by IL-10) did seem to more closely resemble their expression pattern. It can also be seen that these proteins became detectable (by flow cytometry or Luminex) later than the proteins that do behave similarly to their mRNAs. For example, CXCl10 and CD274 do not significantly from unstimulated samples until 240 minutes post stimulation. While conversely, TNFSF9 and TNF both are detectable above unstimulated levels at 120 minutes or before similar to their mRNA. It can be inferred that the LPS/IL-10 cooperative proteins, will be the product of those genes which have high early expression, prior to autocrine signalling feeding back on gene expression.

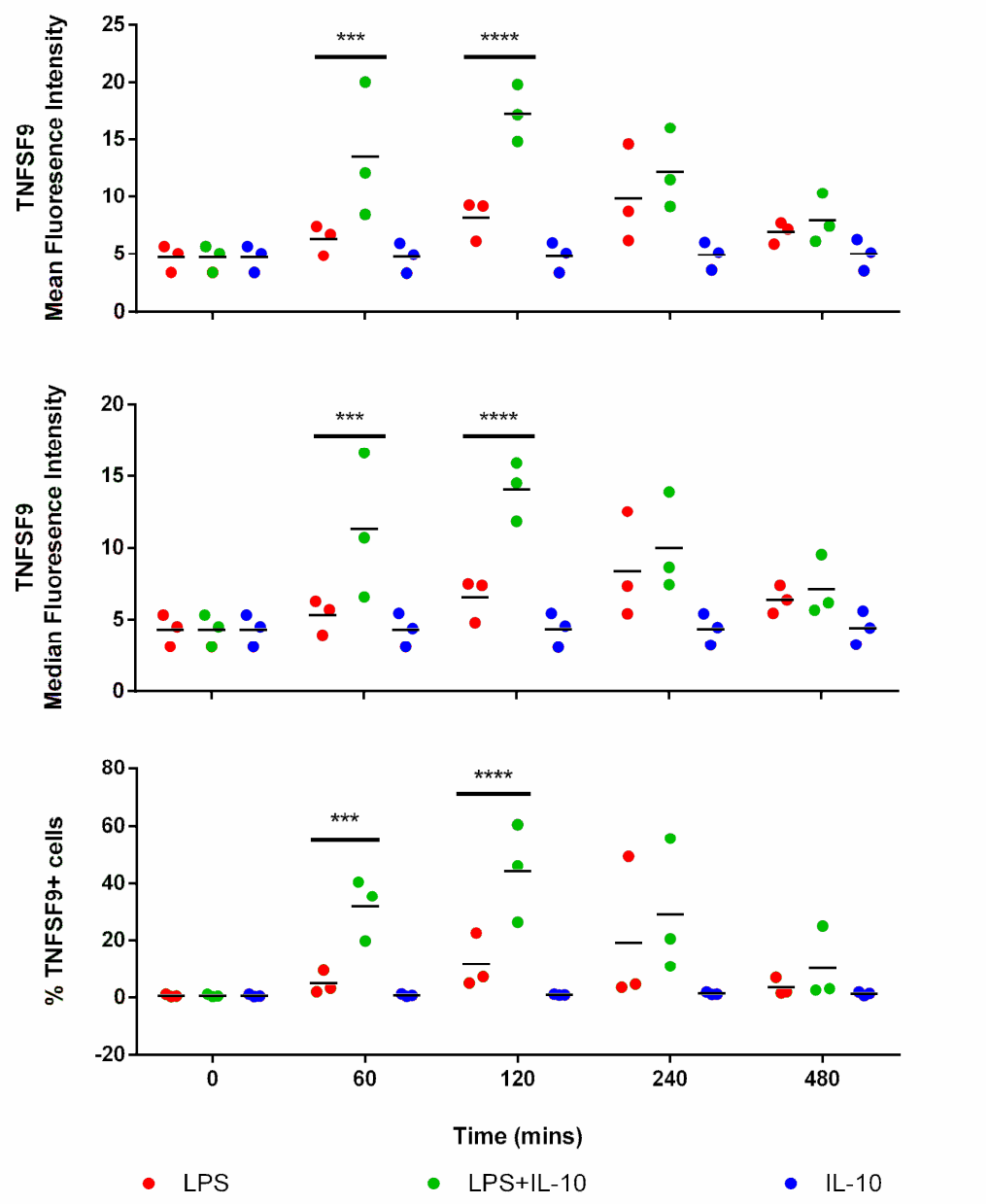

Figure 3.25 – Flow cytometry analysis of TNFSF9 expression after LPS/IL-10 stimulation

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for: 60, 120, 240 or 480 minutes. Cells were then harvested and stained for TNFSF9 or with an isotype control. Cells were then assessed for antibody binding for flow cytometry Gating on single cell events. Statistical significance between the conditions was computed using a matched 2-way ANOVA with Bonferroni correction for multiple comparisons ($p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = ***, $p<0.0001$ = ****)

3.2.6 – IL-10 is produced by LPS-stimulated macrophages

Since many of the genes expressed by macrophages stimulated with LPS and LPS+IL-10, had similar levels of expression at both the protein and mRNA levels after four hours, I examined the production IL-10 by these macrophages, to determine whether IL-10 feedback might be affecting gene expression.

It has previously been shown that IL-10 is itself induced by LPS stimulation in macrophages. Figure 3.26 shows that there was a significant induction of IL-10 by LPS at 4 and 8 hours, roughly equalling 0.5 ng/mL and 2 ng/mL respectively. There was also a small increase of IL-10 production after 120 minutes. However, this was not statistically significant above the baseline production of IL-10 (50 pg/mL).

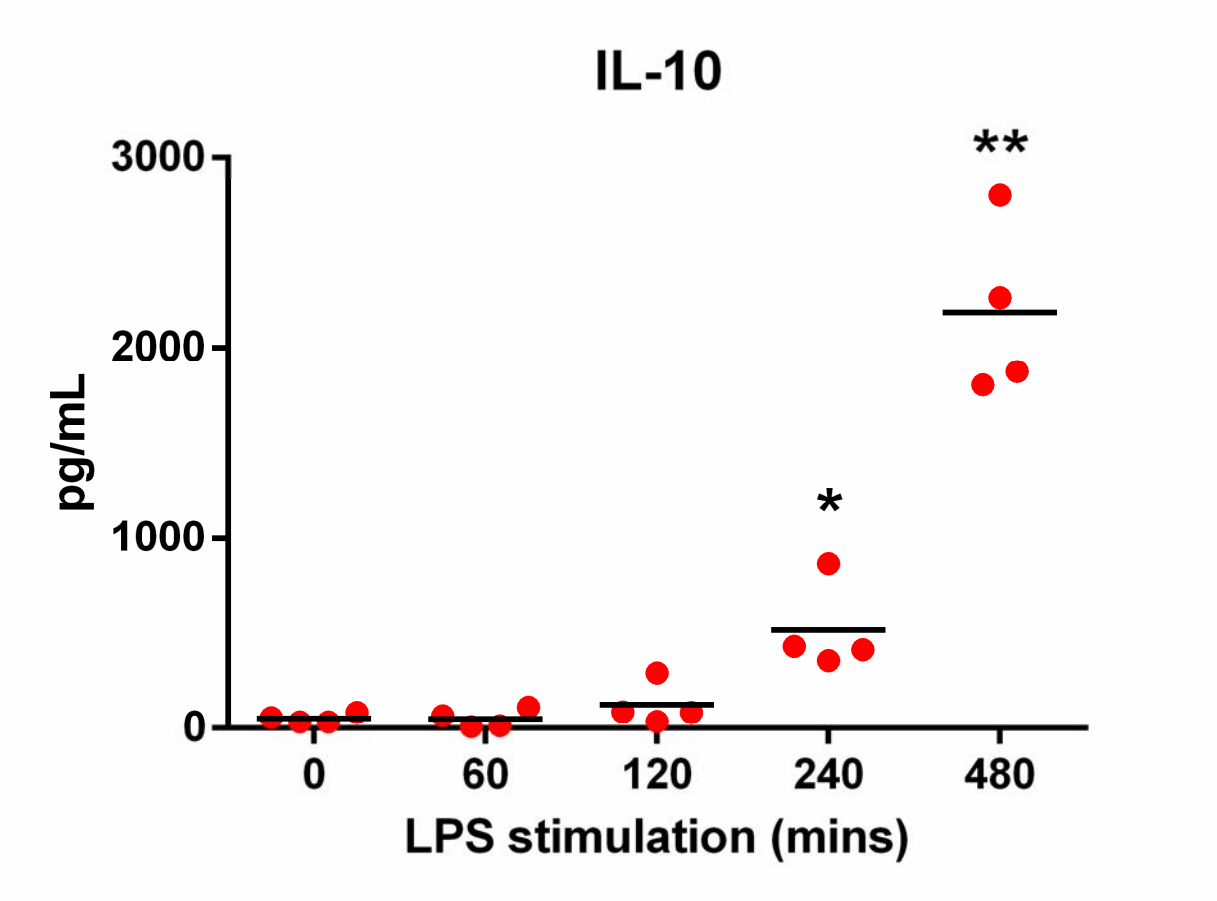

Figure 3.26 – Quantification of IL-10 secretion of LPS-stimulated macrophages

10^6 primary human macrophages were stimulated with 10 ng/mL LPS for: 60, 120, 240 or 480 minutes. Cell culture supernatants were then analysed by ELISA for cytokine secretion. Statistical comparisons between each LPS stimulation and unstimulated cells, was conducted with a Paired students t-test. * $p<0.05$, ** $p<0.01$.

3.3 – Discussion

In this chapter I described that the IL-10 modulation of LPS-induced gene expression was a gene specific effect which can occur at the level of primary transcript. While these gene expression patterns have previously been described in (Murray, 2005), (Smallie et al., 2010) and (Dillow et al., 2014) these results have demonstrated these patterns of gene expression on a transcriptome wide basis. Several of the genes whose expression was inhibited by IL-10 (such as *TNF*, *IL1A* and, *IFNB1*) have been previously described as targets of IL-10 (Fiorentino et al., 1991, Varano et al., 2000). However, *NR4A2* has not previously been described as a target of IL-10-mediated inhibition of gene expression, this is potentially due to its low expression after long LPS stimulations (Data not shown) which are generally selected for gene expression studies.

Results from Murray (2005) agree with the observation of IL-10 inhibiting gene expression at the level of primary transcript and sparing NFKBIA. DIllow et al. (2014) showed also demonstrated an LPS/IL-10 synergy of TNIP3 primary transcript mRNA in THP-1 cells stimulated with LPS/IL-10 for four hours and indicate that this synergy between stimuli required multiple cell signalling pathways including: JAK, PI3K and potentially, JNK. Exactly how these cell signalling pathways interact to produce this synergy is left undescribed; however, LPS and IL-10 are known to activate these signalling cascades (PI3K and JNK for LPS, JAK and PI3K for IL-10). They also note however that a number of cooperatively regulated genes require different signalling cascades, indicating that there are multiple mechanisms at work for a similar effect.

The majority of early induced LPS transcripts are inhibited by IL-10. However, there are a substantial number of transcripts that IL-10 cannot inhibit. GO analysis of each clusters indicates that these clusters represent distinct functional groups of genes, modulating macrophage phenotype to an anti-inflammatory/pro-homeostatic phenotype shown by lower production of inflammatory mediators such as TNFα and IL-6, while at the same time upregulating immune-modulatory molecules (such as TNFSF9) and inhibitors of intra-cellular signalling (TNIP3). The benefit IL-10 exerting these diverse effects as opposed to a global inhibitory effect on LPS-induced gene expression is not immediately apparent. However, considering the number of negative feedback genes and pro-resolution/wound-healing genes upregulated by LPS, a general inhibition of gene expression, could make the return to a homeostasis more difficult. Since the genes required to return to homeostasis (such as IKBα to inhibit NF-κB activation) and would also be inhibited and lead to a cessation of inflammation, without necessarily dealing with changes to the surrounding tissue that would have occurred.

Many genes described at one hour as displaying cooperative induction by LPS and IL-10 in the microarray, later demonstrated an IL-10 insensitivity or were inhibited by IL-10 at four hours. While this could indicate a false result with the one hour data, it is more likely that the four hour data represents a delayed induction of gene expression (illustrated in Figure 3.27). With this behaviour the LPS+IL-10 stimulated cells, reach the peak of gene expression early, which then begins to fall towards a steady state level. Meanwhile, the LPS-stimulated cells, remain at a low level of induction until the later timepoints, when autocrine signalling of cytokines induces a

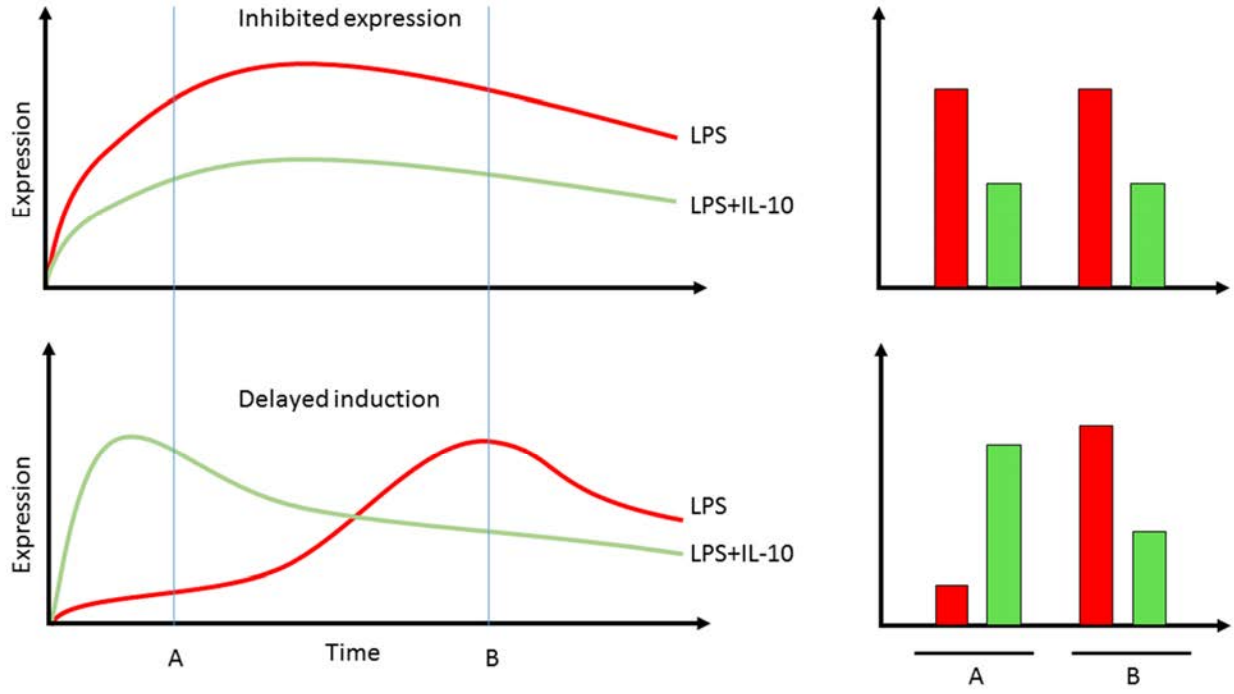

Figure 3.27 - Comparison of inhibited gene expression and delayed induction of expression

Illustration explaining the delayed induction behaviour exhibited by some 1 hour cooperatively regulated genes at later time points. Inhibited gene expression pattern is given for comparison.

similar response to the LPS+IL-10 stimulated samples. Examining two of these timepoints could give a misleading impression of the gene expression pattern.

The data in this chapter demonstrates the differential regulation of the genes *IL1A* and *IL1B* by IL-10. *IL1A/B* are two highly related inflammatory cytokines and (in mouse and humans) are located adjacent to one another on chromosome 2 (Taylor et al., 2002) and co-expressed by macrophages (Mayer-Barber and Yan, 2017, Fenton, 1992). Previous studies have shown the IL-10-mediated inhibition of *IL1A* PT expression (Murray, 2005). However, there is no study examining *IL1B* PT expression in response to IL-10 stimulation. The differential response of *IL1A/B* to IL-10 stimulation is interesting, given that both of these genes share a receptor for signalling exert a similar inflammatory function. There is some evidence to suggest that these two cytokines may differ in their ability to recruit different cell types and are released at different times during inflammation (Rider et al., 2011). In contrast to my observations other studies have shown *IL1B* to be inhibited by IL-10 in peripheral blood polymorphic mononuclear cells (Cassatella MA, 1993) and demonstrates increased expression in *Il10* knockout mouse macrophages (McNab et al., 2014). This could be in part due to the different experimental models: one using a mixed collection of myeloid cells the other using mouse macrophages). It would therefore be interesting to see if this finding could be replicated in other human macrophage models.

The inhibition of secreted CCL8 and CXCL10 by IL-10 is contrary to what was observed at the earlier microarray timepoint (Figure 8.1). However, the pattern of expression of these cytokines is consistent to what was observed at the level of mRNA expression at the four hour timepoint (both inhibited by IL-10). This cannot totally be explained as a delayed induction mechanism, since

IL-10's effect at the protein level is inhibitive and no cooperative regulation of cytokine expression was observed in response to LPS and IL-10. From the IL-10 ELISA data, a significant amount of IL-10 was secreted by macrophages by four hours of LPS stimulation. The amount of IL-10 is roughly equal to 1/20 of the stimulating concentration I have used in my stimulation. However, this is an average value from a supernatant and does not indicate the concentration of IL-10 in proximity to the cells, which will certainly be much higher. This highlights that the four hour dataset in the microarray is unlikely to be showing gene expression induced purely from the stimulation. It is instead likely to be showing the result of many induced cytokines (such as IL-10 (Pattison et al., 2012) and IFNβ1 (Chang et al., 2007)), signalling in an autocrine manner back onto the cells. In order to avoid this potential confounding factor, I selected to focus on the earlier timepoint to dissect the mechanisms of gene expression.

While the focus of my experiments has been examining the effect of IL-10 on LPS induced genes, there are a considerable number of genes whose expression is repressed by LPS stimulation (Figure 3.5B). This phenomenon has been observed in other datasets but has not extensively studied in the literature (Chen et al., 2012, Sharif et al., 2007). The effect of IL-10 stimulation on these genes has not been investigated previously and could prove insightful to the mechanism IL-10 employs to alter gene expression.

Chapter 4 – The contribution of STAT3 and NF-κB to the regulation of gene expression by IL-10

4.1 – Introduction

The mechanism by which IL-10 is able to inhibit gene expression has been a subject of contention for many years, with multiple mechanisms being proposed. The major consensus of the field is that it requires the activity of the protein STAT3 (Williams et al., 2007, Williams et al., 2004a, Niemand et al., 2003).

There have been many studies which have implicated inhibition of NF-κB transcriptional activity, as a mechanism by which IL-10 inhibits inflammatory gene expression (Smallie et al., 2010, Driessler et al., 2004, Schottelius et al., 1999, Wang et al., 1995, Hutchins et al., 2015). Therefore, I hypothesised that a general repression of NF-κB activity would produce a similar specific inhibition of gene expression, as IL-10 stimulation. Depletion of STAT3 was predicted to impair both positive and negative effects of IL-10 on LPS-induced gene expression.

To inhibit NF-κB activity, the inhibitor MLN-4924 was selected. This small molecule inhibitor targets the NEDD8 activating enzyme (NAE) complex, preventing the downstream NEDD8 conjugation (Neddylation) to Cullin-1 (Cul-1). Neddylation of the Skp1: βTrCP: Cul-1 complex is required in order for the ubiquitination of phosphorylated IκBα which is generated in response to pro-inflammatory stimuli such as TLR agonists. In the absence of ubiquitination, IκBα is unable to be targeted by the proteasome and degraded. This leads to NF-κB# subunits being retained within the cytoplasm on TLR stimulation and, ablating NF-κB transcriptional activity (Read et al., 2000, Milhollen et al., 2010, Chang et al., 2012). The inhibition of Neddylation is known to affect cell signalling pathways other than NF-κB. By inhibiting the targeting of Cullin ring ligase to substrates, a number of cell processes and signalling pathways are affected such as the JNK (Zhao et al.,

2011), ERK (Jin et al., 2013)and IRF signalling pathways (Song H, 2016). Therefore, treatment with MLN4924 may not specifically inhibit NF-κB activity.

4.2 – Results

4.2.1 – STAT3 motifs are enriched proximal to transcription start sites of genes cooperatively regulated by LPS and IL-10.

The previous chapter reported that IL-10 modulates the expression of many LPS-induced genes, and that this differential modulation is visible at the level of mRNA primary transcript production. This indicates that IL-10 is able to modify gene expression at the transcriptional level. I therefore hypothesized that these gene expression profiles could be explained by motifs proximal to the transcription start site (TSS), which are likely to have low nucleosome occupancy and therefore allow for transcription factor binding. To detect transcription factor motif enrichment in the different clusters, I extracted the gene lists from each of the clusters and used the software Hypergeometric Optimisation of Motif EnRichment (HOMER) to detect the motifs present in the region -3000bp to +50bp around the TSS compared to randomly selected gene promoters as a background control.

In all of the clusters examined (Table 4.1 – Table 4.3) there was an enrichment of NF-κB p65 consensus binding sites above background. This was expected since NF-κB rapidly induces gene expression on LPS stimulation and the initial gene list was restricted to genes induced by LPS. Both the IL-10 inhibited and IL-10 insensitive clusters also had an enrichment for TATA-box binding. Interestingly, there was an enrichment of the STAT4 motif “TTCCNGGAA” in the LPS/IL-10

Name	Motif	P-value	Benjamini Q-value	% of sequences with motif	
				Target sequences	Background sequences
TATA-Box		0.001	0.038	36.84%	16.07%
NFkB-p65		0.001	0.038	8.77%	0.89%
NFkB-p65		0.01	0.125	21.05%	7.70%
Mef2b		0.01	0.191	21.05%	8.38%

Table 4.1 – Enriched transcription factor motifs proximal to transcription start sites of IL-10 inhibited genes

Areas surrounding the transcription start sites (-300bp to +50bp) of genes inhibited by IL-10 were analysed for transcription factor binding site enrichment using HOMER. The results were then filtered to factors with a p value of less than or equal to 0.01.

Name	Motif	P-value	Benjamini Q-value	% of sequences with motif	
				Target sequences	Background sequences
NFkB-p65		0.00001	0.001	26.56%	7.39%
HOXD13		0.001	0.043	28.12%	11.62%
HOXB13		0.001	0.043	28.12%	11.77%
TATA-Box		0.001	0.070	34.38%	17.49%
SpiB		0.001	0.070	15.62%	4.81%
Atf7		0.010	0.070	23.44%	9.70%
CArG		0.010	0.130	12.50%	3.79%
C/EBP:AP-1		0.010	0.195	18.75%	8.09%
Atf1		0.010	0.225	25.00%	12.89%
Meis1		0.010	0.225	32.81%	19.18%
ISRE		0.010	0.225	6.25%	1.20%
RUNX		0.010	0.234	17.19%	7.64%
IRF2		0.010	0.236	7.81%	2.02%

Table 4.2 – Enriched transcription factor motifs proximal to transcription start sites of IL-10 unaffected genes

Areas surrounding the transcription start sites (-300bp to +50bp) of genes unaffected by IL-10 were analysed for transcription factor binding site enrichment using HOMER. The results were then filtered to factors with a p value of less than or equal to 0.01.

Name	Motif	P-value	Benjamini Q-value	% of sequences with motif	
				Target sequences	Background sequences
NFkB-p65		0.001	0.082	35.00%	6.89%
Oct4		0.001	0.086	30.00%	5.47%
STAT4		0.001	0.086	45.00%	13.31%
ISRE		0.01	0.187	15.00%	1.34%
Foxo1		0.01	0.187	50.00%	23.00%

Table 4.3 – Enriched transcription factor motifs proximal to transcription start sites of IL-10 cooperatively regulated genes.

Areas surrounding the transcription start sites (-300bp to +50bp) of genes cooperatively regulated by IL-10 and LPS were analysed for transcription factor binding site enrichment using HOMER. The results were then filtered to factors with a p value of less than or equal to 0.01.

cooperatively regulated gene cluster (Table 4.3). This binding motif is nearly identical to the STAT3 binding motif (Table 1.1) This would allow the binding of STAT3 to the promoter to induce gene expression.

Table 4.2 shows that a large number of transcription factor binding motifs were enriched at the TSS of the IL-10 unaffected genes. These include another known LPS activated transcription factor, AP-1. The variety of transcription factors in these promoters may indicate that this cluster is heterogeneously regulated.

4.2.2 – Optimising siRNA knockdown of STAT3 in primary human macrophages

Since STAT binding motifs were enriched at the promoters of LPS/IL-10 cooperative genes, I hypothesised that depleting STAT3 would inhibit cooperative gene regulation by LPS and IL-10. To deplete STAT3 in cells, I transfected siRNA oligonucleotides complimentary to the mRNA sequence of STAT3. To avoid potential off target or secondary effects, while allowing an efficient knockdown of STAT3, I titrated the oligonucleotide concentration transfected into the cells. $2x10^6$ primary human macrophages were transfected with 100, 200 or 400nM siRNA specific to STAT3 (siSTAT3) or scrambled control (siControl) on day 3 of differentiation. On day 5 the cells were harvested for protein and these lysates were assessed for STAT3 knockdown by Western blotting.

Figure 4.1 demonstrates that even at 100 nM concentrations, siSTAT3 almost totally depleted the STAT3 from the target cells, while there was little effect on the level of the NF-κB subunit p65. This indicated that depleting STAT3 with siRNA, would effectively deplete STAT3 without affecting the expression of NF-κB and avoid modulating the LPS response.

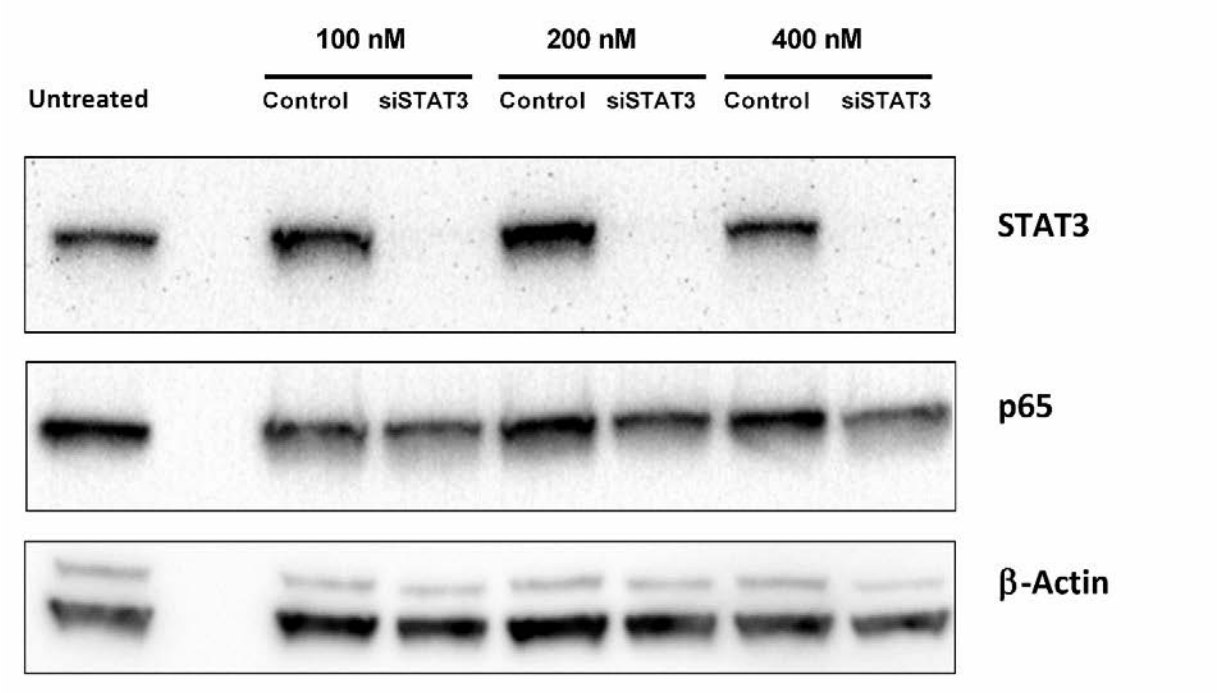

Figure 4.1 – Optimisation of siRNA mediated knockdown of STAT3 protein.

Primary human macrophages were transfected with varying concentration (100, 200 or 400 nM) of oligonucleotides (either specific to the STAT3 mRNA sequence or a scrambled control) 3 days into the differentiation protocol and allowed to recover over a further 2 day period. 20 x 10^6 cells from each point were harvested for western blotting. Representative blot of 2 independent experiment.

4.2.3 – Depletion of STAT3 prevents IL-10 mediated inhibition and cooperative regulation of LPS-induced genes

To reduce off target effects of the siRNA I opted to use the lowest concentration (10 nM) of oligonucleotides for transfection, while retaining a high level of knockdown at the protein level.

After confirming the efficacy of the siRNA depletion, I examined how IL-10 signalling affects LPS-induced gene expression in cells deficient for STAT3. As Figure 4.2 shows, the knockdown of STAT3 greatly changed the patterns of gene expression, in response to IL-10. Both cooperative and inhibitive effect of IL-10 on gene expression were impaired or lost in cells depleted of STAT3 (shown in detail Figure 4.3). This indicates that the effects of IL-10 on LPS-induced gene expression are strongly dependent on STAT3. In contrast the genes that are insensitive to IL-10's effects (*IL1B*, , *NFKBIZ*, *DUSP1*, *NFKBIA*) were unaffected by the depletion of STAT3.

On stimulation with LPS many of the IL-10 inhibited and insensitive genes, exhibited a statistically significant increase in expression, when depleted of STAT3 compared to siControl (Figure 4.3). This highlights that even at this early timepoint there may be IL-10 autocrine feedback in the LPS-stimulated samples. However, in the LPS/IL-10 cooperatively regulated genes, there was no alteration in expression in LPS-stimulated cells, between the siRNA treatments. If IL-10 feedback was occurring significantly, there would have been a downregulation of the LPS response when macrophages were depleted of STAT3 and IL-10 insensitive genes would display no significant

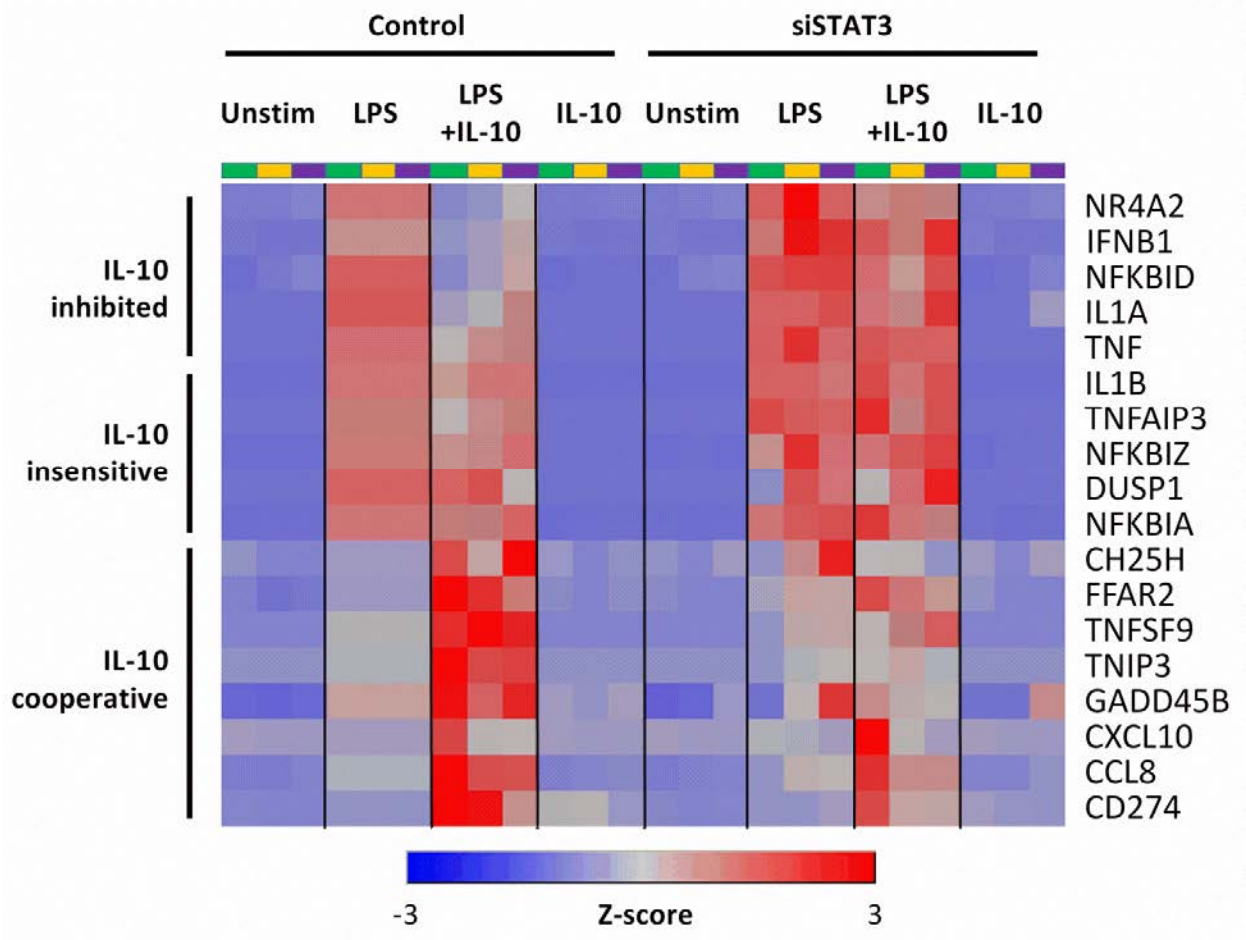

Figure 4.2 – Expression of key LPS/IL-10 responsive genes in primary human macrophages depleted of STAT3.

Primary human macrophages of 3 independent donors were transfected with 10 nM control oligonucleotide or siRNA targeting STAT3, on day 3 of differentiation and left to recover for a further 2 days. On day 5 the cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour. Cells were then harvested for RNA and the expression of genes listed were assessed by SYBR green QPCR and normalised to *GAPDH* for each sample and to LPS treated siControl.

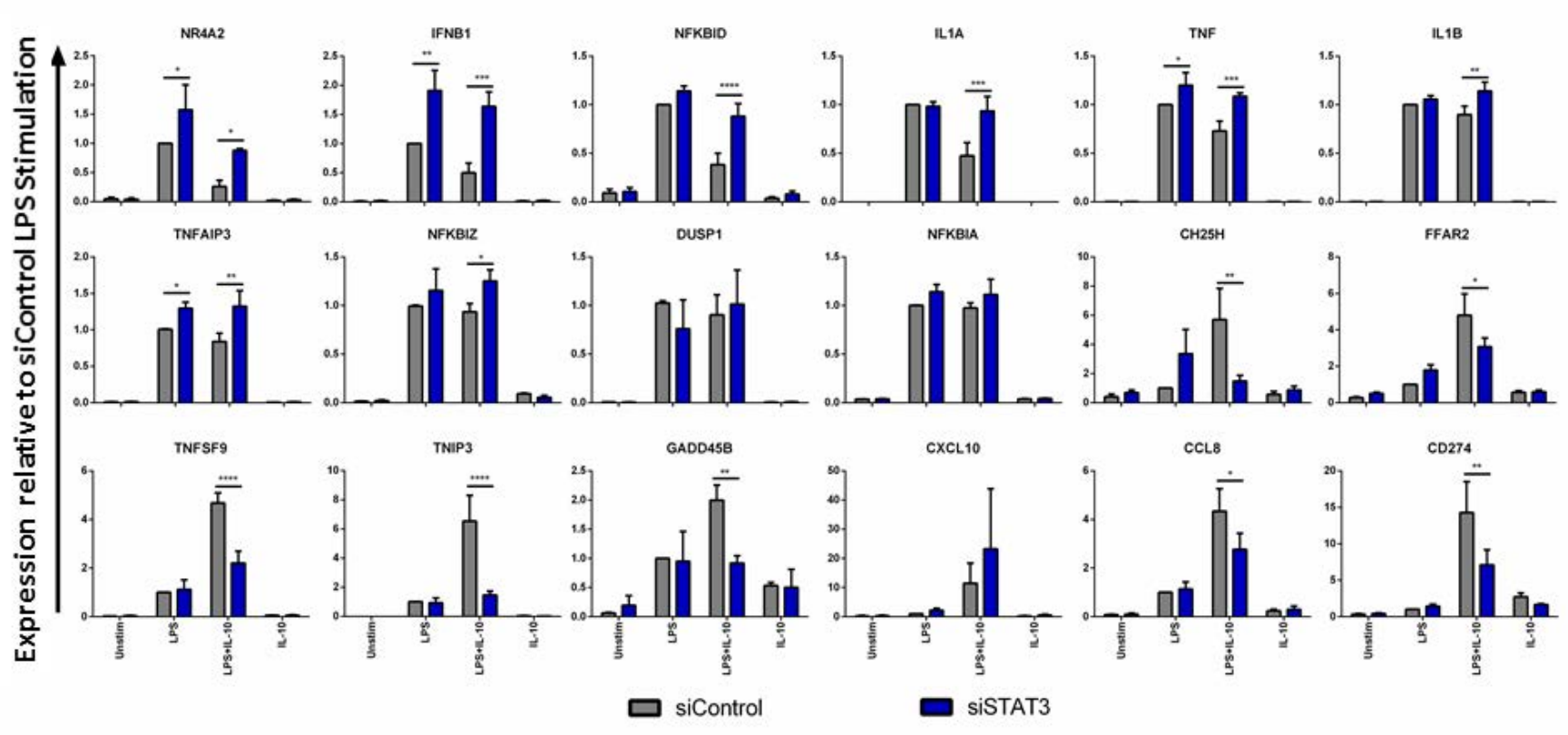

Figure 4.3 – Gene expression in siRNA treated macrophages

Primary human macrophages of 3 independent donors were transfected with 100 nM control 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour. Cells were harvested for RNA and the expression of the genes; *NR4A2*, *IFNB1, NFKBID, IL1A, TNF, IL1B, TNFAIP3, NFKBIZ, DUSP1, NFKBIA, CH25H, FFAR2, TNFSF9, TNIP3, GADD45B, CXCL10, CCL8, CD274* were assessed by SYBR green QPCR. Normalised to *GAPDH* for each sample and to LPS treated siControl sample. Statistical significance was calculated with Students t-test (*= p<0.05, **=p<0.01, ***=p<0.001, ****= p<0.0001).

upregulation. However, this is not the case, as none of the LPS/IL-10 cooperative genes show a statistically significant difference between siRNA treatments after LPS stimulation and of the IL-10 insensitive genes only *TNFAIP3* is significantly upregulated on STAT3 depletion.

Interestingly, the cooperative induction of *CXCL10* mRNA by LPS and IL-10 stimulation remains in macrophages depleted of STAT3. On closer examination of the expression data (Figure 4.4) there is a large variation in induced gene expression between the donors. Between these 3 donors; two display a lesser LPS/IL-10 cooperativity with reduced STAT3, while one donor has an increased LPS/IL-10 cooperativity.

4.2.4 – IL-10 inhibits NF-κB activity as detected by luciferase reporter constructs

Since inhibition of the NF-κB transcription factor activity has been implied by multiple studies to be the main mechanism of IL-10's suppressive activities, I hypothesized that the differential effects of IL-10 on LPS-induced gene expression could be explained by the differential requirement of NF-κB. If this hypothesis was correct, then inhibiting the NF-κB pathway would be able to replicate IL-10's inhibitory activity on gene expression while simultaneously sparing genes that were IL-10 insensitive or demonstrated LPS/IL-10 cooperativity.

To first assess whether IL-10 is able to inhibit NF-κB activity, I transduced an adenoviral luciferase reporter containing 4 artificial NF-κB consensus motifs ("GGGAATTTCC") upstream of the luciferase coding sequence into primary human macrophages. On activation of the NF-κB

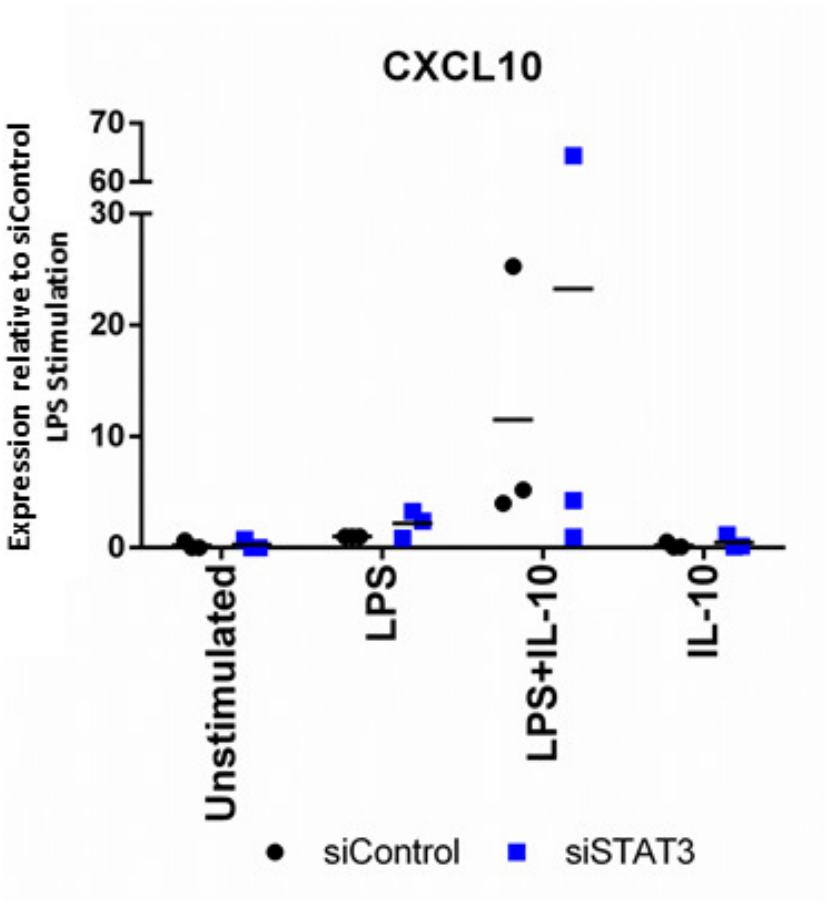

Figure 4.4 – Expression of CXCL10 in cells depleted of STAT3

Primary human macrophages of 3 independent donors were transfected with 10 nM control 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour. Cells were harvested for RNA and the expression of *CXCL10* were assessed by SYBR green QPCR. Normalised to GAPDH for each sample and to LPS treated siControl sample.

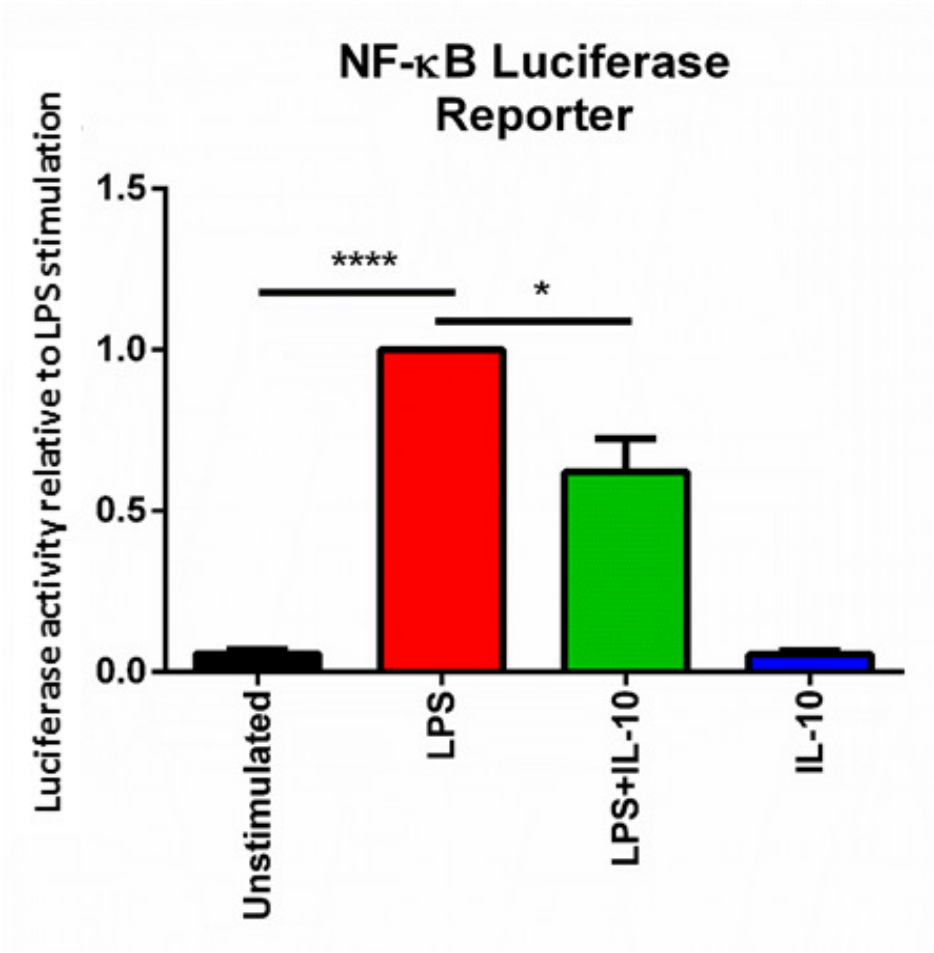

Figure 4.5 – Activity of an NF-κB luciferase reporter construct in response to IL-10

10^5 primary human macrophages were transduced with an NF-κB luciferase reporter encoding adenovirus on day 5 of differentiation and rested overnight. The following day the cells were stimulated with LPS or IL-10 alone or in combination for 120 minutes. Cells were lysed and assessed for luciferase activity. Graph represents 3 independent donors. Statistical significance was determined by unpaired students t-test (*= $p<0.05$, **** = $p<0.0001$).

pathway, the transcription factor is able to be recruited to the viral plasmid and induce the transcription of luciferase. This production of protein can then be assessed by the production of luminescence in the cell lysates, caused by the breakdown of luciferin by luciferase.

On stimulation with LPS (Figure 4.5) the reporter showed a significant ($p<0.0001$) 20 fold induction in luciferase activity, indicating a strong activation of the NF-κB pathway. Stimulation with IL-10 alone did not significantly alter the luciferase activity above unstimulated levels ($p = 0.92$). IL-10 significantly ($p= 0.02$) impaired the activation of the NF-κB reporter by LPS.

4.2.5 – Neddylation inhibitor MLN-4924 can inhibit LPS-induced gene expression on co-treatment, through inhibiting NF-κB translocation to the nucleus

After establishing that IL-10 is able to inhibit NF-κB activity, I wanted to examine whether the general inhibition of NF-κB using a chemical inhibitor could replicate the effects of IL-10 on LPS-induced gene expression. For this purpose, I used the compound MLN-4924, which was previously shown to potently inhibit NF-κB (Chang et al., 2012, Milhollen et al., 2010). To assess the potency of the inhibition, macrophages were transduced with an adenovirus, containing in its genome a luciferase gene with an upstream promoter region containing 4 concatenated NF-κB motifs (κB-luc reporter). Figure 4.6 shows that LPS stimulation caused an increase of the luciferase reporter transcription. This upregulation is then significantly diminished on treatment with MLN-4924, in a dose dependent manner. At the highest two doses (1 and 2 μM) the luciferase activity was almost indistinguishable from the luciferase activity in unstimulated cells.

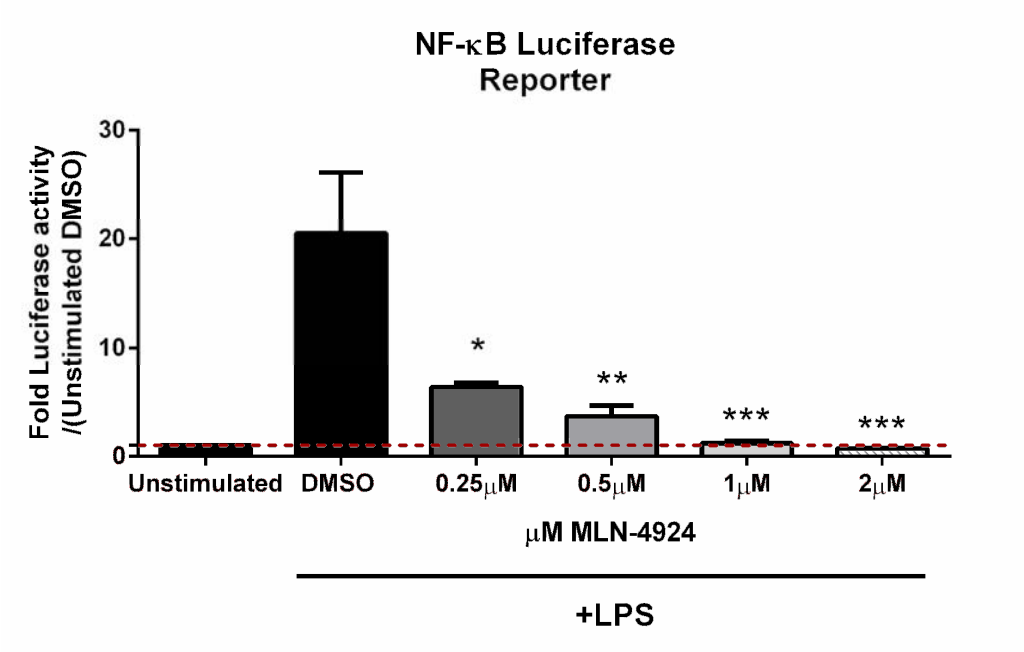

Figure 4.6 – MLN-4924 inhibits an NF-κB luciferase in a dose dependent manner

10^5 primary human macrophages were transduced with an NF-κB luciferase reporter encoding adenovirus on day 5 of differentiation and rested overnight. The following day the cells were stimulated with LPS in the presence of varying doses of MLN-4924 for 120 mins. The cells were then lysed and the lysates were assayed for luciferase activity. Graph shows means of 4 technical replicates for 3 independent donors. Statistical significance compared to the DMSO+LPS treated samples was calculated using 1 way ANOVA (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

It has previously been shown that MLN-4924 treatment is able to block NF-κB induced gene expression through stabilising phosphorylated IκBα and therefore restraining NF-κB in the cytoplasm (Chang et al., 2012). However, these experiments were done with immortalised cell lines and with 6 hour preincubation with the inhibitor prior to LPS stimulation. To examine whether NF-κB was retained in the cytoplasm in our experimental conditions, I elected to treat macrophages with LPS with 2 μM MLN-4924 or a vehicle control for one hour. After stimulation, the cells were harvested and fractionated into their nuclear and cytosolic lysates. These lysates were then analysed by SDS PAGE and western blotting for the NF-κB subunit p65 and loading controls Lamin and Tubulin as indicators of the integrity of nuclear cytosolic fractions.

On LPS stimulation of macrophages there was a clear translocation of p65 into the nuclear fraction, which was heavily abrogated by co-treatment with MLN-4924 (Figure 4.7). In both unstimulated macrophages and macrophages treated with LPS and MLN-4924, a small amount of p65 was detected in the nuclear fraction.

To investigate what other effects MLN-4924 could have on LPS signalling cascades, I stimulated macrophages with LPS in the presence of 2μM MLN-4924 or DMSO control for 15, 30, 60 or, 120 minutes and analysed the phosphorylation of JNK, p38 MAPK and IκBα by western blot (Figure 4.8). The addition of MLN-4924 did not dramatically alter the phosphorylation state of p38 MAPK or JNK before the 2 hour timepoint. After two hours the addition of MLN-4924 led to a sustained JNK phosphorylation compared to the DMSO treated control. On LPS stimulation there was a noticeable loss of IκBα protein in the DMSO treated samples. This loss of IκBα was abrogated by the addition of MLN-4924 and was accompanied by the accumulation of phosphorylated-IκBα in

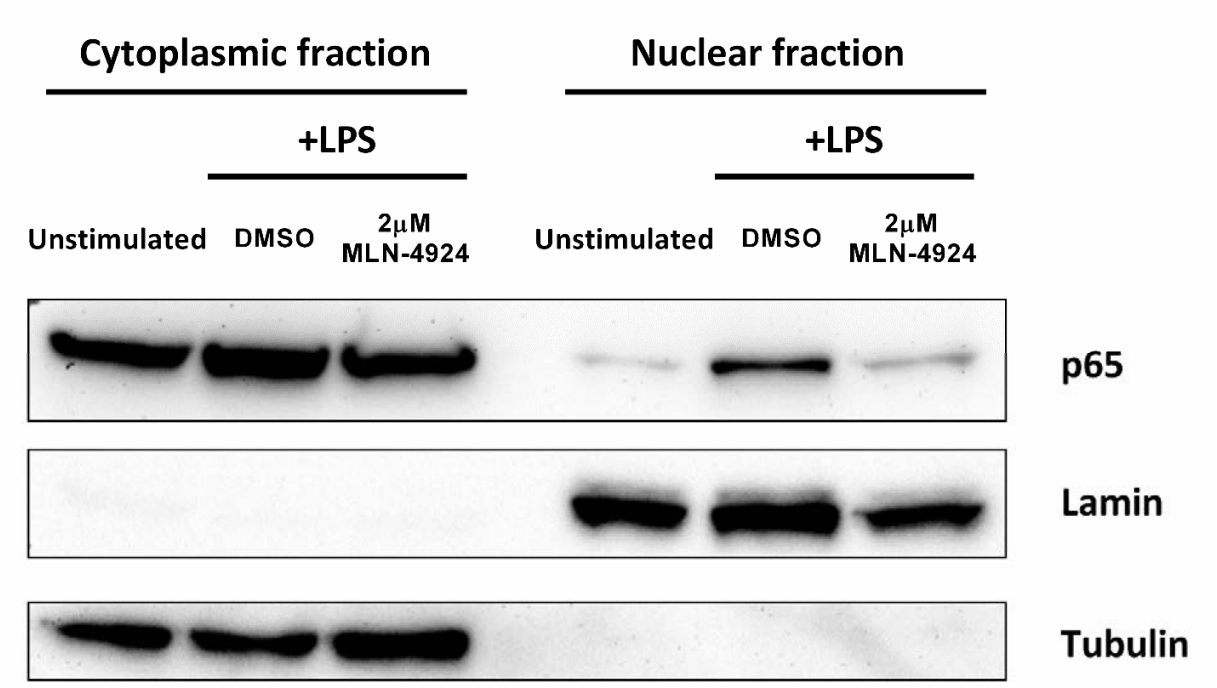

Figure 4.7 – MLN-4924 inhibits LPS-induced p65 nuclear translocation

$2x10^6$ primary human macrophages were stimulated with LPS in the presence of 2 μM MLN-4924 or DMSO control for 1 hour. Cells were then harvested and samples were fractioned into nuclear and cytosolic fractions, which were then analysed for the levels of p65, Lamin and Tubulin by SDS PAGE and Western blot. Representative blot of 3 experiments

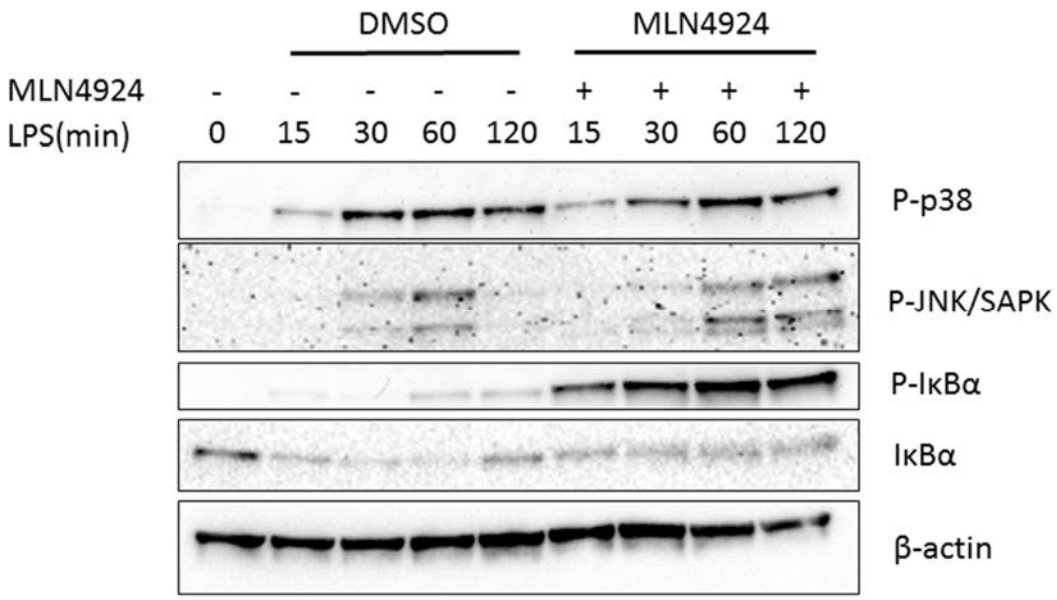

Figure 4.8 –The effect of MLN-4924 on LPS induced signalling cascades

$2x10^6$ MDM macrophages were stimulated with LPS and DMSO or 2 µM MLN-4924 for: 15, 30, 60 or, 120 minutes. Cells were then lysed and the abundance and phosphorylation of MAPK/IKB proteins were assessed by western blotting. Blot is representative of two independent experiments

the cells. These results suggested that prolonged incubation of macrophages with MLN-4924 could result in off -target effects on the JNK pathway, for example by impairing the expression of a negative feedback of this pathway. In order to avoid the effects of sustained JNK signalling (and potential other off-target effects) I elected to examine only the one hour timepoint with this inhibitor.

Since MLN-4924 is able to inhibit the translocation of NF-κB in macrophages by 60 minutes post LPS stimulation, I wanted to investigate whether MLN-4924 was able to inhibit non-reporter gene expression at this time point. To assess this I stimulated macrophages in the presence of DMSO or 2μM MLN-4924 for 60 minutes and then measured secreted TNFα and the expression of *TNF* mRNA. TNF has been previously shown to be highly NF-κB dependent for its transcription and the secretion of the protein by macrophages is detectable by ELISA by 60 minutes (Andreakos et al. (2004), Smallie et al. (2010)). Figure 4.9 shows that on LPS stimulation TNF mRNA and protein were potently upregulated. The addition of DMSO did not alter the expression of TNF at the protein or mRNA level in unstimulated cells, but significantly diminished the response to LPS (reducing TNFα secretion by over 80% and inhibiting TNF mRNA upregulation by 90%).

4.2.6 – MLN-4924 inhibits a significant proportion of the LPS immediate early transcriptome

After optimizing the concentration of MLN-4924 to maximally inhibit NF-κB pathway at the one hour time point, I aimed to determine how much of the LPS response was dependent on the NF-κB pathway and whether the genes that did require NF-κB corresponded to the genes inhibited by IL-10.

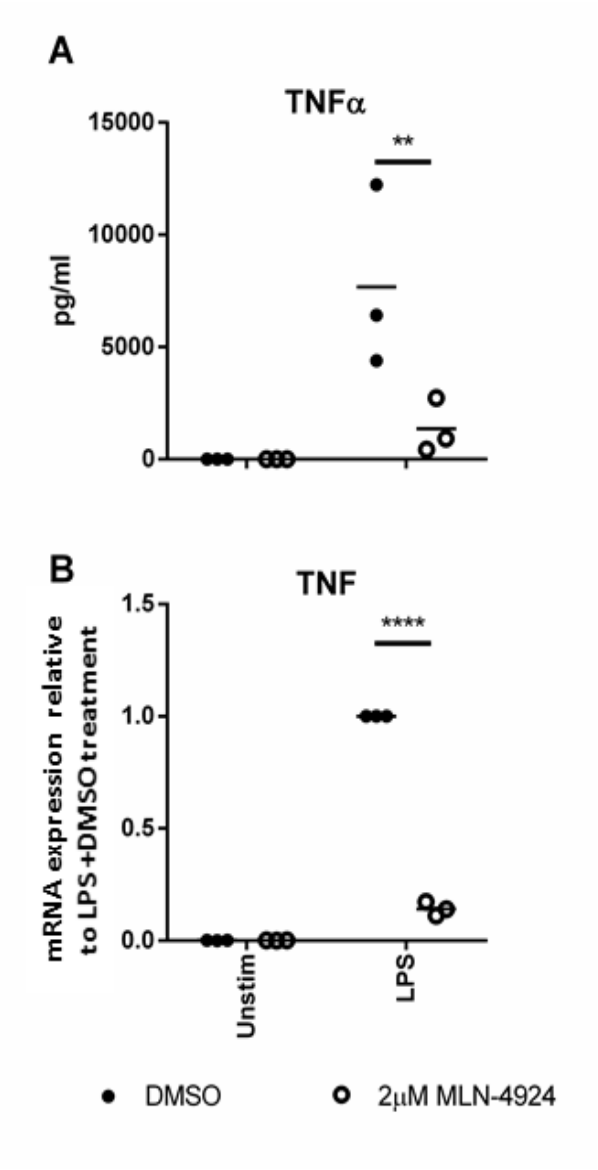

Figure 4.9 – MLN-4924 inhibits TNF gene expression and protein secretion

10^6 primary human macrophages were stimulated with LPS for 60 minutes in the presence of 2 μM MLN-4924 or vehicle control for 60 minutes. A) Cell culture supernatants and assayed for TNF-α production. B) RNA from cells was purified and analysed for TNF gene expression. Graph shows the means of 3 independent experiments. Statistical significance was calculated using Students t-test (**=$p<0.01$, ****= $p<0.0001$).

2x106 primary human macrophages from 3 donors were stimulated for 60 minutes with LPS or LPS in combination with 0.5 μM or 2 μM MLN-4924, after which cell culture supernatants were removed and cells harvested for RNA. The RNA was then processed and analysed by two colour microarray by Oxford Gene Technology. The feature extraction files then analysed by Partek Genomics Suite. The data was quantile normalised to remove cross microarray chip variation (distribution of probes before and after quantile normalisation are shown in Figure 4.10A and B).

In order to assess the similarity of the samples prior to analysis by ANOVA, I performed a Pearson correlation between all samples and hierarchically clustered the results using an average linkage algorithm. Figure 4.11 shows that all samples demonstrated a high level of similarity to one another with all Pearson correlation values exceeding 0.95. It is also apparent from the hierarchical clustering that two of the donors are more similar than the third, which is placed in a separate cluster. This difference is particularly observable between the unstimulated Donor #7 and Donors #5 and #6. However, the variation between all sample conditions was extremely low (similar to the microarray in chapter 3) and therefore the differences between donors may be exaggerated by the hierarchical clustering. The differences between the sample conditions was minimal, but this could be due to a low number of genes being modulated by LPS at this timepoint.

To further assess the sources of variation in this dataset I performed a PCA between all samples (Figure 4.12). In this analysis the first PC segregated donor #7 from donors #5 and #6, while a combination of PC2 and PC3 segregated the unstimulated and the LPS stimulated samples. This indicated that the main source of variation in the dataset is due to differences in gene expression between donors which would weaken the robustness of any statistical comparisons between

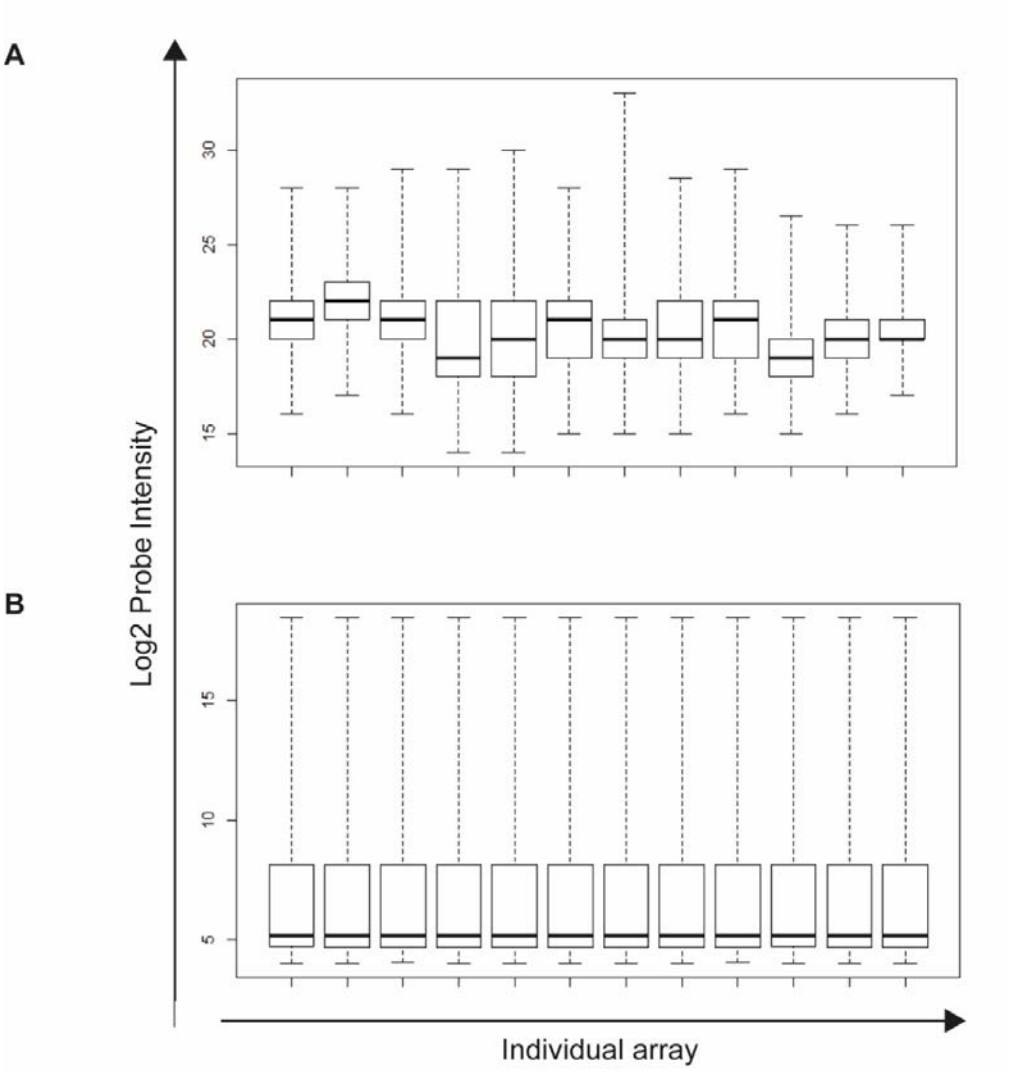

Figure 4.10 – Distribution of probe intensities in the LPS/MLN microarray before and after quantile normalisation

$2x10^6$ MDM from 3 independent donors were stimulated with LPS and DMSO, 0.5 µM or, 2 µM MLN-4924 and RNA extracted for microarray analysis (performed by Oxford Genomic Technologies). Differences in probe intensities between the arrays were normalised using quantile normalisation. A) The variation the probe intensities across the Agilent dataset before quantile normalisation. B) The variation the probe intensities across the Agilent dataset after quantile normalisation.

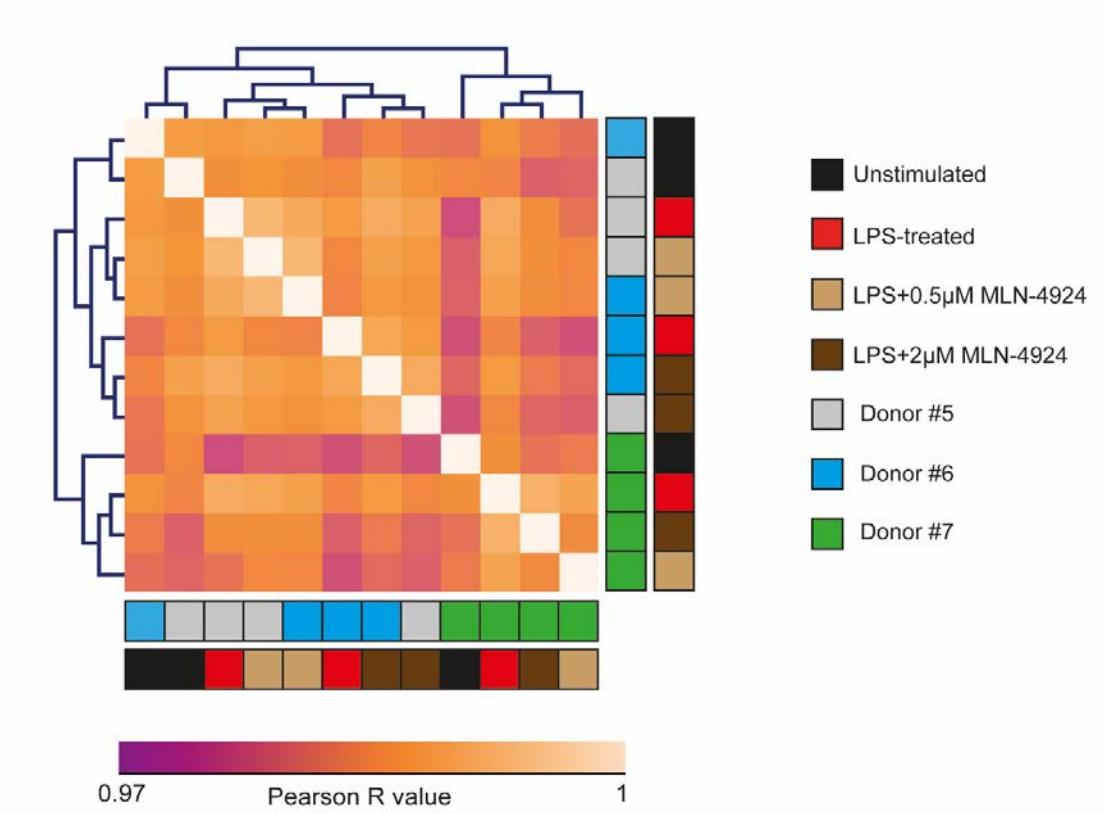

Figure 4.11 – Pearson correlation between samples in the LPS/MLN microarray

$2x10^6$ MDM from 3 independent donors were stimulated with LPS and DMSO, 0.5 μM or, 2 μM MLN-4924 and RNA extracted for microarray analysis (performed by Oxford Gene Technology). Individual samples across the experiment were compared using a Pearson correlation and the resulting correlation coefficient matrix was hierarchically clustered.

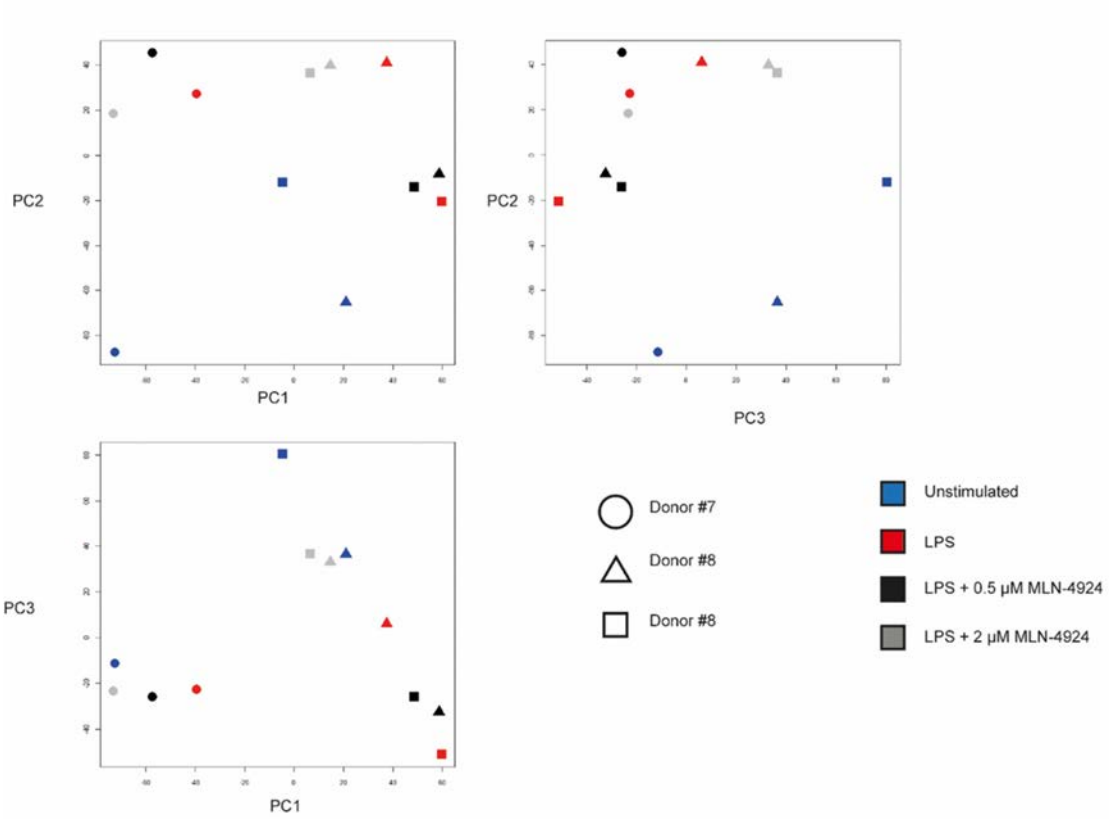

Figure 4.12 – PCA of the LPS/MLN microarray

$2x10^6$ MDM from 3 independent donors were stimulated with LPS and DMSO, 0.5 µM or, 2 µM MLN-4924 and RNA extracted for microarray analysis (performed by Oxford Gene Technology). Data were background corrected, quantile normalised and, the variation in the dataset visualized by PCA (MixOmics package).

treatments. However, there is significant differences in gene expression between stimulated and unstimulated samples indicating that using a statistical comparison between the two conditions would be possible but differences may be masked by donor variation.

To examine the LPS response, probes were selected that had a significant upregulation of gene expression by LPS (>2 fold above unstimulated and ANOVA $p<0.05$), similar to the previous microarray. The probes that passed these criteria (representing 353 unique genes) were then organised into clusters using an average linkage hierarchical clustering using the program Genesis.

Figure 4.13 shows that the results of the hierarchical clustering can be split into 3 major clusters, shown as D, E and F (example genes are shown in Figure 4.14). Cluster D shows a clear dose dependent inhibition of LPS-induced gene expression indicating a strong dependency on NF-κB transcription factor activity. This behaviour of gene expression is exhibited by the genes *IL1A* and *TNFSF9* (shown in Figure 4.14). Cluster E does not show a clear dose dependent sensitivity to MLN-4924 treatment but does contain probes that exhibit a decrease in mRNA abundance on MLN-4924 treatment. This decrease however is only moderate compared to cluster D and does not appear to be dose dependent (highlighted in Figure 4.14 by the genes *KFL6* and *FOS*). Cluster F showed an unexpected pattern of gene expression; MLN-4924 upregulating LPS-induced gene expression in a dose dependent manner (shown in Figure 4.14 by *AREG* and *TNFSF15*).

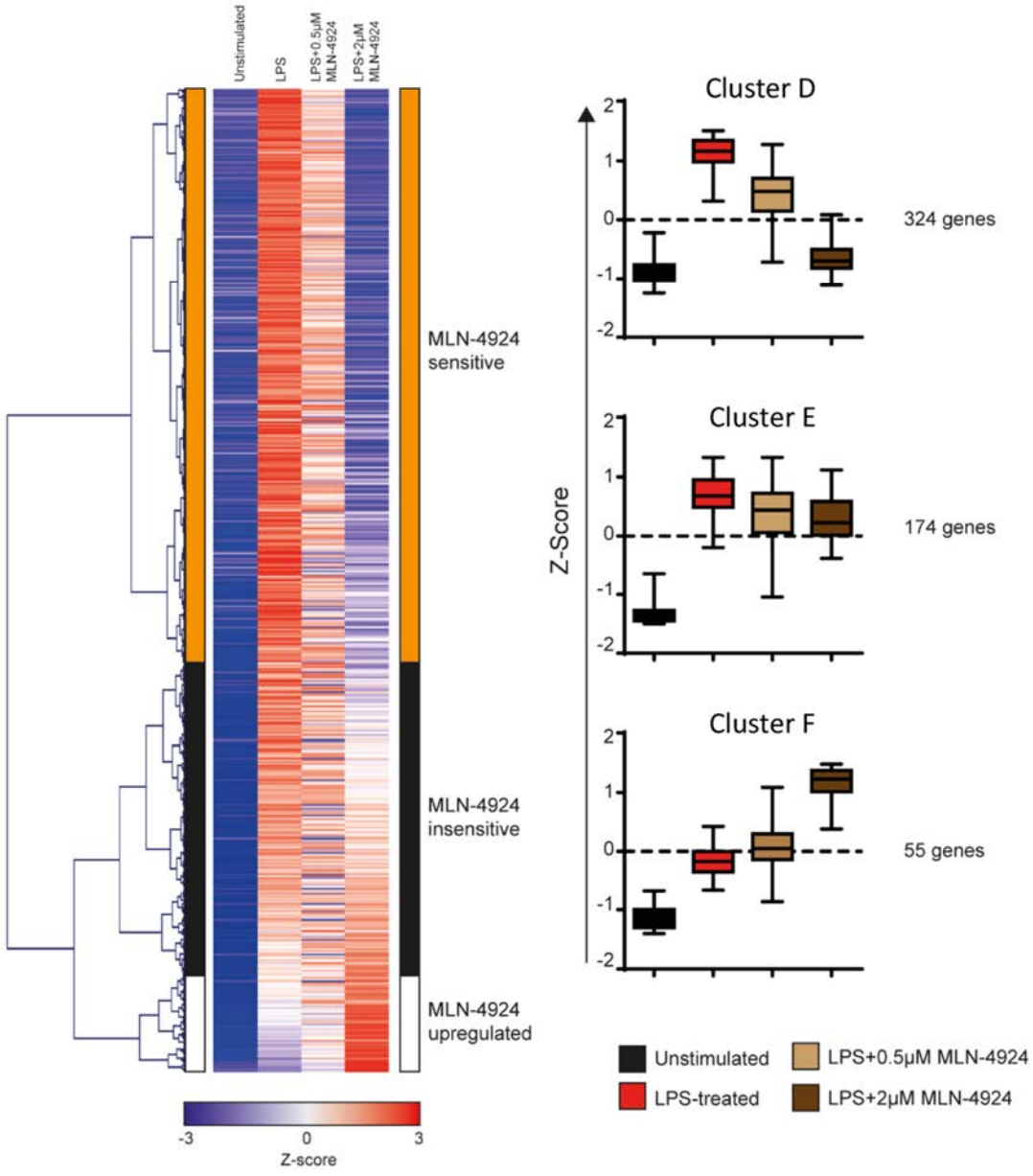

Figure 4.13 – Hierarchical clustering of LPS/MLN microarray

$2x10^6$ primary human macrophages from 3 independent donors were stimulated for 1 hour with LPS in the presence of; DMSO, 0.5 µM or 2 µM MLN-4924. Cells were then harvested for RNA and microarray analysis performed on the samples (Oxford Gene Technology). The raw data from the chips was quantile normalised and the LPS-induced probes (>2 fold above unstimulated, ANOVA p value<0.05) were clustered using average linkage hierarchical clustering.

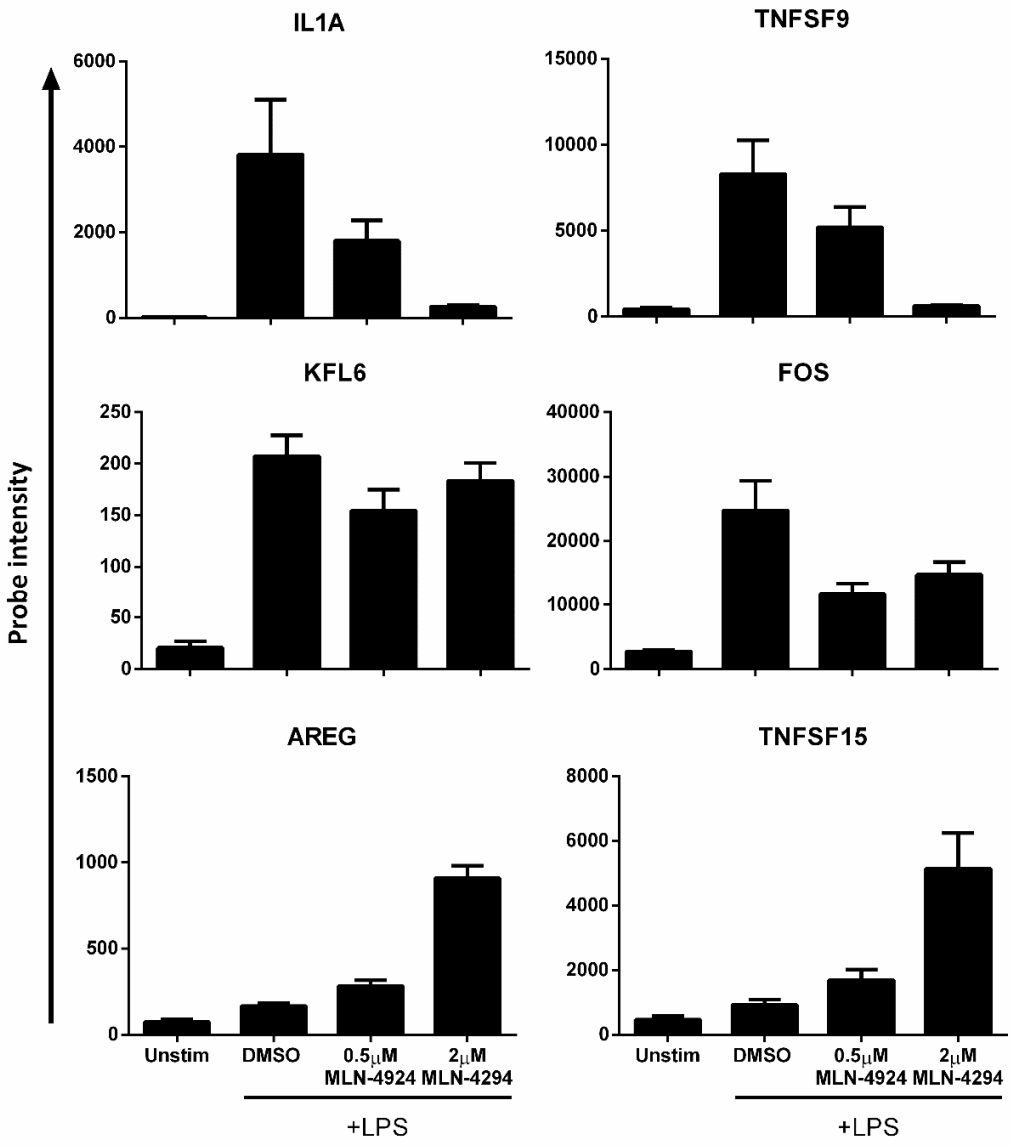

Figure 4.14 – Effect of MLN-4924 on gene expression of select LPS-induced genes

$2x10^6$ primary human macrophages from 3 independent donors were stimulated for 1 hour with LPS in the presence of; DMSO, 0.5 µM or 2 µM MLN-4924. Cells were then harvested for RNA and microarray analysis performed on the samples (Oxford Gene Technology). Select probes from the hierarchical clusters: *IL1A* and *TNFSF9* (MLN-4924 sensitive), *KFL6* and *FOS* (MLN-4924 unaffected), *AREG* and *TNFSF15* (MLN-4924 upregulated).

4.2.7 – Comparing the effect of IL-10 and the inhibitor MLN-4924 on the transcriptome of LPS activated macrophages

On observing that MLN-4924 is able to modulate LPS-induced gene expression both positively and negatively, I wanted to assess the similarity between the LPS+IL-10 and LPS+MLN-4924 transcriptomes to determine whether IL-10's effect on gene expression could be explained by a general inhibition of NF-κB. The initial problem with this comparison is that the experiments were done with different donors, at different times and on different microarray platforms making direct comparisons between the samples inappropriate. To counter this problem; probe IDs of the two microarrays were annotated with the corresponding ENSEMBL transcript ID and the probes that detected transcripts only in one of the microarrays were removed. This filtered down 33297 probes in the Affymetrix (IL-10) microarray and 50599 probes in the Agilent (MLN-4924) microarray to 21372 probes covering common transcripts. This allowed me to examine the common areas of the microarrays more easily and prevented probes unique to one of the platforms from acting as confounding factors in further analysis.

Examining the expression frequency of the common probes between the two platforms (shown in Figure 4.15 for representative donors unstimulated samples), it becomes apparent that the range of expression levels reported by the two platforms, has a strikingly different distribution (seen in Figure 4.15). This effect is most likely due to the differences in the method of detection of RNA-probe hybridization since there are a number of weakly expressed genes in the Agilent array that do not fall in a similar pattern to the Affymetrix array. The data were further filtered to remove probes that reported below the detection threshold in all of the samples of one or both

of the microarray, which altered the distribution of probe intensities to a more comparable state (Figure 4.15 shown as a red line).

As Figure 4.15 shows, even with the filtering of probes below the detection threshold, the range of expression is different between the two platforms (between 5-12 log_2 expression in the Affymetrix and between 2-15 log_2 expressions on the Agilent platform). To account for the differences in reported expression the datasets were normalized by two methods: YuGene and Z-score.

The "YuGene" transformation ranks the absolute expression of each gene and expresses it as a cumulative proportion of the total number of probes, with the lowest expressing transcript being close to 0 and the highest expressed transcript approaching 1. Using this method contracts the differences in the range of expression onto a comparable scale and reduces the differences in absolute expression reported while preserving the pattern. As Figure 4.15 shows, this transformation reduces the data from each of the microarrays with different intensities onto a similar scale.

The "Z-score" normalization expresses each transcript expression as a standard deviation of the whole sample mean". This method again contracts the ranges in expression to comparable scale, but the transformation requires a near Gaussian distribution to prevent distortion toward the extremes edges of the transformation.

After transforming the data, a principal component analysis was performed on the data to assess whether the cross platform variation was reduced to allow a direct comparison between the data

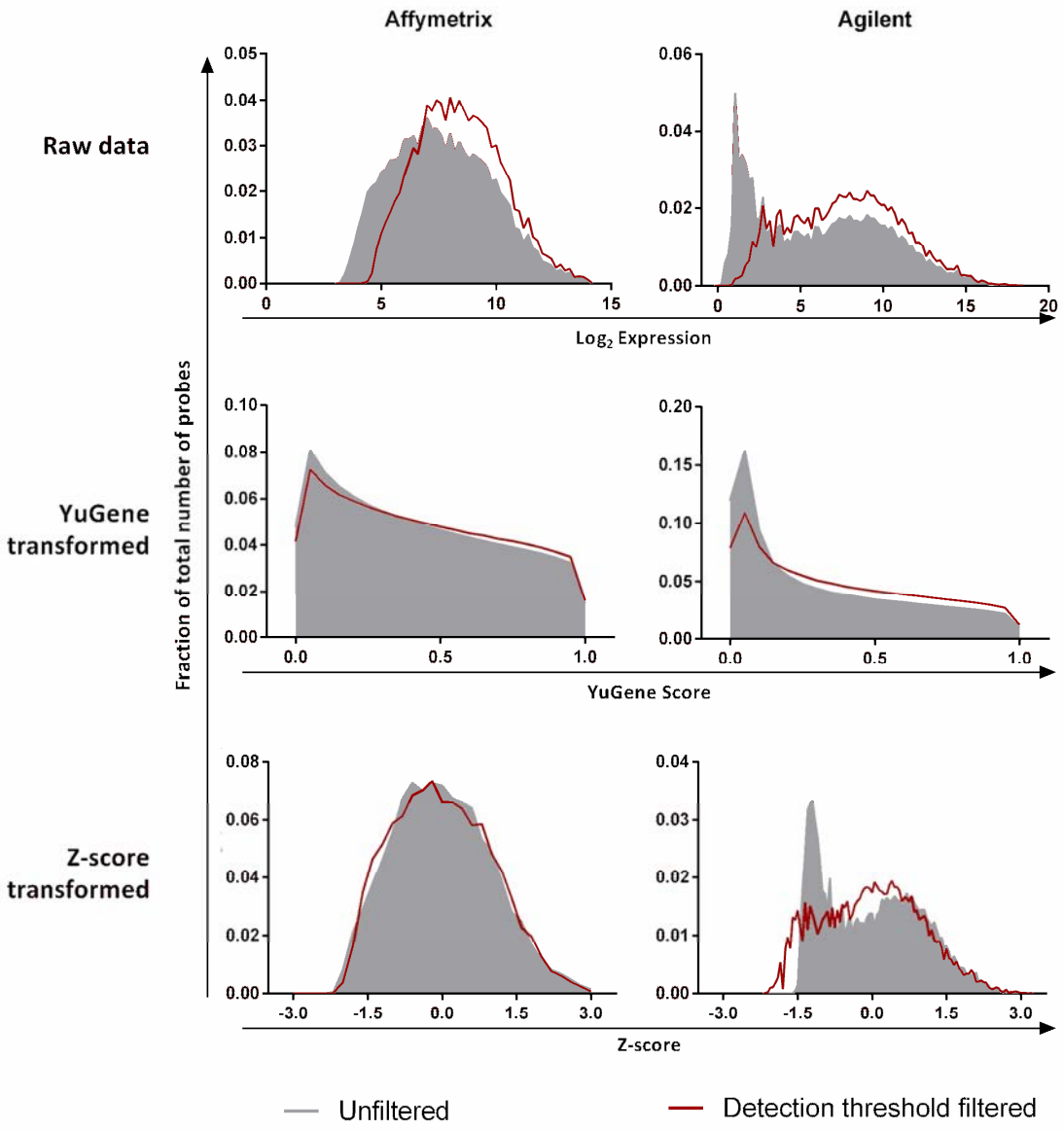

Figure 4.15 – Density plot of gene expression between the platforms, in unstimulated macrophages, before and after transformation.

Microarray probes detecting common RNA transcripts between the two microarray platform (Agilent and Affymetrix) were selected, the expression values of all probes or all those above detection threshold in both microarrays were transformed using either; Yugene or Z-score normalisation. The data was then presented as density histograms with fraction of total number of probes on the Y axis and level of gene expression on the X axis (shown as either Log2 value, Yugene value, or Z-score).

sets. Examining the first 3 principal components of all the data set transformations and filtering in Figure 4.16 I observed that over 90% of the variation can be explained by the first principal component in all of the transformations. Looking at the resulting plots of the PCA data (Figure 4.17) this major source of variation is in fact the array these samples were performed on. This indicates that none of the transformations removes the effect caused by the different platforms and that integrating the datasets together is unsuitable therefore they must be considered in future analysis separately.

Interestingly, the second principal component in this analysis seems to be the stimulus added to the samples, with unstimulated and IL-10 alone samples clustering in the same area of the second principal component distinct from the LPS+ 0.5 μM MLN-4924, LPS+IL-10 and, LPS alone samples with the LPS+ 2 μM MLN-4924 samples clustering between the two groups.

Since the PCA analysis showed significant variance based on the array platform, I decided not to merge the microarrays directly and instead examine the two microarray by comparing the contents of the hierarchical clustering analysis done previously to examine the relationship between IL-10s transcriptional effect and NF-κB dependency.

First the probes that hybridized with common transcripts were selected as before and filtered further to keep transcripts that displayed significant induction on LPS stimulation (>2 fold in both microarrays, ANOVA $p<0.05$). Duplicate probes assessing the expression of the same transcript were filtered out and the highest value probe retained. The comparison between the two

microarrays is shown in Figure 4.18. The transcripts were then sorted into groups based on the hierarchical clustering results of the Affymetrix and Agilent microarrays (Figures 3.8 and 4.13).

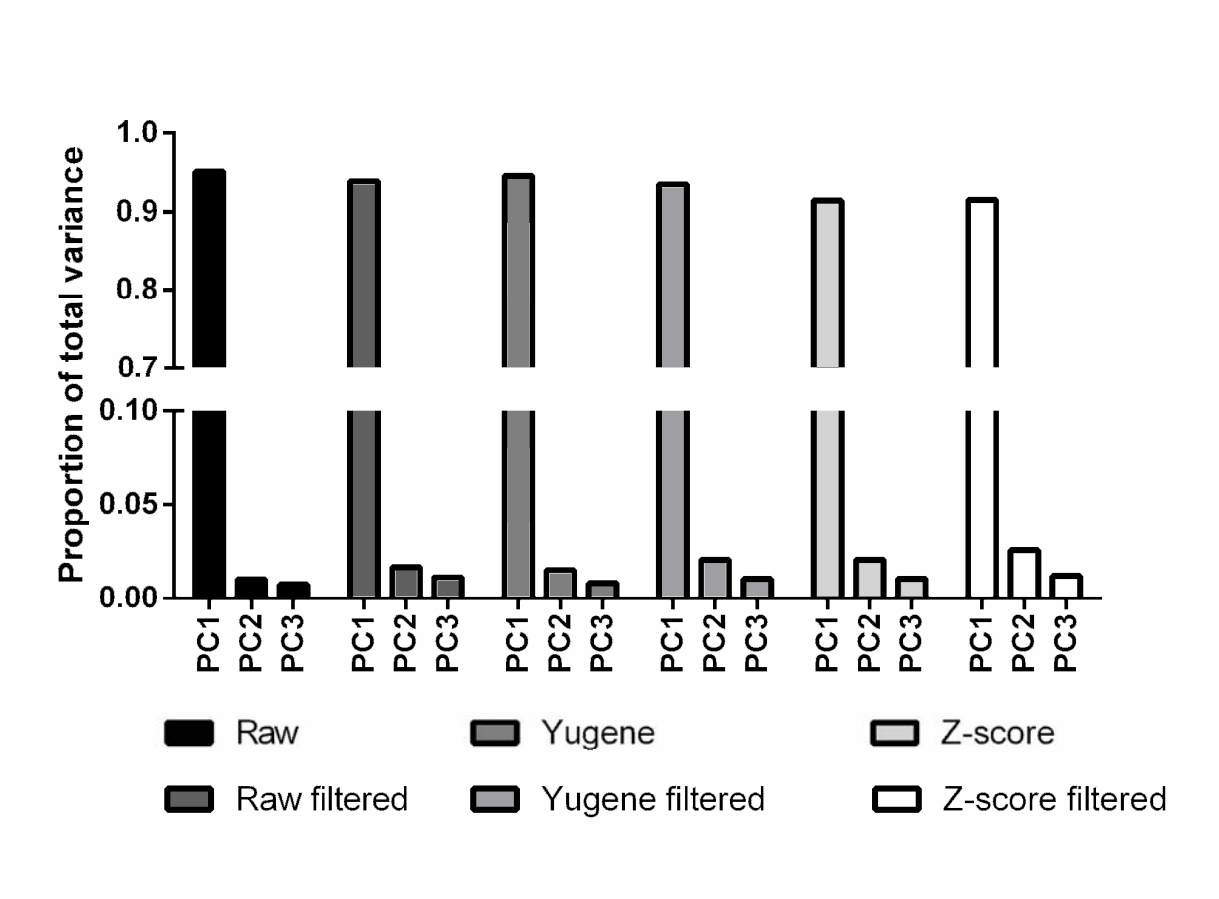

Figure 4.16 – Scree plot showing numbers of principal components in the filtered and transformed data

Microarray probes detecting common RNA transcripts between the two microarray platform (Agilent and Affymetrix) were selected, the expression values of all probes or all those above detection threshold in both microarrays were transformed using either; Yugene or Z-score normalisation. 3 factor Principal component analysis was performed on the data and the eigenvalues of each component were plotted as a fraction of the variance in the whole dataset.

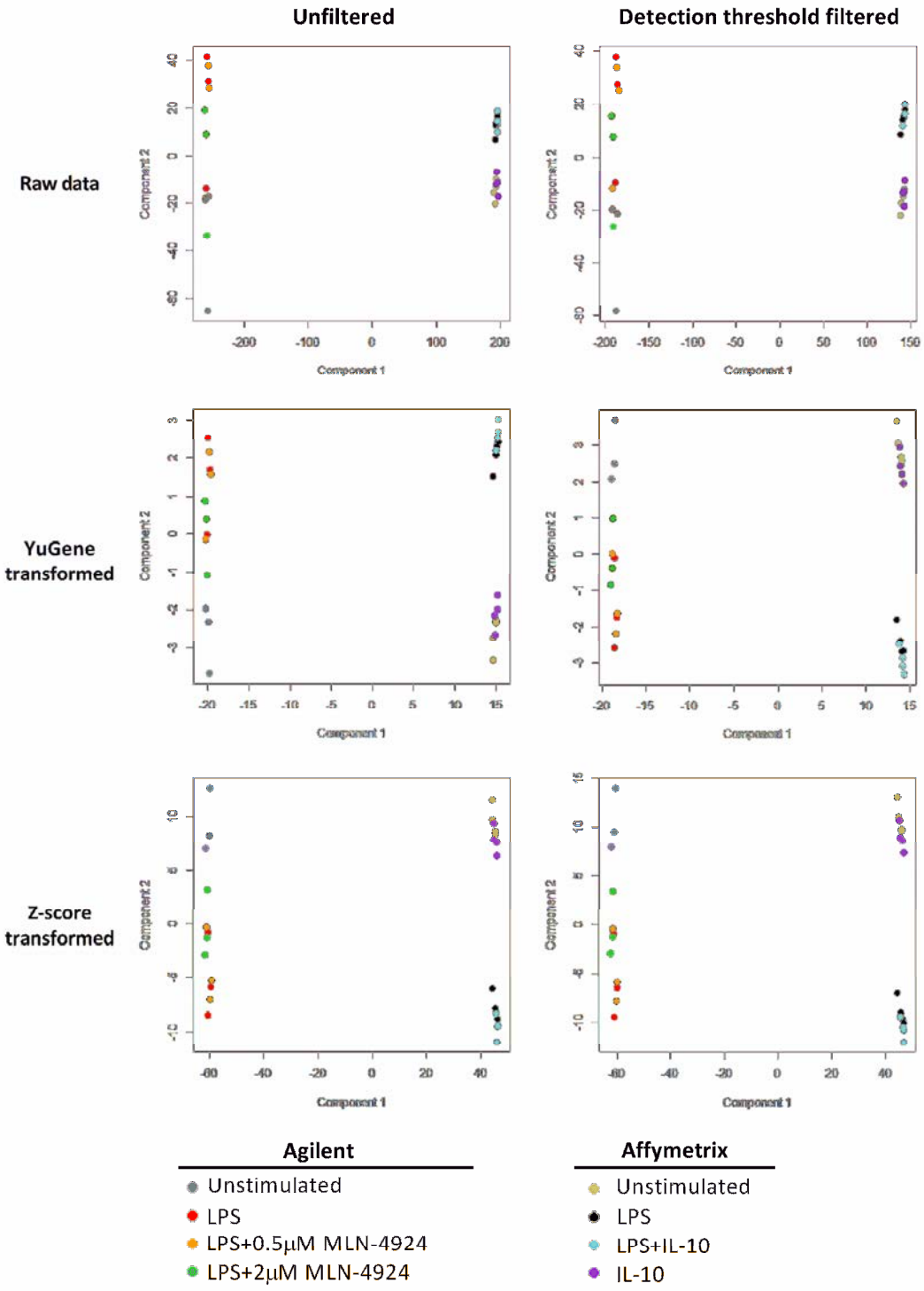
Unfiltered
Detection threshold filtered
Raw data
YuGene transformed
Z-score transformed
Component 1
Component 2
Agilent
Unstimulated
LPS
LPS+0.5μM MLN-4924
LPS+2μM MLN-4924
Affymetrix
Unstimulated
LPS
LPS+IL-10
IL-10

Figure 4.17 – Principal component analysis of the merged datasets.

Microarray probes detecting common RNA transcripts between the two microarray platform (Agilent and Affymetrix) were selected, the expression values of all probes or all those above detection threshold in both microarrays were transformed using either; Yugene or Z-score normalisation. 3 factor Principal component analysis was performed on the data and the samples were plotted according to calculated eigenvector values in the first two principal components.

The clusters in the Affymetrix microarray (IL-10 inhibited, IL-10 unaffected and, LPS/IL-10 cooperative) were assessed for their sensitivity to MLN-4924 as designated by hierarchical clustering (MLN-4924 sensitive, MLN-4924 insensitive and, MLN-4924 upregulated). The clusters in the Agilent microarray were similarly assessed for their response to IL-10 the results of these analyses are shown in Figure 4.19 and 4.20).

Between the Affymetrix microarray clustered transcripts there was no significantly different pattern of response to MLN-4924. Conversely looking at the clusters from the MLN-4924 microarray (Figure 4.20), there was no significant pattern to the changes in gene expression observed when stimulating with LPS+IL-10. Neither of these showed significantly different patterns of MLN-4924 sensitivity or IL-10 responsiveness compared to the whole data set (χ^2 $p>0.05$). Examining the breakdown of MLN-4924 clustering in the IL-10 inhibited group (Figure 4.20) shows that just under half of the transcripts inhibited by IL-10 had an LPS response which was sensitive to MLN-4924 and that the IL-10 inhibited transcripts account just over 40% of the total number of MLN-4924 sensitive genes. This indicated that IL-10's ability to modulate gene expression in a positive or negative manner is not recapitulated by a general inhibition of NF-κB.

4.2.8 – MLN-4924 alters the magnitude of the LPS response but does not alter the effect of IL-10 on gene expression

Since many of the LPS/IL-10 cooperative genes were dependent on NF-κB for LPS induction, I investigated whether the pattern of response to IL-10 (IL-10 inhibited, unaffected and, LPS/IL-10 cooperative) was affected by geneal inhibition of NF-κB by MLN-4924. To do this I stimulated

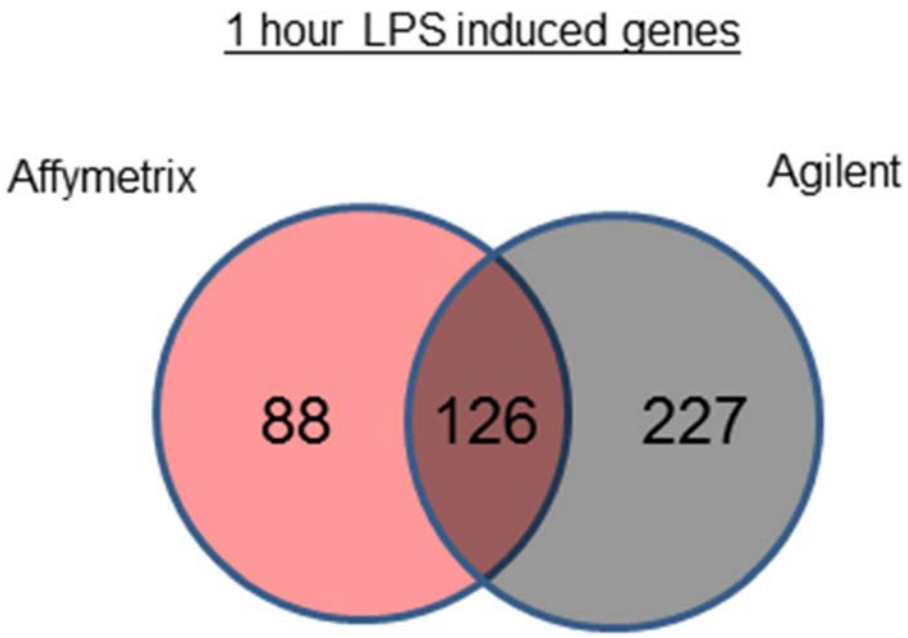

Figure 4.18 – Comparison of LPS-induced probes between Affymetrix and Agilent microarrays

Probes significantly induced by LPS ($p<0.05$, >2 fold induction above unstimulated) for unique transcripts common between the Affymetrix and Agilent array samples were compared.

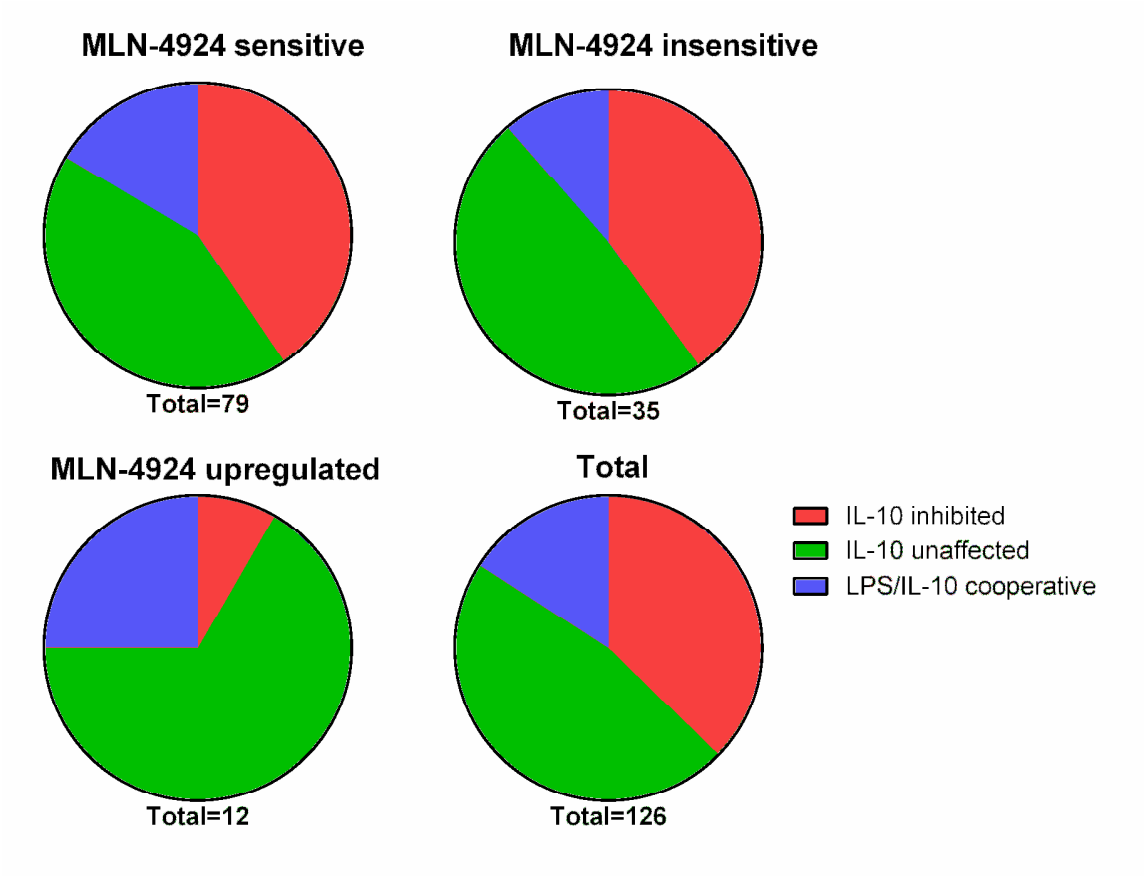

Figure 4.19 – Distribution of IL-10 microarray clusters paired with MLN microarray data

LPS-induced transcripts (>2 fold above unstimulated, ANOVA p value <0.05) common to both microarray platforms were selected and separated into previously observed clusters of gene expression in response to MLN-4924 and then assessed for the response to IL-10.

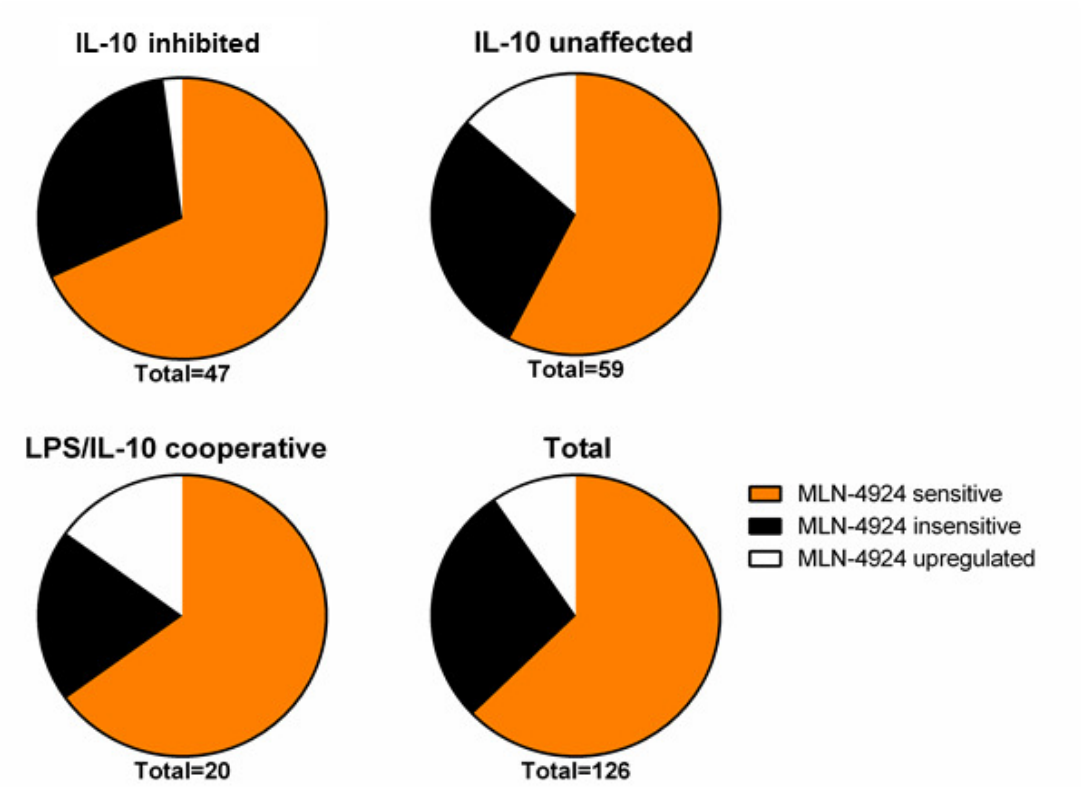

Figure 4.20 – Distribution of MLN microarray clusters paired with IL-10 microarray data

LPS-induced transcripts (>2 fold above unstimulated, ANOVA p value <0.05) common to both microarray platforms were selected and separated into previously observed clusters of gene expression in response to IL-10 and then assessed for the response to MLN-4924.

primary human macrophages with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for one hour in the presence of 2 μM MLN-4924 or DMSO vehicle control and examined the expression of select LPS induced genes.

Figure 4.21 shows that the many of the genes selected were highly sensitive to MLN-4924 treatment. Out of the select genes only two (*NR4A2* and *CD274*) had an LPS induction which was unaffected by MLN-4924 treatment (highlighted in Figure 4.22). To examine whether the gene expression pattern of the genes sensitive to MLN-4924 treatment was altered, I normalized the data to the LPS stimulated samples. Figure 4.23 clearly shows that despite large scale inhibition of gene expression by MLN-4924 many genes retain their gene expression pattern (Shown in Figure 4.24). The only exception to this was the gene *TNFSF9* which required NF-κB activity in order for LPS and IL-10 to cooperatively regulate its expression.

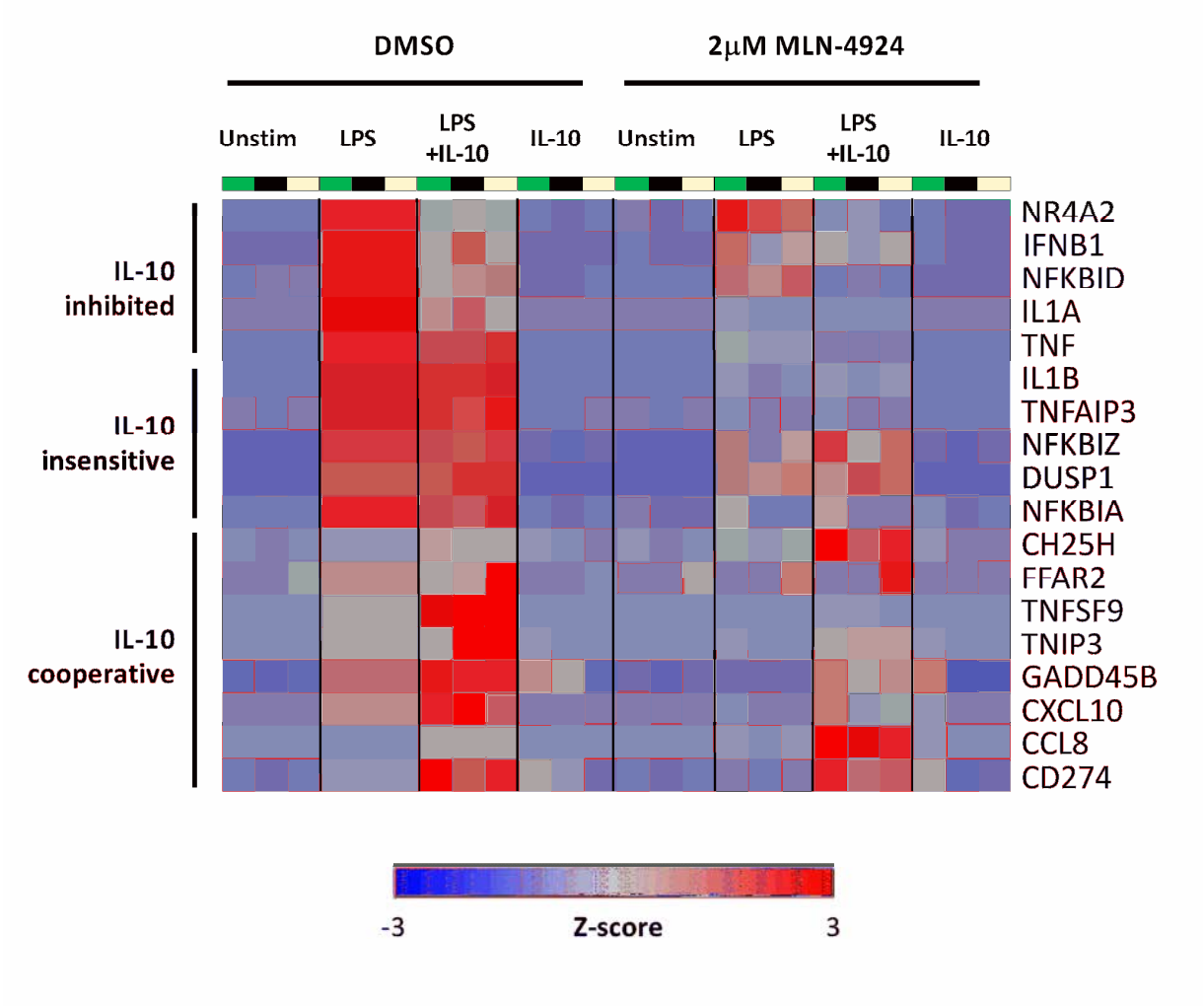

Figure 4.21 – Heat map showing the effect of MLN-4924 on LPS/IL-10 gene expression

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour in the presence of 2 μM MLN-4924, DMSO control or media control. After stimulation cells were harvested for RNA and expression of genes listed were assessed by SYBR green QPCR and normalised to *GAPDH* for each sample and to LPS treated media control.

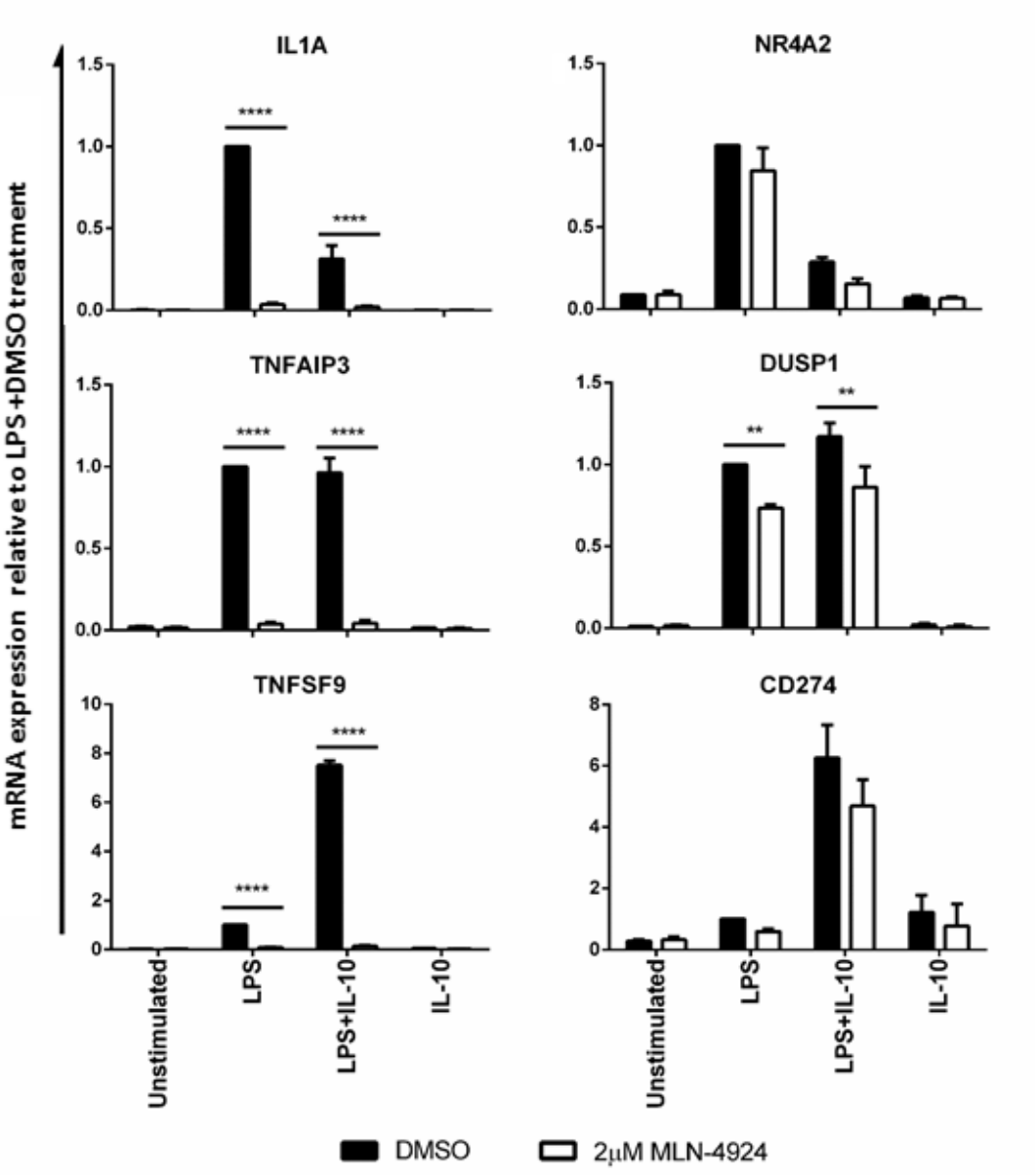

Figure 4.22 – Expression of *NR4A2*, *IL1A*, *TNFAIP3*, *DUSP1*, *TNFSF9* and *CD274* in MLN-4924 treated cells

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour in the presence of 2 μM MLN-4924, DMSO control or media control. After stimulation cells were harvested for RNA and expression of *NR4A2*, *IL1A*, *TNFAIP3*, *DUSP1*, *TNFSF9* and *CD274* were assessed by SYBR green QPCR and normalised to *GAPDH* for each sample and the LPS and DMSO treated sample. Graphs represent data from 3 independent experiments

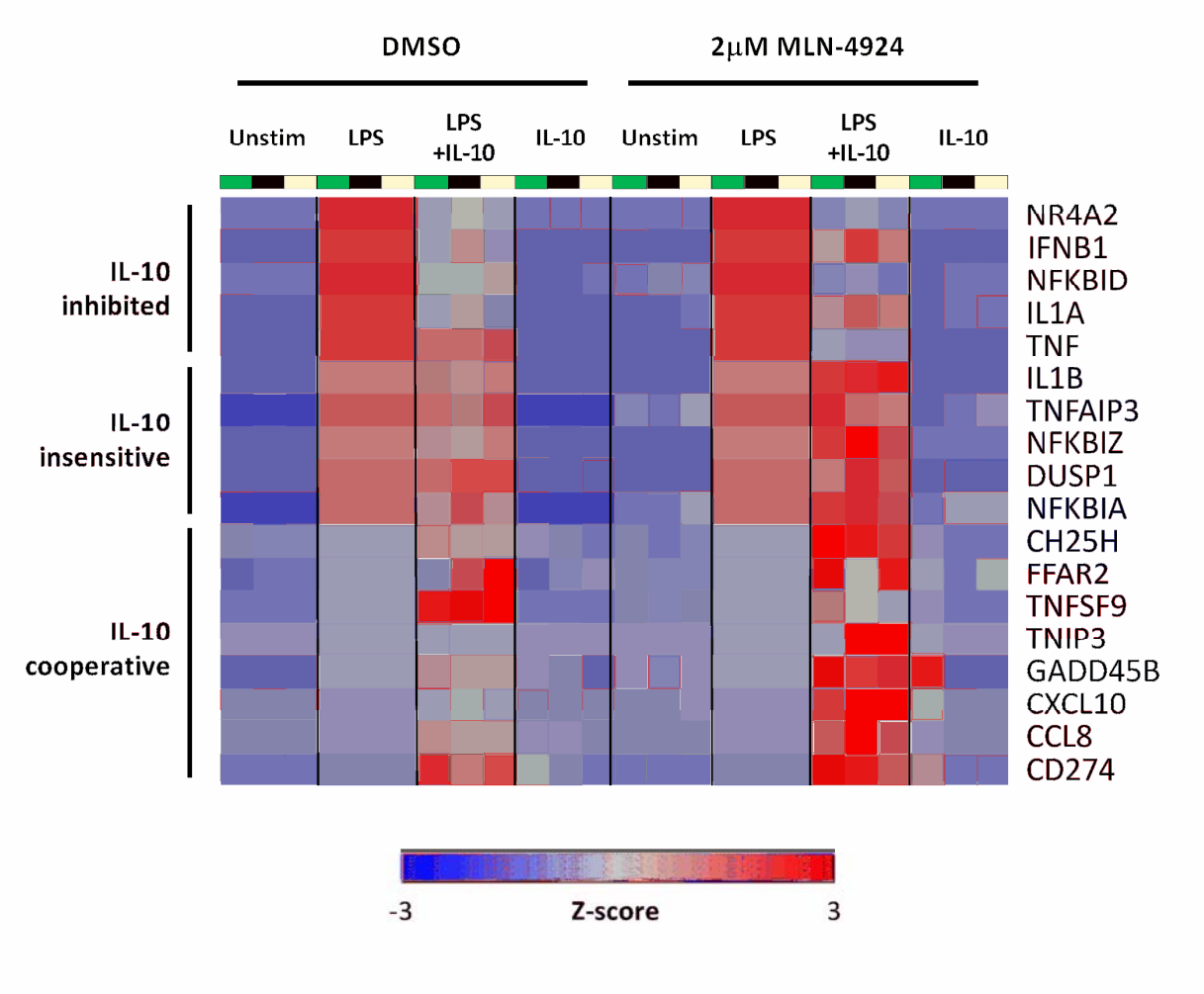

Figure 4.23 – Heat map showing the effect of MLN-4924 on LPS/IL-10 gene expression patterns

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour in the presence of 2 μM MLN-4924, DMSO control or media control. After stimulation cells were harvested for RNA and expression of genes listed were assessed by SYBR green QPCR and normalised to *GAPDH* for each sample and the LPS treated sample of each treatment.

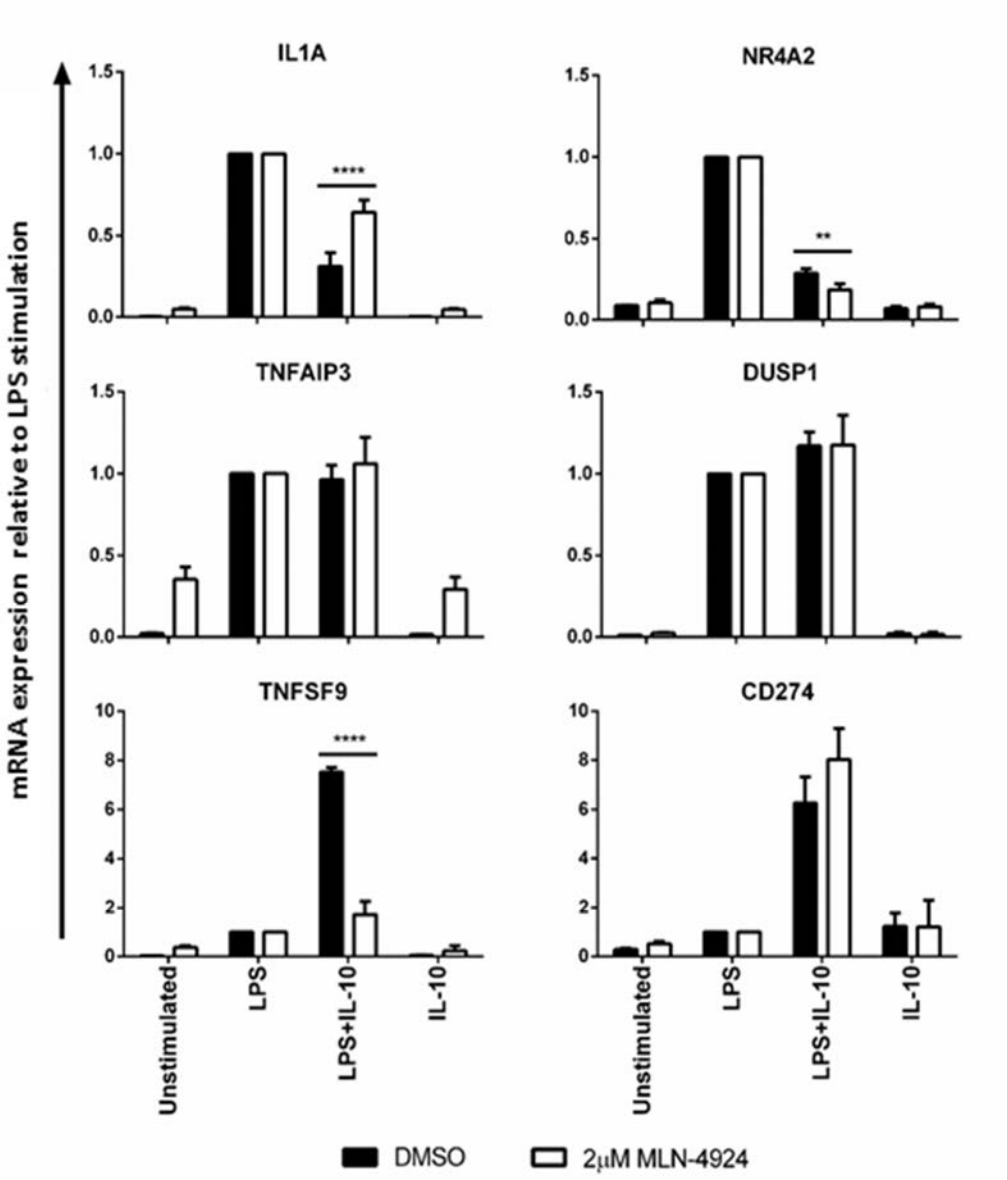

Figure 4.24– Expression patterns of *NR4A2*, *IL1A*, *TNFAIP3*, *DUSP1*, *TNFSF9* and *CD274* in MLN-4924 treated cells

10^6 primary human macrophages were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 1 hour in the presence of 2 μM MLN-4924, DMSO control or media control. After stimulation cells were harvested for RNA and expression of *NR4A2*, *IL1A*, *TNFAIP3*, *DUSP1*, *TNFSF9* and *CD274* were assessed by SYBR green QPCR and normalised to *GAPDH* for each sample and the LPS stimulation of DMSO and MLN-4924 treated samples. Graphs represent data from 3 independent experiments.

4.3 – Discussion

It is well known that NF-κB activity is responsible for the induction of a proportion of gene expression by LPS (Sen and Baltimore, 1986). However, given the ability of IL-10 to inhibit NF-κB dependent transcription (Smallie et al., 2010, Castellucci et al., 2015) it was unexpected to find an enrichment of the NF-κB consensus motifs in all of the clusters. An interesting find was the enrichment of a broad range of transcription factor binding motifs in promoters of the IL-10 unaffected genes. This could potentially be indicative of the broad range of stimuli that induce the expression of negative feedback genes (such as *TNFAIP3* or *NFKBIA)* a number of which are in this cluster (Murray and Smale, 2012).

In this chapter I have shown that the modulation of LPS-induced gene expression by IL-10 can be attributed to the activity of the transcription factor STAT3. The requirement of STAT3 for IL-10 to inhibit gene expression has been previously described in human macrophages by Williams et al. (2004). However, the requirement for STAT3 in LPS/IL-10 cooperative gene expression, has not been shown before. Recently (Dillow et al., 2014) demonstrated a requirement for JAK signalling in LPS/IL-10 cooperativity in a macrophage like cell-line, but did not show that the signalling pathway acted through STAT3 to alter gene expression.

One hypothesis to explain the IL-10 unaffected gene expression pattern, was that IL-10 could generally inhibit the recruitment of transcription factors such as NF-κB to chromatin (Smallie et al., 2010), but at specific genes STAT3 could bind to chromatin and induce expression to a similar level to LPS stimulation alone. From the STAT3 siRNA data shown in this chapter this hypothesis can be rejected since the IL-10 unaffected genes do not change on STAT3 depletion. Furthermore,

these genes also lack induction on IL-10 stimulation and are not enriched for enrichment for STAT consensus binding motifs in their promoters.

On closer examination between IL-10-inhibited and cooperative genes, it is apparent that the loss of STAT3 from macrophages causes a greater dysregulation in the inhibited genes than the cooperative genes, with inhibited genes (such as *NR4A2* and *IL1A*) losing the IL-10 mediated inhibition after STAT3 depletion. Meanwhile, LPS/IL-10 cooperatively-regulated genes such as *TNFSF9* and *CD274* lose only a fraction of their LPS/IL-10 cooperative induction. This differential sensitivity to STAT3 depletion, could be due to a differential requirement for *de novo* protein synthesis in modulating gene expression(Murray, 2005).

It is clear from the intersection of the LPS/MLN-4924 and the LPS/IL-10 treated microarrays, that the hypothesis that IL-10's inhibitive actions are solely a result of broad NF-κB inhibition can be rejected, since all clusters of gene expression behaviours in response to IL-10 contained approximately similar proportions of MLN-4924 sensitive and insensitive genes. Furthermore, the spread of the data in the PCA analysis separates the LPS-stimulated samples with the higher dose of MLN-4924 form the LPS + IL-10, further indicating that the conditions are not equivalent.

The luciferase reporter data however does suggest that IL-10 is capable of inhibiting NF-κB transcription. This could also be explained however if the protein complexes linking NF-κB to increasing transcription, were altered in the presence of IL-10

Many studies have suggested IL-10's gene specific inhibition derives from an inhibition of NF-κB based transcription (Smallie et al., 2010, Hutchins et al., 2015) and the enrichment of LPS activated transcription factors that are not NF-κB (such as AP-1 or c-JUN) at the promoters of

genes, not inhibited by IL-10, would have supported this as a potential explanation of the gene expression patterns.

Microarray analysis of the NF-κB dependence of the macrophage LPS response highlighted a number of LPS-induced genes which showed a moderate drop in expression when treated with MLN-4924. This drop in expression also did not appear to be dependent on MLN-4924 concentration as increasing the dose of MLN-4924 did not inhibit expression more effectively.

This moderate reduction in LPS-induced gene expression by MLN-4924 could be explained by one of three hypotheses:

1) NF-κB responsive enhancive elements contribute to the induction of gene expression but other LPS-activated transcription factors (such as c-Jun or AP-1) are dominant in the LPS induction of these genes.

2) MLN-4924 blocks the induction of an NF-κB dependent mRNA stabilising protein and this reduction in transcript stability could be shown as increased mRNA abundance in the microarray.

3) MLN-4924 is able alter the activity of transcription factors or mRNA stabilising proteins.

MLN-4924 has recently been shown to prevent the activity of the transcription factor IRF3 by preventing its recruitment to chromatin independently of its inhibition of neddylation manner (Song H, 2016). The exact mechanism of this inhibition is yet to be clearly defined but it highlights that the inhibitor may have undescribed effects on other transcription factors. However, by using multiple MLN-4924 concentrations in the microarray and focusing on the genes that only operate in a dose dependent manner, such side effects should be avoided in interpreting the microarray results.

Chapter 5 – An upstream evolutionarily conserved region mediates the cooperative regulation of *TNFSF9* by LPS and IL-10

5.1 – Introduction

The requirement for STAT3 in IL-10's inhibition of gene expression has been long established (Williams et al., 2004a, Williams et al., 2007, Bosmann et al., 2014). However few studies have attempted to interrogate the mechanisms of LPS/IL-10 cooperative induction of gene expression. Work in (Denys et al., 2002) and (Smallie et al., 2010), established that IL-10 is able to inhibit luciferase reporters with cloned sections of the TNF locus in MDM. The implication of these experiments is that the gene-specific inhibitory effects of IL-10 require the regulatory elements for that gene. In the case of *TNF*, the small size of the locus and highly conserved regions of intergenic DNA highlight the local elements required for its transcriptional regulation.

IL-10's effect on transcription (as detailed in the previous chapters) is not completely antagonistic to inflammatory signalling. Instead, a subset of genes are able to be induced synergistically, which is visible at the level of primary transcripts. In the (Dillow et al., 2014) study, these LPS/IL-10 cooperatively regulated genes were characterised in human and mouse myeloid cell lines. The study also cloned a 2.5kb fragment upstream of the human TNIP3 gene (a target of LPS/IL-10 cooperativity) into a GFP reporter construct. This reporter demonstrated increased expression in response to stimulation with LPS and LPS+IL-10, but not IL-10 alone. Therefore I hypothesised that other LPS/IL-10 cooperatively-regulated genes, at the level of transcription, are controlled by distinct regulatory elements which can operate in isolation.

The intracellular signalling pathways contributing to LPS/IL-10 cooperativity are largely unknown as stimulation of cells with LPS or IL-10 induce a broad range of signalling pathways. Many of these signalling pathways are known to activate transcription factors, or modulate the activity of

transcription factors, to fine-tune a cellular response to a stimulus. In order to assess the role of some of the major LPS and IL-10-activated signalling pathways, I utilised small molecule inhibitors to abrogate downstream signalling of pathways. These inhibitors were: MLN-4924 (NF-κB transcriptional inhibitor), BAY 11-7082 (inhibitor of IKK), SB2035580 (p38 MAPK inhibitor), JNK-in8 (JNK inhibitor), Ruxolitinib (JAK1/2 inhibitor), Wortmannin and, LY290002 (inhibitors of PI3K activity). The IKK, p38, JNK and, PI3K signalling pathways are activated by LPS in macrophages(Lu et al., 2008). This activation of signalling has been shown by previous studies to occur rapidly after the addition of stimulus (Sato et al., 2005, Bauerfeld et al., 2012). JAK1 activation has been previously shown as the dominant signalling pathway induced by IL-10 (Pattison et al., 2012, Finbloom and Winestock, 1995). However, the PI3K/Akt pathway has been shown to be activated by IL-10 stimulation, which contributes to the anti-apoptotic functions of IL-10 (Antoniv and Ivashkiv, 2011, Zhou et al., 2001, Crawley et al., 1996). The inhibition of JAK1/2 activity has been recently explored as a therapeutic target for inflammatory disease and as such a new generation of specific inhibitory compounds for use in the clinics have been synthesised and described.

5.2 – Results

5.2.1 – IL-10 cooperative regulation, but not inhibition of transcription is a primary event that is independent of *de novo* protein synthesis

A previous study (Murray 2005) used the protein synthesis inhibitor cycloheximide to try to determine whether IL-10's inhibition of inflammatory genes required new protein. However, previous studies have noted that inhibiting protein synthesis has a stabilising effect on the cellular

pool of mRNA by preventing mRNA regulator protein production (Han et al., 1991b, Han et al., 1991a, Beutler et al., 1986). To counteract these effects on mRNA stability, I utilised ChIP to investigate RNA polymerase II (Pol II) enrichment at downstream coding regions of genes, to assess the transcriptional elongation.

10^7 primary human macrophages were pre-treated with DMSO control or 5μg/mL cycloheximide to inhibit *de novo* protein synthesis prior to stimulation with LPS, IL-10 or in combination for 60 minutes. The cells were then assayed for Pol II enrichment in the downstream regions of the genes: *TNF*, *NFKBIA*, *TNFSF9* and, *IL6*.

To assess whether the treatment of cycloheximide was effective at blocking protein synthesis at these time points, the cell culture supernatants of stimulated cells were analysed by ELISA for the secretion of TNFα. Figure 5.1 shows that TNFα production in macrophages pre-treated with cycloheximide was completely abrogated, with all the cycloheximide samples containing less than the detection threshold of the ELISA (<13pg/mL). After determining the effectiveness of the cycloheximide treatment, I assessed the rate of transcription by examining the downstream regions of: *TNF*, *NFKBIA, TNFSF9* and *IL6* (schematic of the QPCR primer locations shown in Figure 5.2). *TNF*, *NFKBIA, TNFSF9* were selected for analysis by ChIP due to the differential effects of IL-10 on their LPS-induced gene expression (inhibited, insensitive and cooperatively regulated respectively), while *IL6* was selected to confirm the effectiveness of the cycloheximide, since the transcription of *IL6* requires *de novo* protein to initiate transcription. (Ramirez-Carrozzi et al., 2009). In the downstream regions of *TNF*, *NFKBIA* and *IL6* a clear LPS-induced enrichment of Pol II was observed. *TNFSF9* on the other hand demonstrated no enrichment of Pol II at the

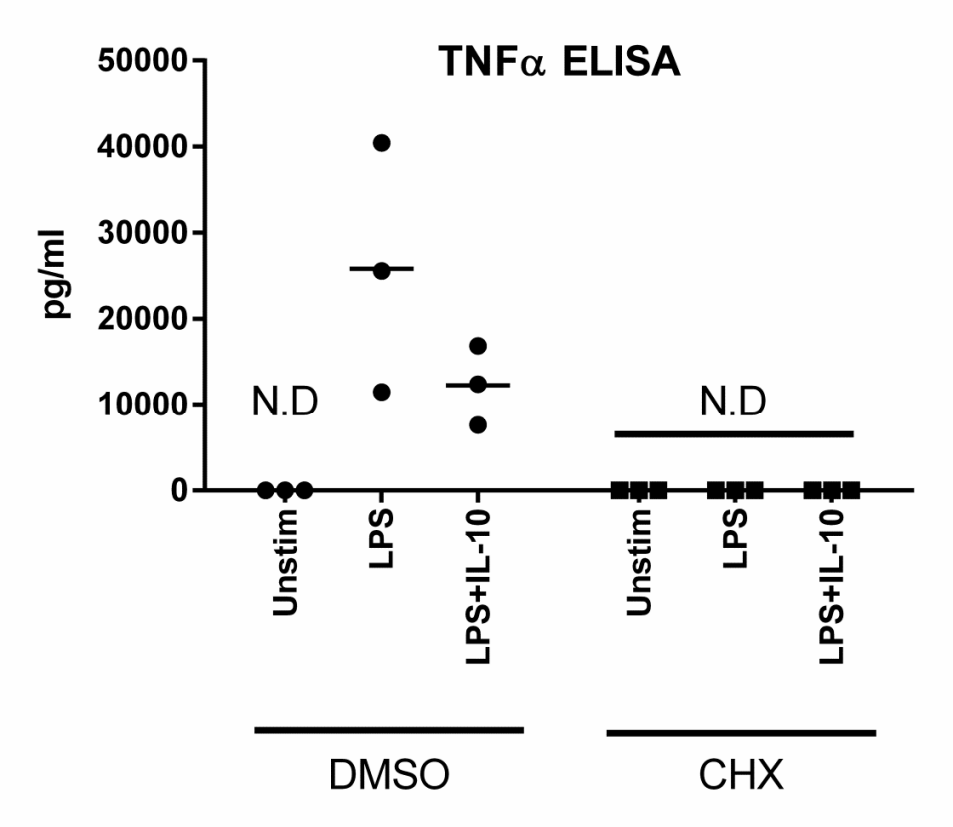

Figure 5.1 – Cycloheximide treatment of primary human macrophages blocks TNFα release

10^7 primary human macrophages were pre-treated with DMSO or 5µg/mL cycloheximide for 20mins prior to stimulation with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 60 minutes. Cell culture supernatants were then removed and analysed for TNFα production by ELISA. N.D, No TNF detectable.

downstream regions (Figure 5.3). On cycloheximide treatment, the enrichment of Pol II on LPS stimulation was not affected at the downstream regions of *TNF* and *NFKBIA*. At the downstream region of *TNFSF9* there no statistical increase of Pol II enrichment compared to DMSO control. At the downstream region of *IL6*, the LPS-induced enrichment of Pol II was blocked by cycloheximide treatment, remaining at the level of Pol II in unstimulated cells. This stands to reason as *IL6* is a secondary response gene on LPS stimulation and requires *de novo* protein synthesis to initiate transcription.

On addition of IL-10 in combination with LPS, there was a statistically significant inhibition of Pol II enrichment at the *TNF* downstream region, while simultaneously there was a cooperative enrichment of Pol II in the downstream region of *TNFSF9,* mirroring the effect seen at the mature and primary transcript level in chapter 3. Pre-treatment of macrophages with cycloheximide completely abolishes the ability of IL-10 to inhibit Pol II enrichment downstream of TNF. This loss of IL-10's inhibitive ability was distinct from its cooperative regulation at *TNFSF9*, which was unaffected by the addition of cycloheximide.

The inability of IL-10 to inhibit Pol II recruitment to the TNF locus in the presence of cycloheximide can be explained in one of two ways. Either the inhibition of TNF by IL-10 requires new protein synthesis or the cycloheximide pre-treatment was able to interfere with IL-10 signalling to prevent the activation of STAT3, which was required to inhibit TNF. To investigate whether cycloheximide interferes with IL-10 signalling, $2x10^6$ primary human macrophages were pre-treated for 20min with DMSO or 5μg/mL cycloheximide, prior to stimulation with LPS, IL-10 or in combination for 30 or 60 minutes. Cells were then harvested for protein and analysed by western blot for STAT3 tyrosine 705 phosphorylation and total STAT3 levels.

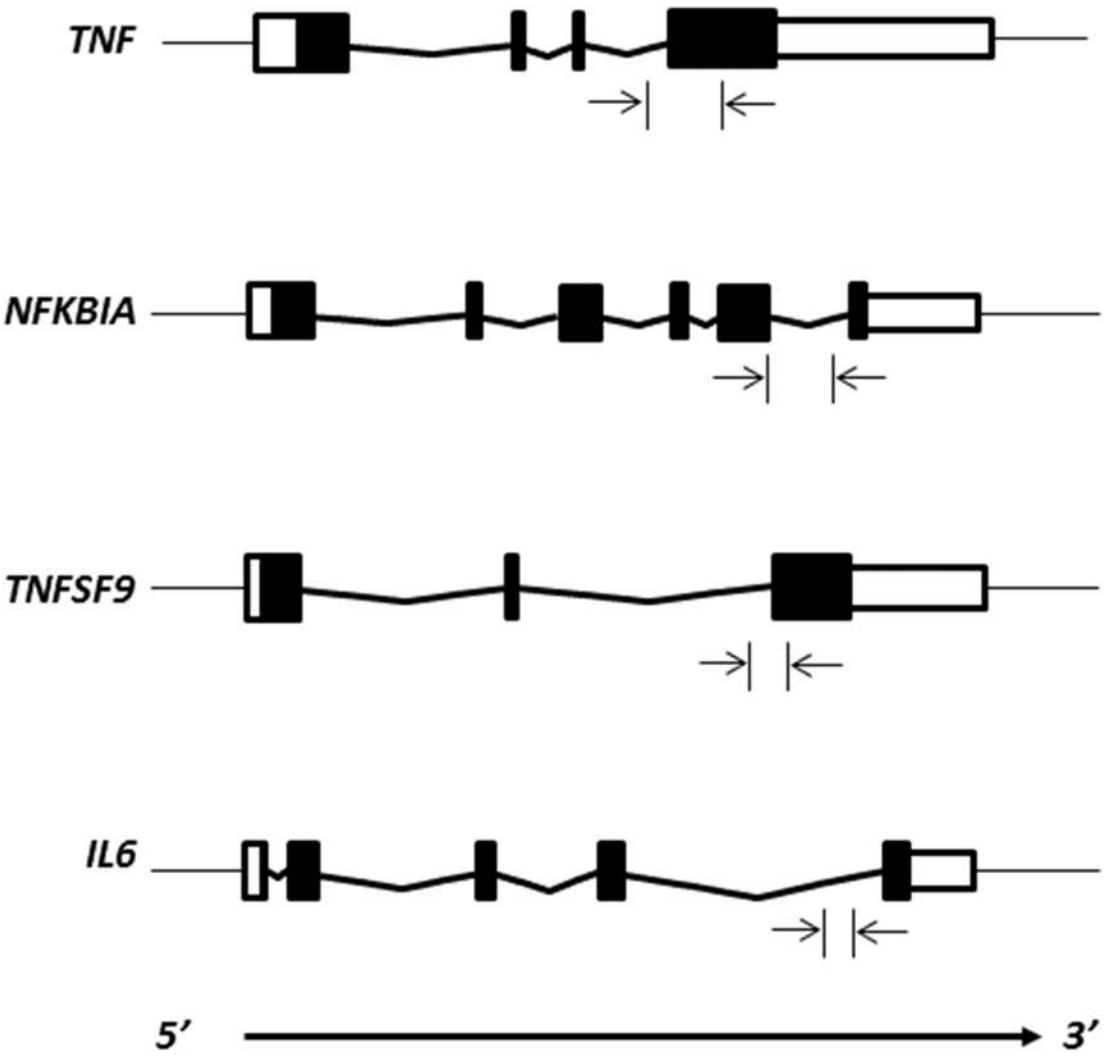

Figure 5.2 – Schematic representation of Pol II ChIP assay locations

Schematic of the intron-exon structure of genes selected for analysis by ChIP. Black bars indicate exons, white bars indicate untranslated regions and bent lines denote introns. Arrows show the locations of the amplicons.

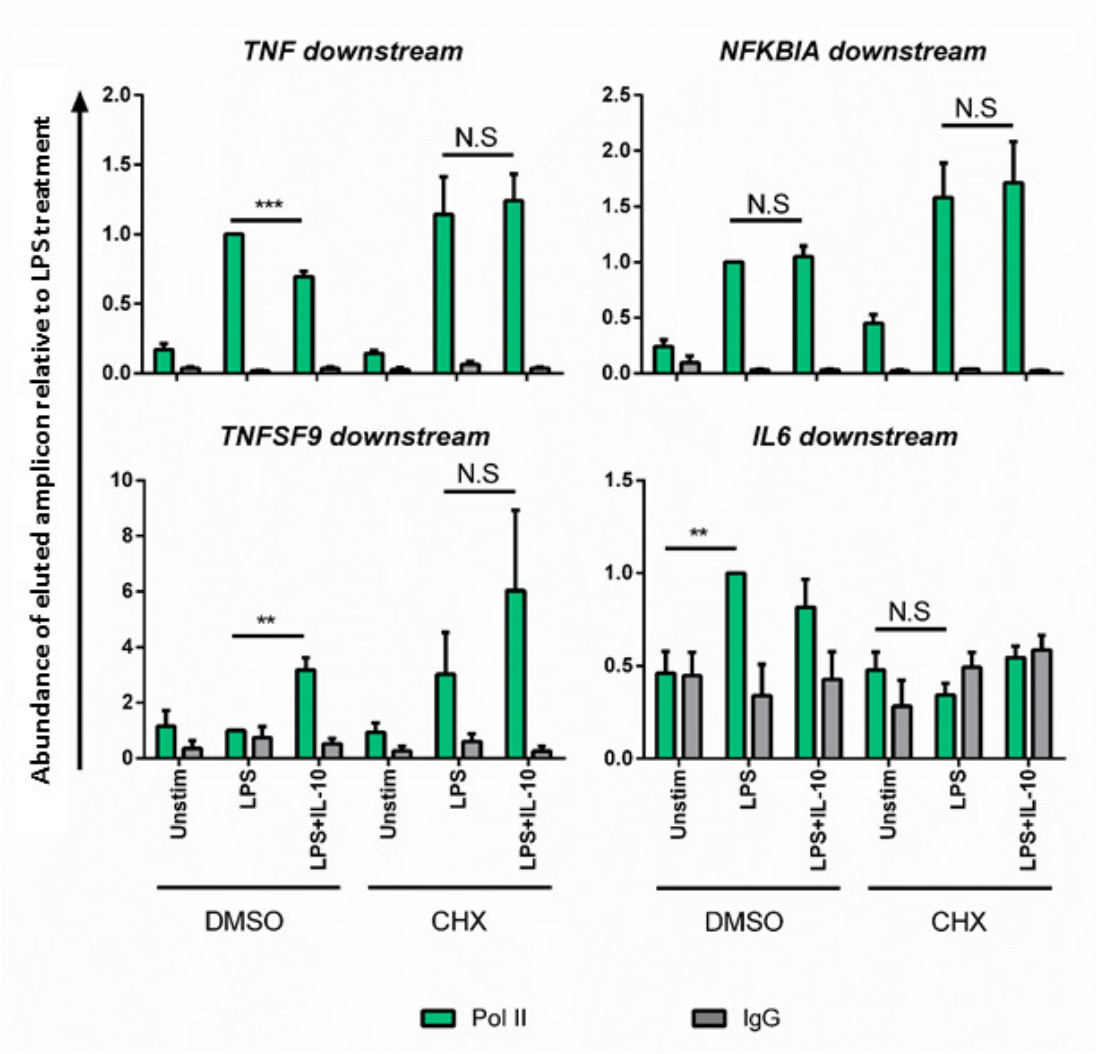

Figure 5.3 – Cyloheximide blocks IL-10 inhibition of RNA polymerase II enrichment at downstream regions of *TNF* but not *TNFSF9* and *NFKBIA*

10^7 primary human macrophages from 3 independent donors, were pre-treated with DMSO or 5µg/mL cycloheximide for 20mins prior to stimulation with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 60 minutes. Cells were analysed by ChIP for RNA polymerase II (Pol II) or Isotype control (IgG) at downstream regions of genes. Enrichment of Pol II was normalised to the LPS and DMSO treated sample. Graphs represent data from 3 independent experiments. Error bars represent SEM and statistical significance calculated by paired Student's t-test ** $p<0.01$, *** $p<0.001$, N.S $p>0.05$

Figure 5.4 show that the addition of IL-10 induced a strong phosphorylation of the tyrosine 705 residue at both 30 and 60 minutes. This phosphorylation is required for SH2-dependent STAT3 dimerisation, therefore it is an indicator of STAT3 activation. There was also a weak phosphorylation of Y705 after 60 minutes of LPS stimulation, possibly indicating that LPS-induced IL-10 production was beginning to induce signalling in the macrophages. This was supported by the observation that the LPS-induced phosphorylation was blocked on treatment with cycloheximide, which would block *de novo* IL-10 production.

5.2.2 – An evolutionarily conserved region upstream of TNFSF9 is able to cooperatively regulate gene expression in response to LPS and IL-10

Since the cooperative effect of LPS and IL-10 on TNFSF9 expression was observable at the level of primary transcript production and was independent of protein synthesis, the elements that control the expression of *TNSF9* must be readily available to bind signal-activated transcription factors in macrophages.

To first narrow the search around the TNFSF9 locus, I used the mouse macrophage like cell line RAW264.7 (RAW) to determine if the cooperative activity of LPS and IL-10 on TNFSF9 was conserved. Figure 5.5 shows that similar to IL-10 does not significantly induce *Tnfsf9* expression on its own, but cooperated with LPS to induce a higher level of *Tnfsf9* expression at 60 minutes. However the cooperative expression was not sustained and was lost by 120 minutes of stimulation. Despite the lower levels of cooperative expression in this cell line, it can be

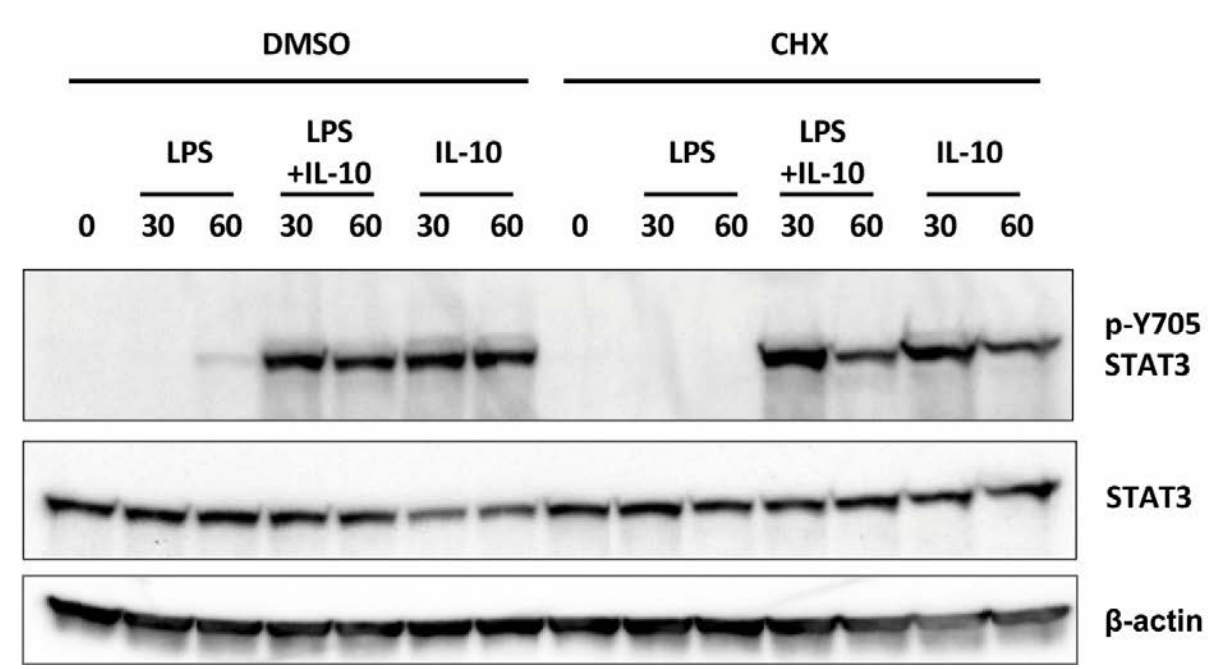

Figure 5.4 – Cycloheximide does not alter STAT3 tyrosine 705 phosphorylation

$2x10^6$ primary human macrophages were pre-treated with DMSO or 5µg/mL cycloheximide for 20mins prior to stimulation with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30 or 60 minutes. Cells were harvested and analysed for STAT3 activation by SDS PAGE and western blot. Representative blot of 2 independent experiments

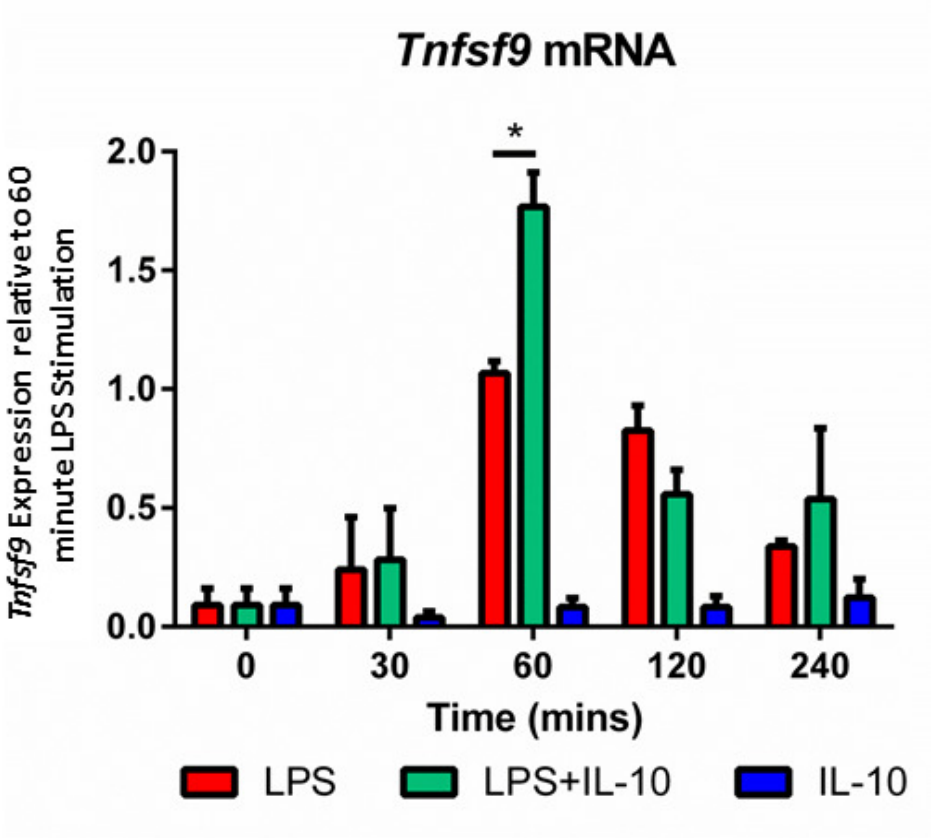

Figure 5.5 – *Tnfsf9* is cooperatively regulated by LPS/IL-10 in RAW 267.4 macrophages.

$0.8x10^6$ RAW 264.7 cells seeded overnight and were then stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120 or 240 minutes. Cells were then harvested for RNA and analysed for *Gapdh* and *Tnfsf9* expression by SYBR green QPCR.The expression of *Tnfsf9* was first normalised to the *Gapdh* values in each sample, then normalised to the LPS-stimulated sample. Statistical significance calculated between LPS and LPS+IL-10 treated samples using students t-test (*= $p<0.05$) Graph represents 3 independent experiments.

hypothesised that the DNA element or elements controlling the cooperativity are conserved between mouse and human.

Examining the conservation of the *TNFSF9* locus between multiple species (Figure 5.6) it is clear that the locus only showed one clear evolutionary conserved region (ECR) between mouse and human. This ECR was highly conserved between species, even when there was a low conservation between the protein coding regions. Due to this level of conservation between species and the proximity to *TNFSF9* TSS in humans and mouse I hypothesised that this region of DNA had a functional role in the transcription of *TNFSF9*.

To assess whether this ECR could be accessible for transcription factor binding in human macrophages, I used publically available ChIP-seq and Dnase-seq datasets from similarly derived macrophages to assess the chromatin accessibility and transcriptional activity of the *TNFSF9* locus. I examined markers of chromatin accessibility (DNase-seq and PU.1 ChIP-seq) and histone marks associated with transcriptionally active regions (H3K4 mono and tri methylation (H3K4me1/H3K4me3 respectively) and H3K27 acetylation (H3K27ac)). The H3K4me1/3 and DNase-seq data was acquired from the BLUEPRINT consortium (*Saeed* et al. (2014)) "macrophage" dataset, while PU.1 and H3K27ac tracks were acquired from *Pham* et al. (2012) "monocyte-derived-macrophage (MAC)" dataset.

Figure 5.7 shows that in these macrophages there are high levels of H3K27ac and H3K4me3 around the transcription start site of *TNFSF9*, indicating a transcriptionally active or poised region. There was also H3K27ac enriched regions surrounding a PU.1 binding site, immediately upstream of the ECR. Unlike the TSS, this region lacks H3K4me3 marks and instead was enriched for high

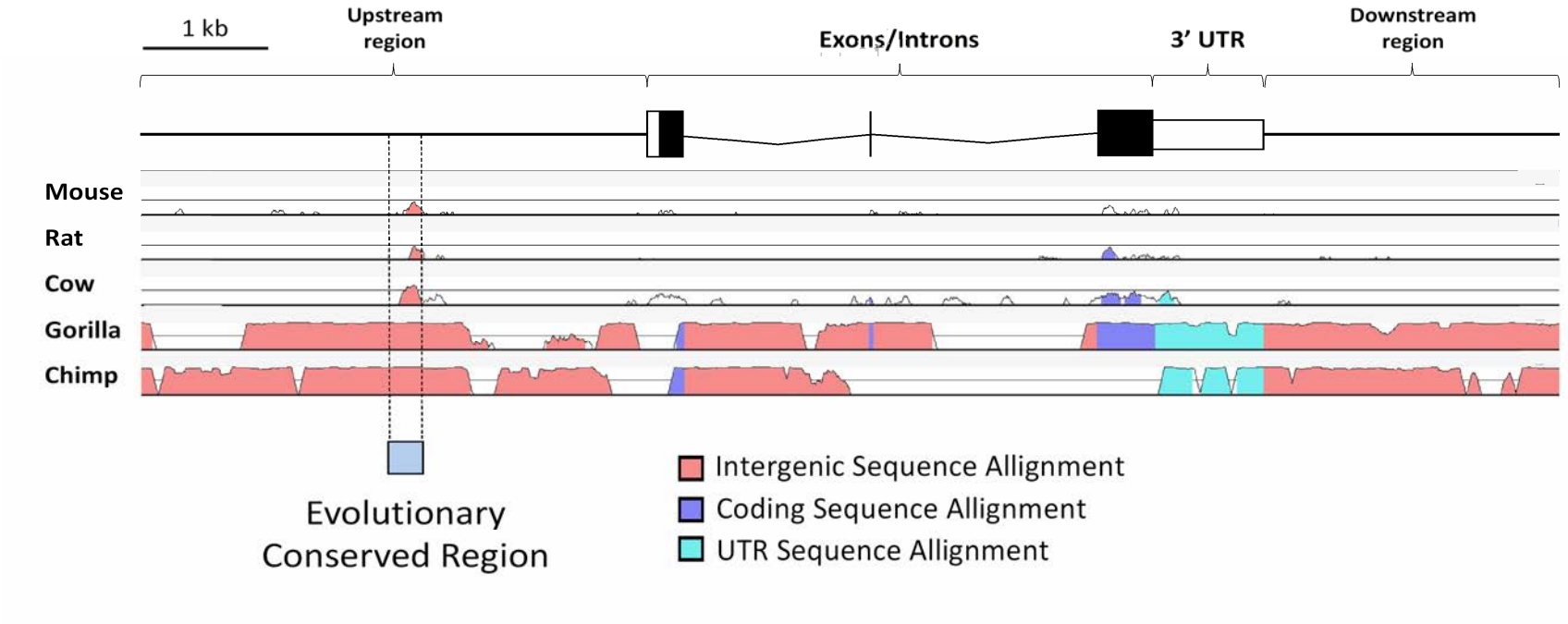

Figure 5.6 – *TNFSF9* gene locus conservation

Comparison of Mouse, Rat, Cow, Gorilla and Chimp alignments to the Human *TNFSF9* locus. Y axis showing % identity to the human reference sequence and X axis representing the nucleotide position. The dotted lines bisecting the conservation plots highlights a region of high evolutionary conservation.

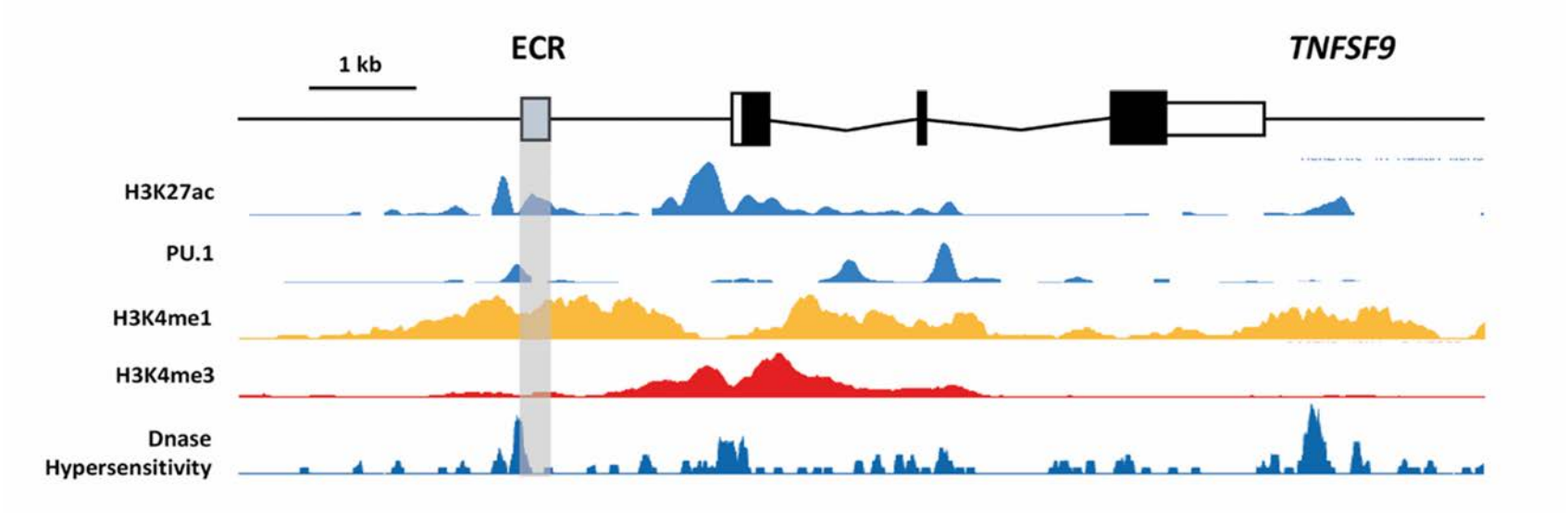

Figure 5.7 – TNFSF9 ECR contains marks of a poised enhancer in macrophages

ChIP-seq data from *Pham* et al (2013), the BLUEPRINT consortium and, UCSC, showing histone marks, PU.1 binding ,DNase hypersensitivity peaks and, sequence conservation between 100 vertebrate species. X axis showing aligned nucleotide position and Y axis represents the number of aligned reads from samples immunoprecipitated for the proteins shown, or the conservation score calculated by UCSC.

levels of H3K4me1. The combination of these histone marks has been previously suggested to signal an active transcriptional enhancer. The ECR also overlaps with part of a DNase hypersensitive region in macrophages indicating there are areas of chromatin that are accessible for transcription factors to bind.

To interrogate potential transcription factor binding sequences in the ECR the human reference sequence for the region (hg19) and syntenic sequences from: chimp (PanTro4), gorilla (gorGor3), mouse (mm10), rat (rn5) and cow (bosTau7) were downloaded from UCSC genome browser and aligned with each other using the ClusterOmega alignment tool.

Figure 5.8 shows that after the divergence of these species there have been several insertions and deletions the ECR upstream of TNFSF9. The Chimp and Gorilla sequences had high similarity to the human sequence. The Chimp sequence of this region was identical to the human sequence and the Gorilla sequence contained only one base substitution in the region. The sequences for the other mammals selected (mouse, rat and cow) all showed lesser degrees of identity compared to the human sequences (), with mouse and rat only having roughly 50% identity compared to the human sequence due multiple insertions and deletions. The cow sequence on the other hand had a higher similarity score to the human and great ape sequences than the rodent sequences. This was due to a number of substitutions in the sequence as well as a 9 nucleotide deletion at positions 36 to 44 (Figure 5.8).

The major area of sequence conservation of the ECR is located between bases 112 and 234 of the alignment in Figure 5.8. This area contains a very few small indels and had sections of high sequence similarity. Analysiis of these sequences through the transcription factor motif finder

JASPAR, identified two NF-κB sites (highlighted in the red boxes) and one STAT motif (highlighted in blue).

The first NF-κB motif (sequence "GGGAATTTCTC" in the human sequence) was highly conserved between the selected species, with only the rat genomic sequence varying from the consensus sequence. The second detected NF-κB site contains more variation across different species, with cow, rat and mouse sequences containing; 1, 3 and 4 nucleotide substitutions respectively. This could be because it may not represent a complete NF-κB binding motif and instead resembles part of the canonical NF-κB consensus sequence. Similarly, the detected STAT site (positions 184-194 of) had multiple nucleotide substitutions between the primate, mouse, rat and cow sequences, indicating that there may have been less selective pressure to retain this putative STAT binding site.

In order to examine other potential transcription factor binding sites within the TNFSF9 ECR, I utilised the transcription factor binding motif program ALIBABA2 (Figure 5.9). A number of transcription factor binding sites were observed including the proposed NF-κB site at position 137 (which equates to position 128 in Figure 5.8). The second NF-κB site was also detected (positon 228) as two individual NF-κB motifs overlapping. These two "half-sites" form a less than ideal consensus motif and therefore it is unlikely NF-κB may bind directly to the sequence.

It's also notable that the STAT3 motif was absent in this analysis and in its place is a motif for MPBF (a previous name for STAT5 (Burdon et al., 1994, Hughes and Watson, 2012). The difference in motif between STAT3 and 5 is only a few nucleotides occurring in the centre of the motif, which

	Human	Gorilla	Chimp	Mouse	Rat	Cow
Human		100%	100%	50%	51%	75%
Gorilla	100%		100%	50%	51%	75%
Chimp	100%	100%		50%	51%	75%
Mouse	50%	50%	50%		90%	52%
Rat	51%	51%	51%	90%		52%
Cow	75%	75%	75%	52%	52%	

Table 5.1 – Percent identity matrix of the TNFSF9 ECR between species

Syntenic sequences of the human *TNFSF9* ECR were extracted from UCSC genome browser and aligned according to ClusterOmega algorithm. Percent identity matrix was then computed by UGENE with a gap penalty.

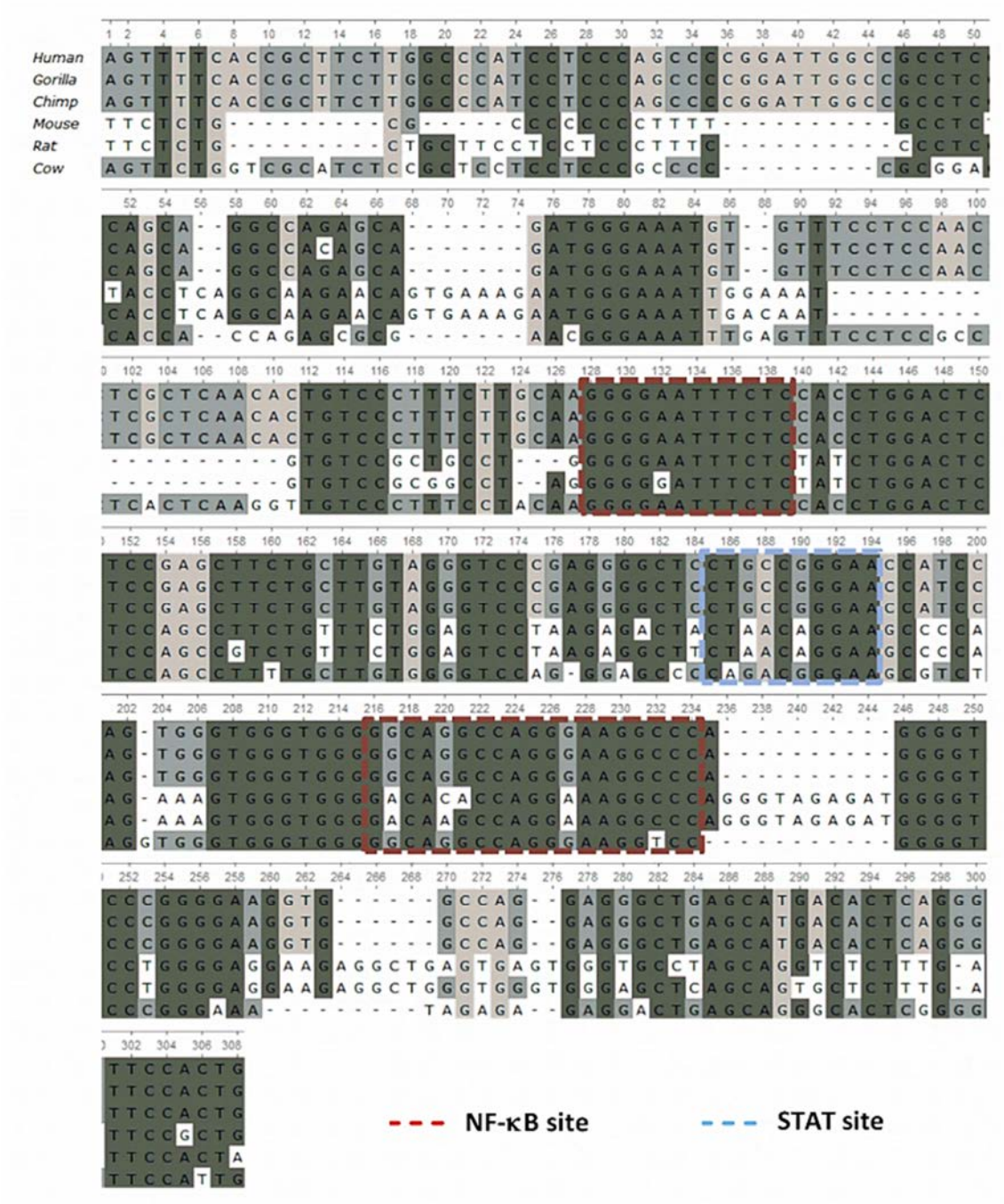

Figure 5.8 – TNFSF9 ECR contains conserved transcription factor binding sites

Gorilla, chimp, mouse, rat and cow *TNFSF9* conserved region sequences aligned to human reference sequence. Computed NF-κB and STAT3 binding sites are highlighted. The binding location of the primers used to amplify the human genomic sequence are indicated by the arrows.

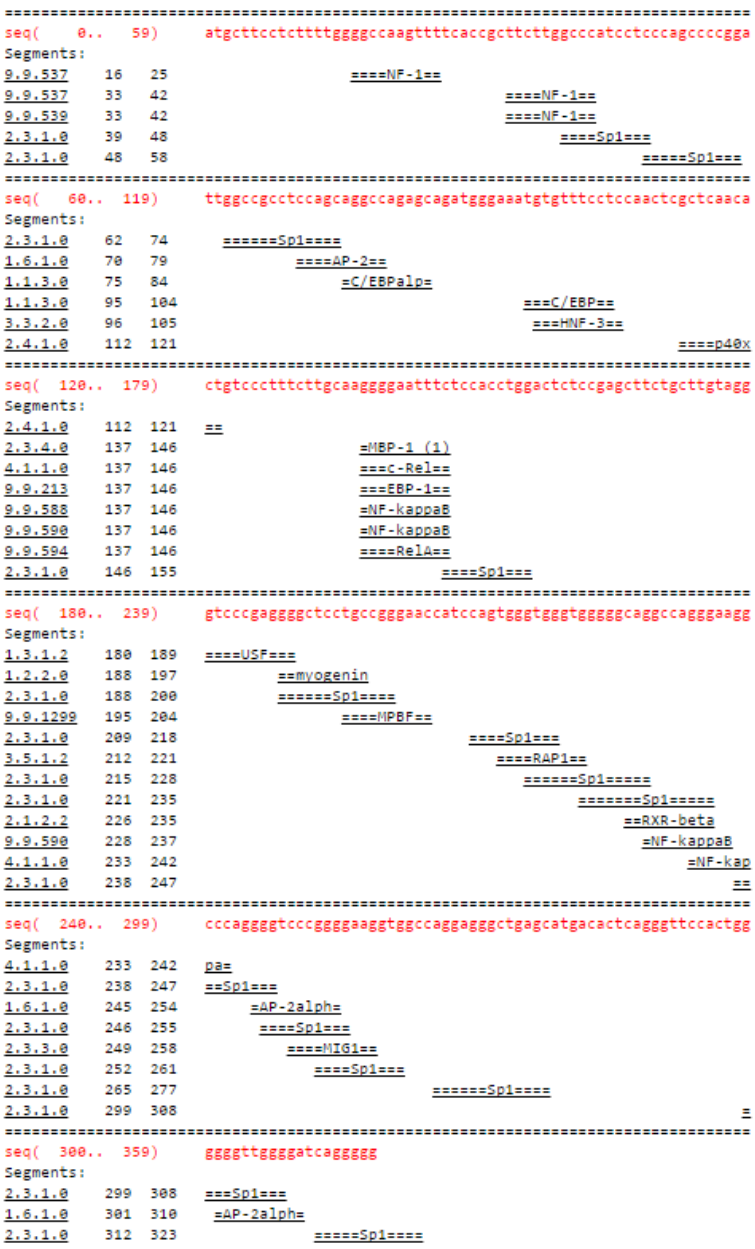

Figure 5.9 – Motif analysis of the TNFSF9 ECR

Transcription factor binding motifs present in the conserved TNFSF9 ECR were computed and visualised by the program ALIBABA2. Motifs were detected provided the exhibited a minimum of 75% sequence conservation with the JASPAR motif position weight matrix.

shows greater variability. This difference in nucleotide sequence could be enough to prevent binding.

To assess if the *TNFSF9* ECR was responsive to LPS and IL-10 stimulation, I subcloned the ECR from human genomic upstream of a luciferase open reading frame in a reporter plasmid containing a minimal promoter (pGL4.26). The plasmid containing the ECR or the vector alone was then transfected into RAW 264.7 cells overnight, before the cell culture medium was replaced and the cells stimulated with LPS and IL-10 for 120, 240 or 480 minutes.

Figure 5.10 shows a large difference between the luciferase activity of the ECR containing plasmid and the minimal promoter control in unstimulated cells. The minimal promoter control had a very low basal activity (231 luciferase units) while the ECR plasmid had a basal luciferase activity of approximately 3500 units (Two-way ANOVA p value of 0.009). This could indicate that the TNFSF9 ECR fragment had an innate enhancive effect on the reporter's transcription. On stimulation with LPS and IL-10, the ECR plasmid retained a consistently higher activity than the minimal promoter, throughout all time points.

After LPS+IL-10 the luciferase activity produced by the ECR plasmid increased significantly above the basal luciferase activity after 240 minutes and continued to increase until 480 minutes. The minimal promoter on the other hand was not able to increase the luciferase activity in a consistent manner. There was a slight increase in luciferase activity at 120 and 240, which was lost by 480 minutes.

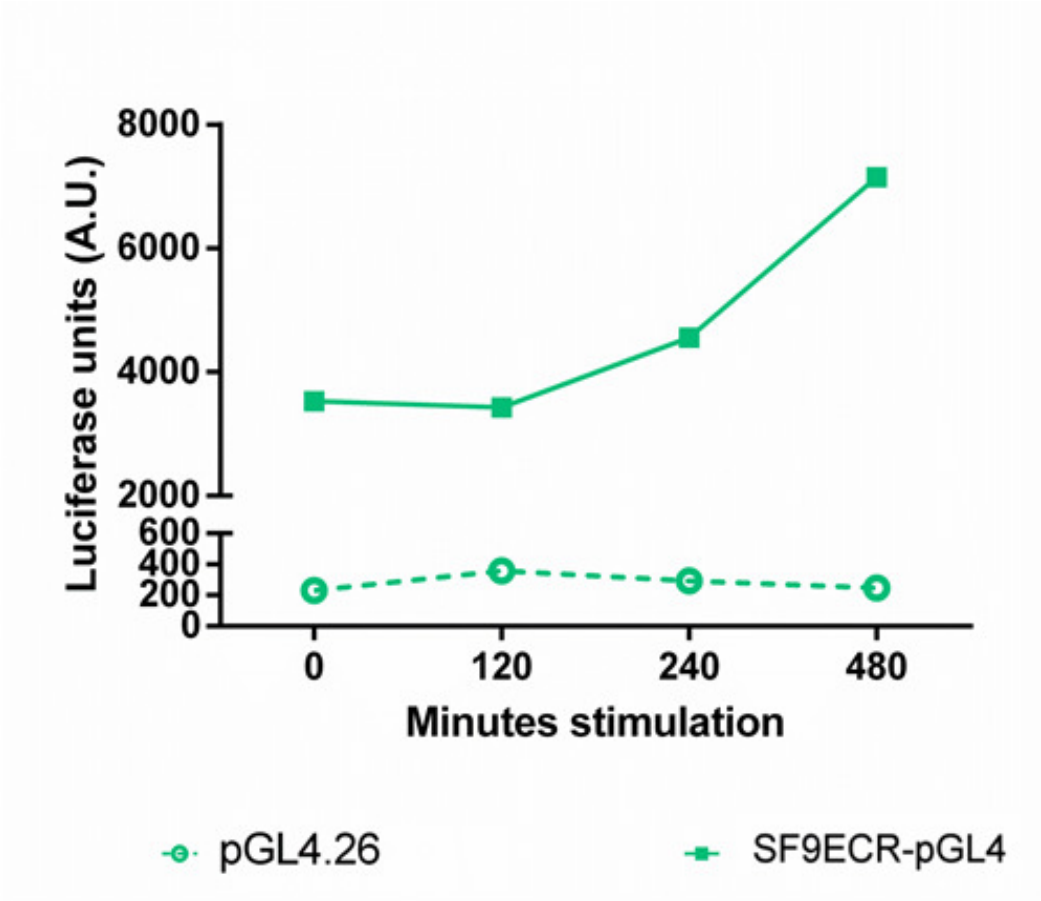

Figure 5.10- LPS+IL-10 time course of TNFSF9-ECR luciferase reporter

RAW 264.7 cells were transfected with empty PGL4.26 vector or TNFSF9-ECR luciferase reporter construct. The following day these cells were stimulated with 10 ng/mL LPS and 10 ng/mL IL-10 for; 120, 240 or 480 minutes. Cells were then harvested and analysed for luciferase activity. Data shown is a single experiment and error bars represent the standard deviation of 3 technical replicates.

Since the ECR containing plasmid was responsive to LPS+IL-10 stimulation in transfected RAW cells, I aimed to determine whether this responsiveness was a cooperative effect between LPS and IL-10 signalling or whether one signalling pathway had a dominant effect on the transcription of the reporter. To do this I stimulated plasmid-transfected RAW cells with LPS, IL-10 or in combination for 480 minutes and analysed the luciferase activity.

In Figure 5.11 there was a clear induction of luciferase activity when the ECR reporter-transfected RAW cells were stimulated with LPS, which did not occur in cells treated with IL-10 alone sample. However, on LPS+IL-10 stimulation there was a statistically significant increase in luciferase activity compared to the LPS alone stimulation. While there was a synergistic effect between LPS and IL-10 with this reporter, it was to a lesser extent than the maximal amount observed at the mRNA level. This could be due to changes to mRNA stability or changes in other areas of chromatin which allow *Tnfsf9* transcription to be enhanced.

Earlier in this study, I noted that *TNFSF9* expression was strongly impaired by inhibiting NF-κB activity with the inhibitor MLN-4924.Therefore I wanted to examine whether NF-κB activity contributed to the upregulation of the TNFSF9 ECR reporter by LPS. To inhibit NF-κB activity in RAW264.7 cells I used the inhibitor MLN-4924 and examined the expression of *Tnfsf9* after stimulation with LPS/IL-10 at 60, 120 and 240 minutes.

As can be seen in Figure 5.12, the treatment of RAW 264.7 cells with MLN-4924 leads to an inhibited production of *Tnfsf9* mRNA on stimulation with LPS and LPS+IL-10 at all timepoints. The cooperative induction seen at 60 minutes was heavily reduced in magnitude, after treatment with MLN-4924. However, the pattern of gene expression still persisted at this timepoint.

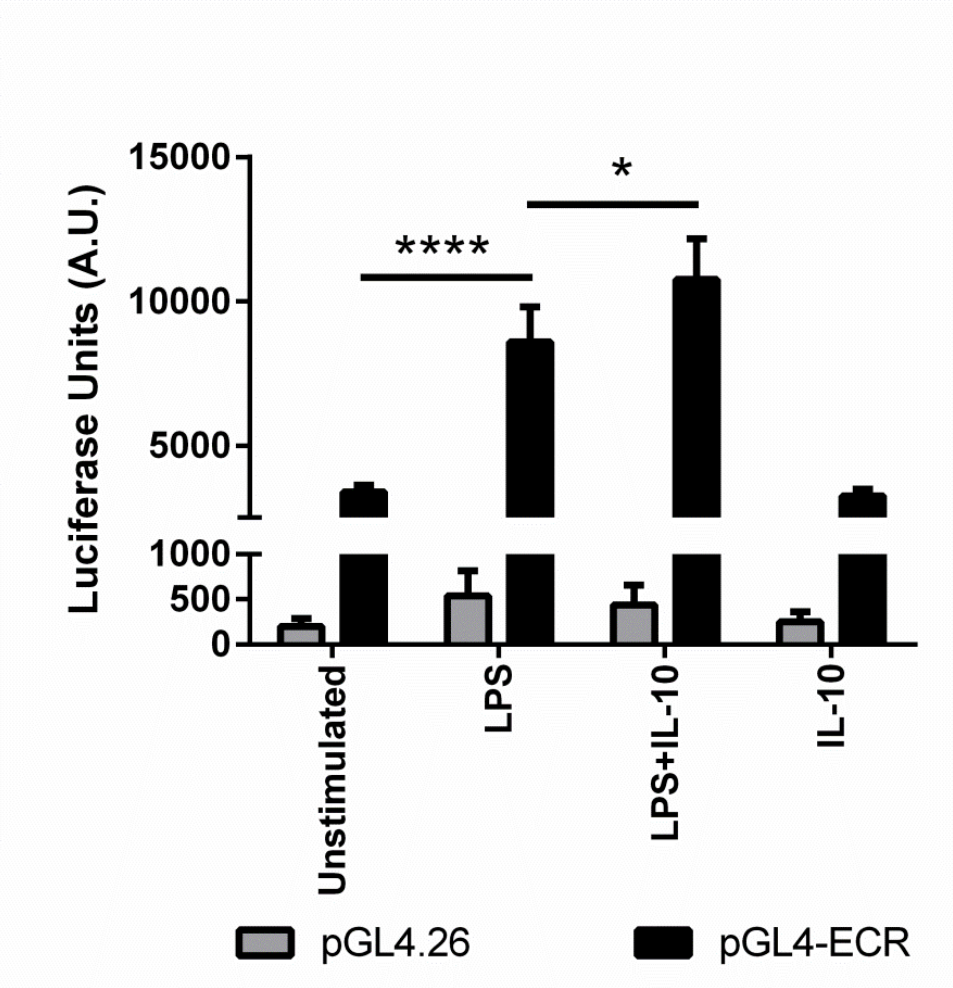

Figure 5.11 – Luciferase assay of TNFSF9-ECR reporter

RAW 264.7 cells were transfected with empty PGL4.26 vector or TNFSF9-ECR luciferase reporter construct. The following day these cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 480 minutes. Cells were then harvested for and analysed for luciferase activity. Statistical significance calculated using two-way ANOVA (* = $p < 0.05$, **** = $p < 0.0001$). Graph represents 5 independent experiments.

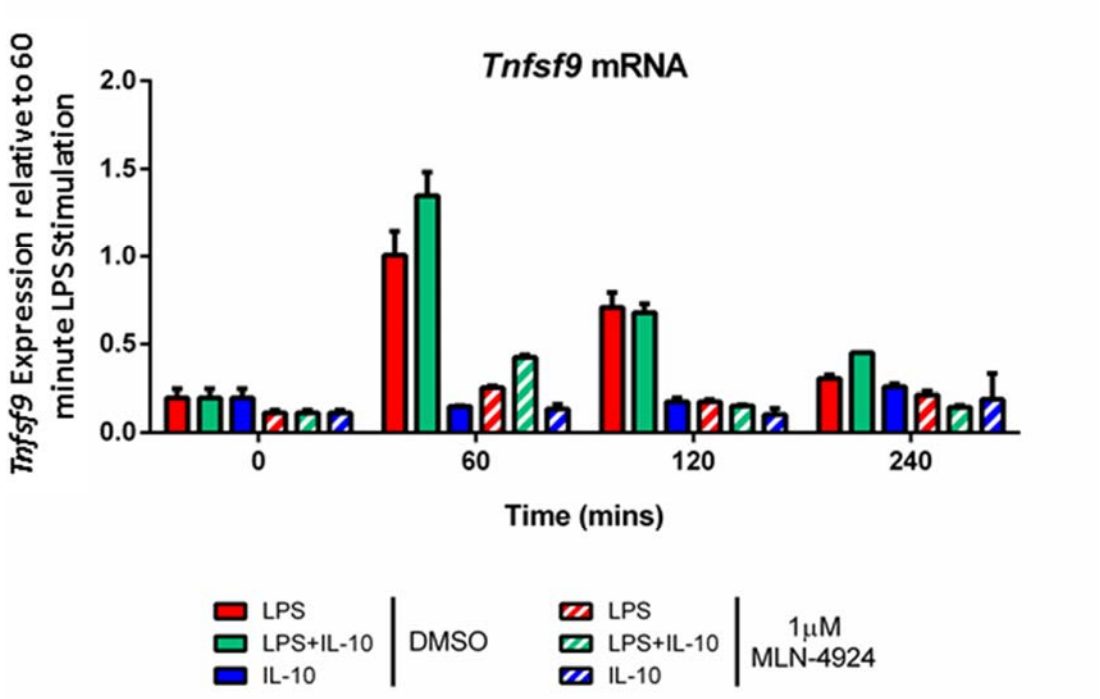

Figure 5.12 – MLN-4924 inhibits LPS induction but not LPS/IL-10 cooperativity of *Tnfsf9* expression in RAW 264.7 cells

10^6 RAW 264.7 cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 30, 60, 120 or 240 minute in the presence of DMSO or 1 μM MLN-4924. Cells were then harvested for RNA and analysed for *Gapdh* and *Tnfsf9* expression by SYBR green QPCR. Graph represents 1 experiment, triplicate measurements.

I then examined the effect of MLN-4924 on the TNFSF9 ECR reporter. RAW 264.7 cells were transfected with the TNFSF9 ECR reporter or minimal promoter overnight, before the cell culture medium being changed. The cells were then stimulated with LPS/IL-10 or in combination in the presence of DMSO or 1 μM MLN-4924. Cells were then lysed and analysed for luciferase activity. The high basal activity of the ECR reporter appeared to be independent of NF-κB since it was unaffected by the addition of MLN-4924 (Figure 5.13). Activation of the ECR reporter by LPS was abolished by MLN-4924, as was its cooperative regulation by LPS and IL-10. Both of these responses are therefore likely to be dependent on NF-κB.

Data detailed in chapter 4 also highlighted STAT3 activation as a critical factor induction of TNFSF9 gene expression by LPS and IL-10 but not by LPS alone. To inhibit STAT3 phosphorylation in these cells, I used the JAK1/2 inhibitor Ruxolitinib, which has previously been shown to inhibit phosphorylation of STAT3 in response to IL-10 (Pattison et al., 2012). When treated with Ruxolitinib the cooperativity between LPS and IL-10 was completely lost while the LPS induction of the reporter remained intact (Figure 5.14).

These experiments indicate that the LPS induction of this ECR reporter absolutely requires NF-κB, and when NF-κB transcriptional activity is compromised, cooperative regulation of the reporter plasmid is abolished. JAK1/2 activity is also required in order for the cooperative induction of this reporter by LPS and IL-10, but is dispensable for the induction by LPS alone.

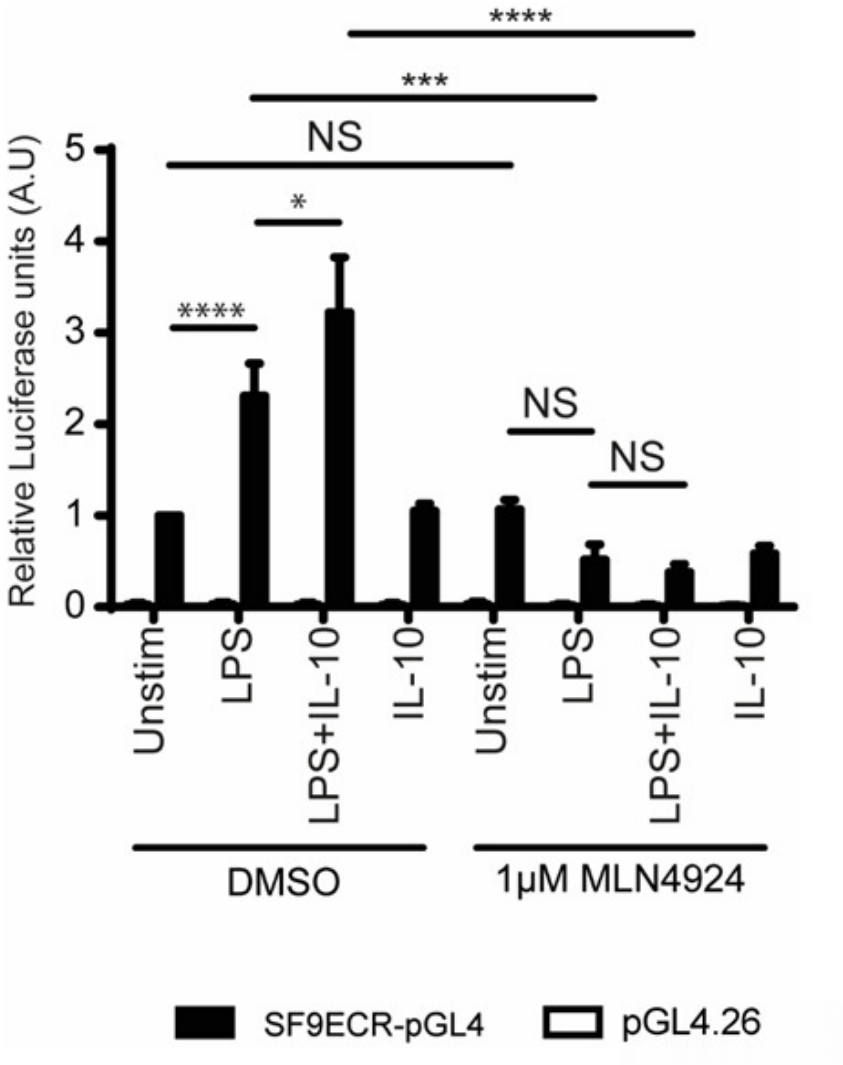

Figure 5.13 – MLN-4924 inhibits TNFSF9 ECR luciferase reporter LPS/IL-10 cooperativity

RAW 264.7 cells were transfected with empty pGL4.26 vector or TNFSF9-ECR luciferase reporter construct. The following day these cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 480 minutes in the presence of 1 μM MLN-4924 or DMSO control. Cells were then harvested for and analysed for luciferase activity. Two-way ANOVA (**** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, *=$p<0.05$, N.S = $p>0.05$). Graph represents 3 independent experiments

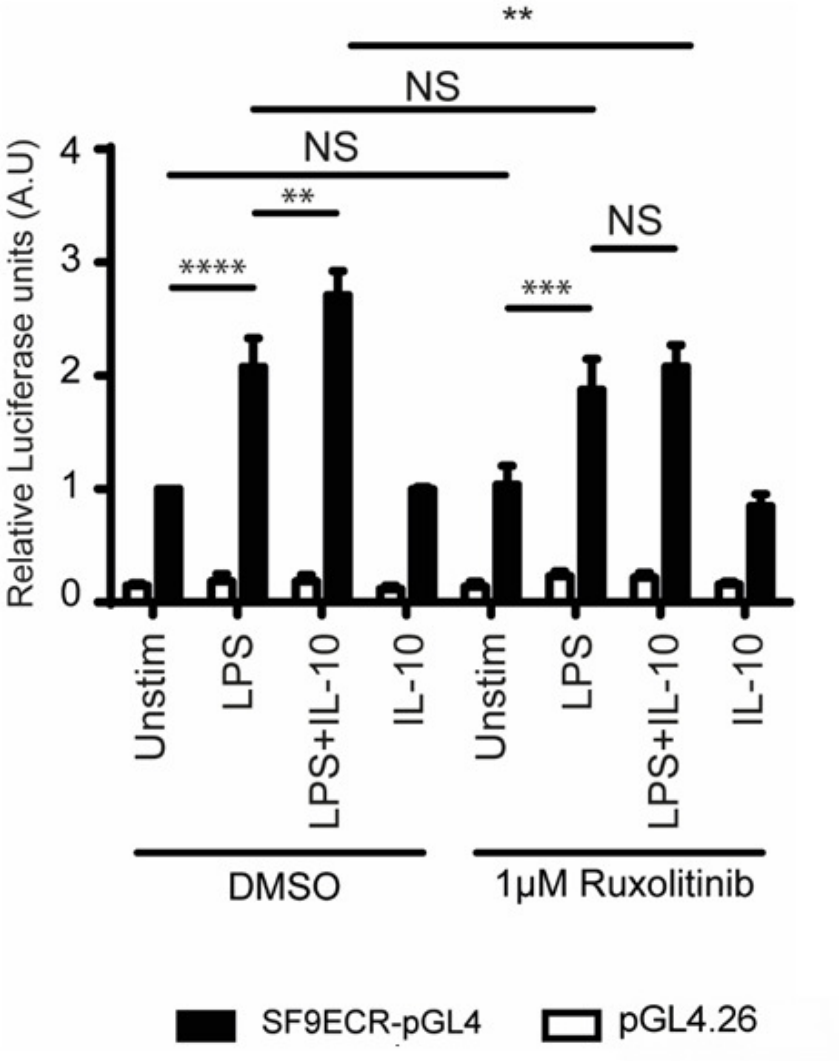

Figure 5.14 – Ruxolitinib inhibits TNFSF9 ECR luciferase reporter LPS/IL-10 cooperativity

RAW 264.7 cells were transfected with empty PGL4.26 vector or TNFSF9-ECR luciferase reporter construct. The following day these cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 or in combination for 480 minutes in the presence of 1 μM Ruxolitinib or DMSO control. Cells were then harvested for and analysed for luciferase activity. Statistical significance calculated by Two-way ANOVA (**** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, N.S = $p>0.05$). Error bars represent the SEM of 3 independent experiments.

5.2.3 – A STAT3 luciferase reporter exhibits LPS/IL-10 cooperativity

After observing the effect of IL-10 on the NF-κB reporter in chapter 2, I wanted to ascertain whether the transcriptional activity of STAT proteins was affected by the co-stimulation of LPS and IL-10. To do this I transduced primary human macrophages with an adenoviral STAT3 reporter (gifted by Dr Lynn Williams, University of Oxford). This reporter contained 4 repeats of the sequence: "GGTTCCCGTAAATGCATCA" (STAT3 binding site underlined) upstream of a TATA-like promoter and firefly luciferase coding region. After resting from transduction overnight, the cells were stimulated with LPS, IL-10 or in combination for 2 hours, before being lysed and assessed for luciferase activity.

Figure 5.15 demonstrates that after stimulation with LPS or IL-10, there is a statistically significant (p value = 0.018 and 0.0091), 2 fold upregulation of luciferase activity. When co-stimulated by LPS and IL-10, there is a significant 5.8 fold increase in luciferase (p value = 0.0008) activity compared to unstimulated cells. This cooperative effect is significantly more potent than the effect of LPS or IL-10 stimulation alone (p = 0.0036 and p = 0.0036 for LPS and IL-10 vs LPS+IL-10 treatments, respectively).

Previous work by Dillow et al. (2014) demonstrated that LPS/IL-10 cooperative regulation was sensitive to small molecule inhibitors of multiple cell signalling pathways. Therefore, I undertook to examine whether this luciferase reporter cooperativity was dependent on similar cell-signalling pathways. I treated the cells with the inhibitors: MLN-4924 (Nedd8/NF-κB pathway inhibitor), SB203580 (p38 MAPK inhibitor), JNK-in8 (JNK pathway inhibitor), BAY 11-7082 (IKK inhibitor), Ruxolitinib (JAK1/2 inhibitor), Wortmanin and LY294002 (PI3K inhibitors).

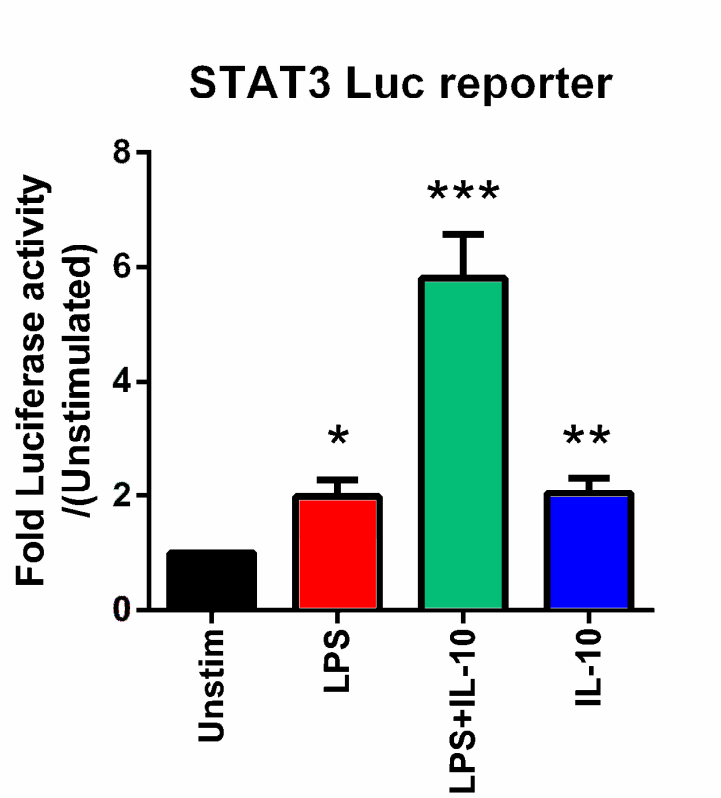

Figure 5.15 – STAT3 reporter activity on LPS/IL-10 stimulation

10^5 primary human macrophages were transduced with 100 M.O.I of a STAT3 luciferase reporter construct containing 4 STAT3 consensus motifs for two hours, before the cell culture media was changed. The next day the cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 ng/mL or in combination for 120 minutes. Cells were then harvested and assessed for reporter activity by luciferase assay. Statistical differences between unstimulated and stimulated samples were calculated by unpaired t-test for 4 independent donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Ruxolitinib completely blocked the activation of the reporter by LPS or IL-10 and its synergistic activation by both agonists, as well as slightly decreasing its basal activity (Figures 5.16 and 5.17). The involvement of JAK/STAT signalling in reporter responses to IL-10 were expected. The inhibition of the LPS response by Ruxolitinib indicates, unexpectedly, that this also depends on STAT activation. Neither MLN-4924 nor the IKK inhibitor BAY 11-7082 significantly affected reporter activity under any condition, demonstrating a lack of involvement of NF-κB in any of the responses tested. A JNK inhibitor (JNK-in8) also failed to influence reporter activity under any condition, but the MAPK p38 inhibitor SB203580 inhibited the response to LPS or LPS and IL-10 in combination, as well as slightly decreasing the basal activity of the reporter. This result suggests a convergence of between the MAPK p38 pathway and STAT3. Two PI3K pathway inhibitors, Wortmanin and LY29002 had contradictory effects on the reporter. Whilst LY29002 reduced basal LPS-induced and LPS + IL-10 induced activity, Wortmanin significantly reduced only the basal activity. The involvement of the PI3K pathway in the activation of the STAT reporter therefore remains unclear and requires further investigation.

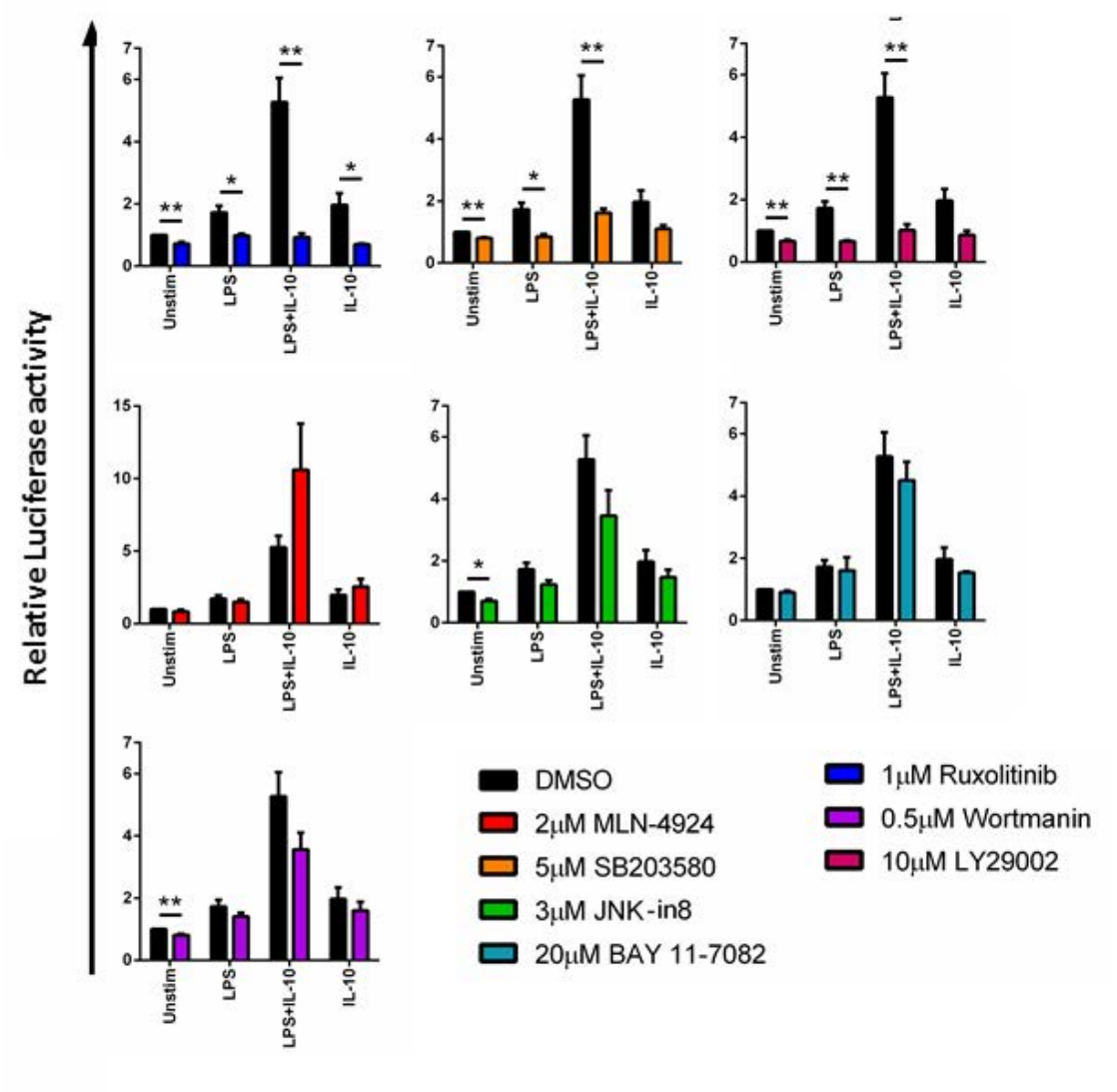

Figure 5.16 – Comparison of cell signalling inhibitors on STAT3 reporter activity

10^5 primary human macrophages were transduced with 100 M.O.I of a STAT3 luciferase reporter for two hours, before the cell culture media was changed. The next day the cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 ng/mL or in combination for 120 minutes with the shown concentration of inhibitor or DMSO control. Cells were then harvested and assessed for reporter activity by luciferase assay. Luciferase activity was then normalised to the DMSO treated unstimulated sample. Statistical differences between DMSO and inhibitor treated samples were calculated by unpaired t-test for 3 independent donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

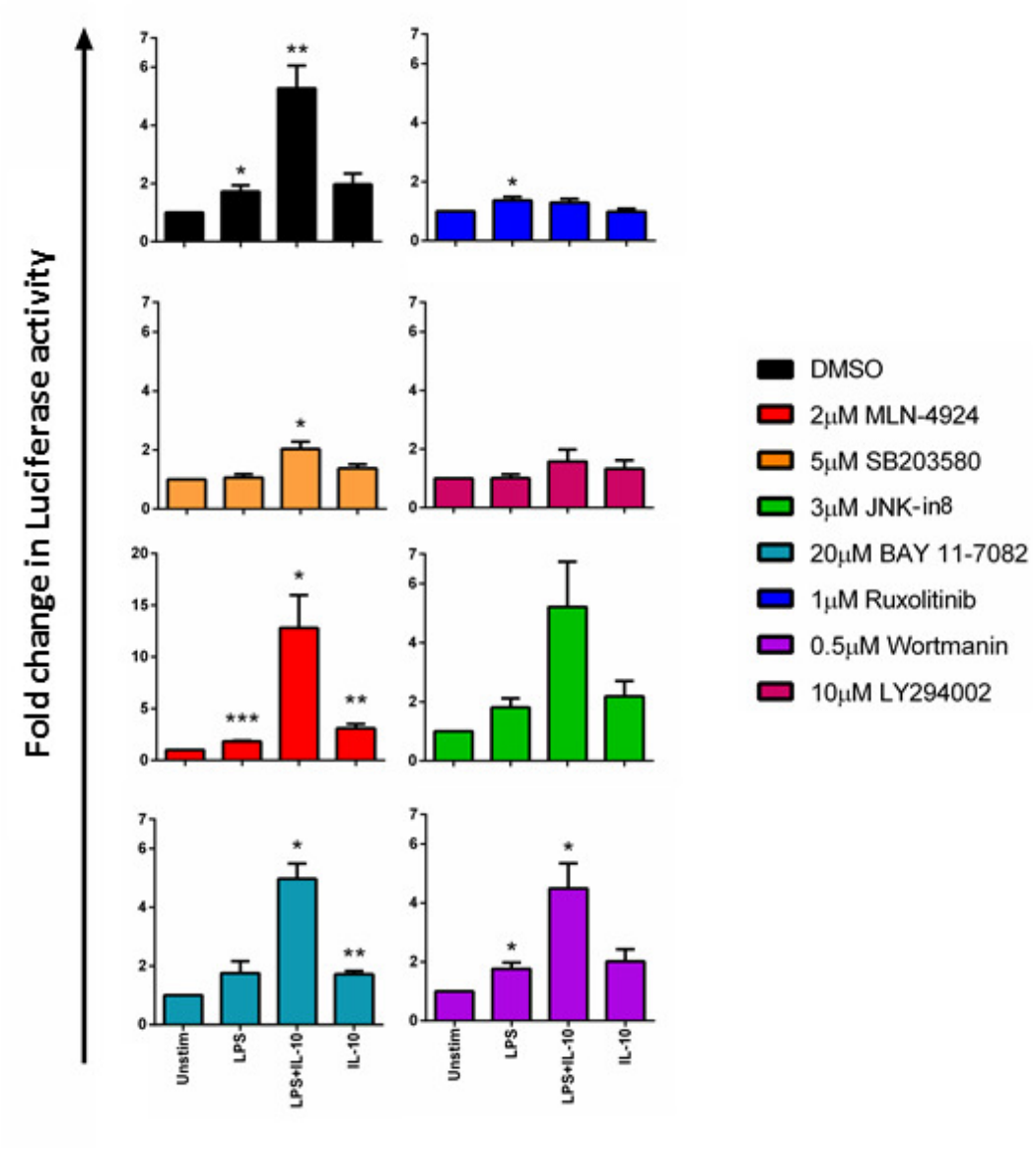

Figure 5.17 – Effect of chemical inhibitors of signalling pathways on STAT3 reporter activity

10^5 primary human macrophages were transduced with 100 M.O.I of a STAT3 luciferase reporter for two hours, before the cell culture media was changed. The next day the cells were stimulated with 10 ng/mL LPS, 10 ng/mL IL-10 ng/mL or in combination for 120 minutes with the shown concentration of inhibitor or DMSO control. Cells were then harvested and assessed for reporter activity by luciferase assay. Luciferase activity was then normalised the unstimulated sample for each treatment group. Statistical differences between unstimulated and stimulated samples were calculated by unpaired t-test for 3 independent donors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

5.3 – Discussion

5.3.1 – Differential sensitivity of IL-10-modulated gene expression to cycloheximide

Murray (2005) previously concluded that the inhibitory activity of IL-10 on macrophage gene expression required *de novo* gene expression, based on the production of primary transcript mRNA from cells treated with cycloheximide. I show for the first time that the rate of Pol II transcriptional elongation is directly affected by protein synthesis inhibition and that IL-10's inhibitory action is un-coupled to its ability to cooperatively regulate gene expression which, in the case of *TNFSF9*, is a direct effect of cell signalling. *NFKBIA* and *TNFAIP3* showed no alteration in Pol II enrichment in the downstream coding regions of these genes on stimulation with IL-10 or treatment with cycloheximide .These observations at *TNFSF9*, *TNF*, *NFKBIA* and *TNFAIP3* can be interpreted as IL-10 having a gene specific effect on transcriptional elongation, since all three patterns of gene expression (IL-10 inhibited, insensitive and cooperatively regulated) of IL-10's effect on LPS-induced genes can be observed.

The inhibitive effect of IL-10 has been previously described as extremely rapid (Williams et al., 2004b). It can be therefore, assumed that the gene (or genes) responsible for inhibiting gene specific Pol II elongation on stimulation with IL-10 is poised for transcription or translation.

The cooperative regulation of *TNFSF9* at the level of transcriptional elongation by LPS and IL-10 has not been described previously. Some studies have previously noted cooperative effects of LPS/IL-10 on gene expression at other genes, but not in primary human cells.

There is also slight increase in Pol II enrichment in the downstream region enrichment after cycloheximide treatment at the downstream regions of *TNF*, *NFKBIA* and, *TNFSF9* which could be

due to cycloheximide's described effect of super-inducing NF-κB transcriptional activity by preventing the resynthesis of IκBα (Sen and Baltimore, 1986). However, this did not reach the level of statistical significance with the number of donors used, but may have with further replicates.

5.3.2 – *TNFSF9* ECR

The identification of an enhancer element upstream of *TNFSF9* has not previously been described. However, the external data sets from *Saeed* et al. (2014) and *Pham* et al. (2012) show distinct enhancer marks at this location in similarly derived cells. This data combined with the observation that the *TNFSF9* ECR induces a reporter plasmid in response to LPS indicates that this region can act as a transcriptional regulator in macrophages.

There were a number of other transcription factor binding motifs in this ECR (such as SP1, AP-2 and, C/EBPα) which are known to regulate transcription in response to LPS stimulation. The sensitivity of the reporter to the inhibitor MLN-4924 supports the hypothesis that this reporter is driven by NF-κB activity, however MLN-4924 can also affect the activity of transcription factors and signalling pathways (Park et al., 2016). MLN-4924 has been previously shown to inhibit the expression of C/EBPa (Park et al., 2016). This transcription factor has a known role in macrophage function(Zhang et al., 1996, Pham et al., 2007) and its consensous motif is present in the ECR reporter. MLN-4924 has also been shown to block the inactivating sumolyation of the transcription factor AP-2 (gene name *TFAP2A*)(Bogachek et al., 2016, Bogachek et al., 2014). The consensus motif for this transcription factor does appear in the ALIBABA motif analysis but since the addition of MLN-4924 does not increase the activity of the ECR reporter, this protein is

unlikely to play a role in the induction of the reporter. In order to establish whether NF-κB is responsible for the LPS induction of the reporter NF-κB binding of the ECR must be examined by ChIP.

The low induction of the ECR reporter plasmid by LPS and cooperativity when stimulated with LPS and IL-10 in RAW 264.7 cells could be reflective of the lower level *TNFSF9* expression generally observed in this cell line. This cell line was initially selected due to its ability to respond to both LPS and IL-10 and its myeloid cell phenotype, but more importantly it has been previously used in similar studies to demonstrate human macrophage enhancer function, since primary human macrophages are notoriously difficult to transfect without using viral vectors.

The identity of which STAT family member is able to induce transcriptional activity at the reporter is a key question from my experiments. While the ECR does contain a detectable STAT motif it is more similar to a STAT5 motif which has been indicated to be activated by IL-10 signalling (Wehinger et al., 1996). Work in the previous chapter has shown that *TNFSF9* expression is sensitive to STAT3 depletion however, in order to conclusively prove STAT3 is binding to this ECR to drive transcription, ChIP of the ECR for both STAT3 and STAT5 should be performed.

To assess the role of the NF-κB and JAK/STAT3 signalling pathways on the reporter induction, I used the inhibitors MLN-4924 and the JAK1/2 inhibitor Ruxolitinib. I observed that the NF-κB pathway was highly required for both the LPS induction of the reporter, as well as the cooperative induction by LPS and IL-10 co-stimulation, while the JAK1/2 pathways was only responsible for the cooperative effect when cells were co-stimulated with LPS and IL-10.

This phenomenon can be explained by two potential hypotheses (shown in Figure 5.18 and Figure 5.19):

1) Dependent on the reorganization of local chromatin

2) Independently or chromatin remodelling

If chromatin remodelling were required (for example if the STAT3 motif was blocked by histones), NF-κB would be required to bind first, to induce the recruitment of chromatin remodelling complexes, or histone modifiers, to increase the accessibility of the DNA within the ECR and reveal a STAT motif previously masked by chromatin.

Alternatively STAT3 may not be able to bind to the exposed motif due to its underlying sequence, without NF-κB present to stabilize the binding, or recruit more transcriptional machinery.

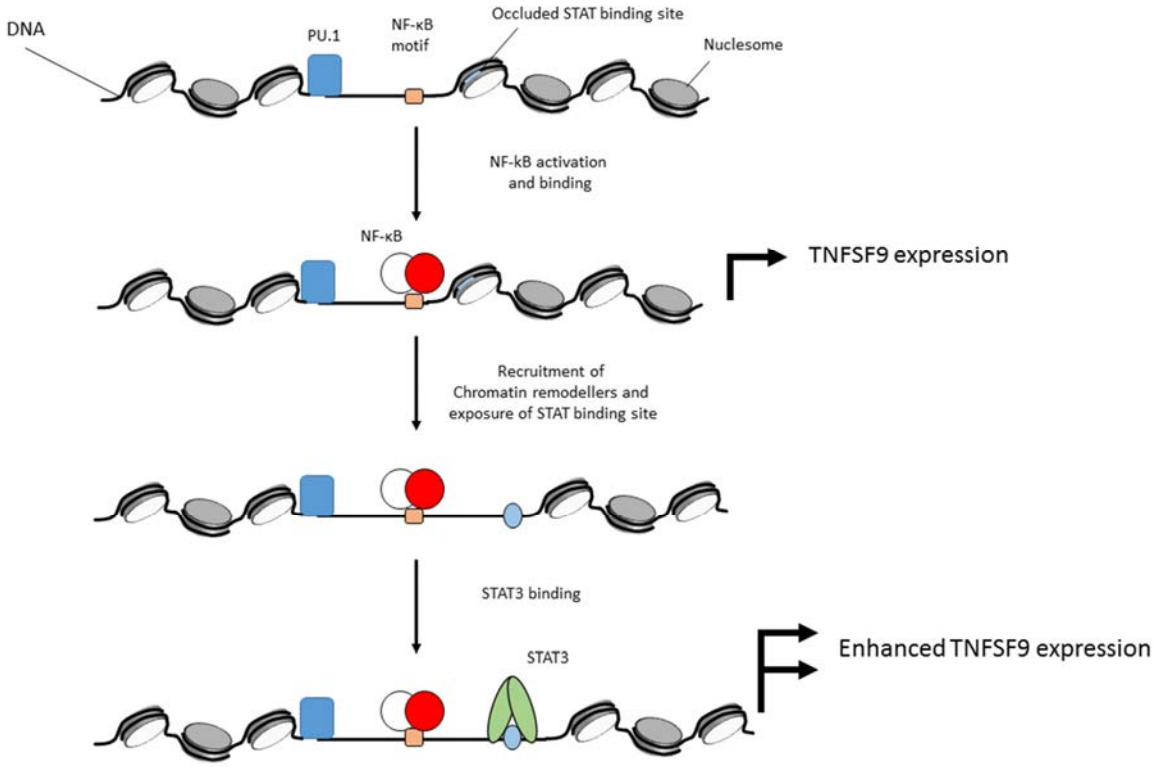

Figure 5.18 – Chromatin remodelling dependent hypothesis of LPS/IL-10 cooperativity at TNFSF9 ECR

Diagram illustrating a hypothesis of how the IL-10 mediated enhancive effect of LPS-induced *TNFSF9* transcription may occur, through local remodelling of chromatin. The lineage factor PU.1 is bound proximal to the ECR allowing local unwinding of chromatin to expose the NF-κB motif, but not the STAT3 binding site, which remains occluded by surrounding histones. The activation of NF-κB by LPS causes the recruitment of NF-κB to chromatin and causes the local remodelling or shuffling of nucleosomes, exposing the STAT3 binding site and allowing the enhancer to respond to IL-10 signalling.

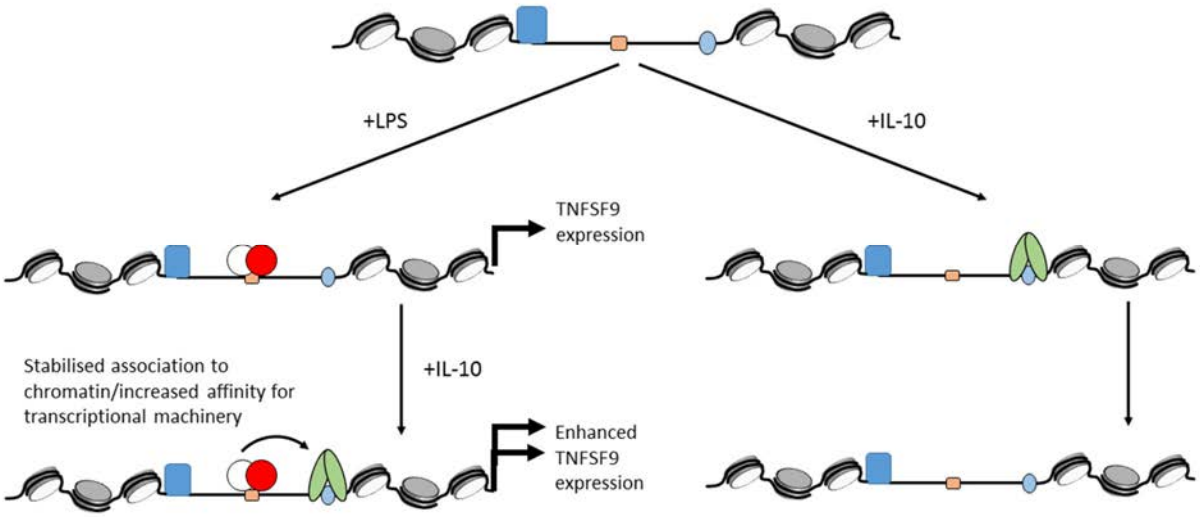

Figure 5.19 – Chromatin remodelling independent hypothesis of LPS/IL-10 cooperativity at TNFSF9 ECR

Diagram illustrating a hypothesis of how the IL-10 mediated enhancive effect of LPS-induced *TNFSF9* transcription may occur independently of the modification of the local chromatin landscape. The lineage factor PU.1 is bound proximal to the ECR allowing local unwinding of chromatin to expose the NF-κB motif and STAT3 binding site. The activation of NF-κB by LPS causes the recruitment of NF-κB to chromatin and is able to either stabilise the association between STAT3 and chromatin, or increase the recruitment of transcriptional machinery. Without NF-κB, STAT3 is unable to associate with chromatin or possibly recruit the transcriptional machinery to initiate transcription

To determine these hypotheses, ChIP could be used to ascertain whether STAT3 is able to bind the ECR under IL-10-stimulated and LPS/IL-10-stimulated conditions. If it is unable to bind under IL-10 alone stimulation, DNase hypersensitivity assay or formaldehyde-assisted isolation of regulatory elements (FAIRE) can be utilized to determine whether the STAT motif is free of nucleosomes or occluded.

5.3.3 – STAT3 luciferase reporter

There are several possible explanations for increased luciferase activity of the STAT3 reporter on LPS stimulation. Firstly LPS signalling could be inducing type I interferons which would feedback on the cell and activating STAT1, which has been shown to occur previously (Ohmori and Hamilton, 2001). An alternate explanation is that the LPS-stimulated macrophages are producing IL-6 or IL-10 and that is leading to increased STAT3 activity. However, considering the production of IL-6 and IL-10 on LPS stimulation in macrophages has been linked to NF-κB activity and both NF-κB inhibitors in the screening experiment (BAY 11-7082 and MLN-4924) do not block the synergy or the LPS-induced reporter activity, so are therefore unlikely. TLR4 stimulation has previously been shown to phosphorylate STAT1 through a PI3K dependent mechanism (Rhee et al., 2003). Since the LPS induction of the reporter is blocked by LY294002, this could be an indication of STAT1 phosphorylation activating the reporter. To assess this in future experiments IFNγ stimulation could be used as a positive control.

The suppression of the STAT3 luciferase reporter by Ruxolitinib, was an expected result. The phosphorylation of Y705 of STAT3 by JAK1 (a target of Ruxolitinib) is required for nuclear import and DNA binding activity of STAT3. Therefore inhibiting JAK1 kinase activity with Ruxolitinib, will prevent any activation of STAT3 and therefore prevent the production of luciferase from the reporter.

The inhibition of reporter activity on stimulation by the inhibitors; SB203580 and LY294002 was unexpected. Inhibition of PI3K has been previously shown to inhibit IL-10 induced gene expression in a manner independent of *de novo* protein synthesis (Antoniv and Ivashkiv, 2011). While this phenomenon would explain the loss of activity on IL-10 stimulation, the mechanism by which PI3K contributes to IL-10 induced gene expression to examine whether this mechanism also explains the inhibition of LPS and LPS+IL-10 induction. In Dillow et al.(2014) the LY294002 inhibitor did affect the expression of multiple cooperative genes, while SB203580 only affected a small subset of LPS/IL-10 cooperatively regulated genes. p38 MAPK has previously been shown to phosphorylate STAT3 and modulate its transcriptional activity(Huang et al., 2014, Miyakoshi et al., 2014, Darnell, 1997, Decker and Kovarik, 2000). This phosphorylation could explain both the synergistic induction of the reporter on LPS and IL-10 co-stimulation and the sensitivity of this reporter to p38 MAPK inhibition.

There is a discrepancy in the inhibition of the reporter by Wortmanin and LY294002, which both inhibit the PI3K family. This could be due to Wortmanin needing a different time of treatment to establish maximum potency. However LY294002 has recently been described as an inhibitor of BET bromodomains (Dittmann et al., 2014). Inhibitors of the BET bromodomain proteins, have been shown to antagonize signal-induced gene expression.

It should be noted that while the STAT3 reporter has been shown to bind STAT3 by EMSA (Horvath et al., 1995) the consensus sequence of the reporter is not an ideal motif for STAT3 ("TTCCCGTAA" in the reporter and an ideal STAT3 motif of "TTCCNGGAA").. The discrepancy between the two sequences occur a critical region of the STAT binding motif and would probably affect the affinity of the STAT dimer for the reporter. Therefore future work could investigate whether STAT3 is binding to the reporter through ChIP or knockdown of STAT3 with siRNA. However, this work would be more insightful if the TNFSF9 enhancer region or other cooperatively regulated regions were used in an adenoviral construct to investigate the requirement of these cell signalling pathways on this phenomenon.

Chapter 6 – Discussion

6.1 – IL-10-mediated inhibition of LPS-induced gene expression

The ability of IL-10 to suppress the expression of inflammatory genes has long made it an attractive target to mimic therapeutically and multiple hypotheses have been posited on the mechanism that this suppression occurs. Several recent studies have highlighted modulation of NF-κB activity as a potential method by which IL-10 may exert its inhibitive effects in macrophages. Several mechanisms have been proposed including: inhibition NF-κB recruitment to chromatin (Smallie et al., 2010), inhibition phosphorylation of the p65 subunit, blocking the recruitment of histone acetylases and P-TEFb (Castellucci et al., 2015) , or generally inhibiting the expression of NF-κB family members mRNA (Hutchins et al., 2015).The mechanisms hypothesised in Smallie et al. (2010) and Hutchins et al. (2015) are not wholly consistent with our observations. General inhibition of NF-κB activity using the small molecule inhibitor MLN-4924, did not replicate IL-10s effect. Additionally, the expression of a number of genes that were unaffected by IL-10 stimulation (including the negative regulators of TLR and NF-κB signalling: *TNFAIP3* and *NFKBIA*) was heavily inhibited when cells were treated with the compound.

Castellucci et al. (2015) recently demonstrated in monocytes that IL-10 blocked the phosphorylation of serine 276 of NF-κB p65 subunit, as well as the acetylation of lysine 310 on the same molecule. The inhibition of these post-translational modifications decreased the recruitment of bromodomain protein Brd4 at the *CXCL8* and *TNF* loci but not at the *NFKBIA* locus. Brd4 has been shown previously (Huang et al., 2009) to recruit P-TEFb to acetylated NF-κB to stimulate transcriptional elongation and blocking its recruitment prevents production of mRNA at specific loci (Nicodeme et al., 2010, Chan et al., 2014). This differential blocking of Brd4 recruitment to chromatin in gene expression could explain the differences between IL-10

inhibited and IL-10 unaffected genes. In order to test this further, ChIP-seq of phosphor-s276 p65 and Brd4 should be performed in macrophages stimulated with LPS or LPS+IL-10 and compared to the gene expression data.

The results of this thesis are able to shed new light on the expression conditions of the IL-10 induced suppressor(s) of gene expression. It has previously established that STAT3 was absolutely required for the IL-10's inhibitory activity (Williams et al., 2007, Williams et al., 2004a) which I confirmed using siRNA in primary cells. Here I show for the first time in primary human cells, that this effect on *TNF* gene transcription requires de novo protein synthesis from IL-10 signalling to inhibit transcriptional elongation. I also show that the IL-10 is still able to inhibit gene expression even in the presence of NF-κB inhibitor MLN-4924 and therefore would be contained in the NF-κB independent section of our datasets.

Interestingly, while not all NF-κB dependent genes were inhibited by IL-10 there was a decrease NF-κB luciferase reporter activity in LPS-stimulated cells co-stimulated with IL-10. This phenomenon has two possible explanations. Firstly IL-10 signalling could be acting on this reporter, similarly to the TNF locus in Smallie (2010) or Castelucci (2015), or inhibiting the recruitment or compromising the ability of NF-KB to recruit P-TEFb to initiate transcription. Secondly this effect could be in part due to the cooperative induction of the poly-ubiquitin binding protein TNIP3, which is able to bind the ubiquitin editing enzyme TNFAIP3 (which is also upregulated by LPS). This complex is then able to target and breakdown the polyubiquitin chains which act as a scaffold to the TLR4 signalling complex (Wagner et al., 2008, Wullaert et al., 2007). TNIP3 is also able to inhibit NF-κB activation independently of its TNFAIP3 binding ability (Weaver et al., 2007, Verstrepen et al., 2008).

6.2 – IL-10 insensitive genes

The IL-10 insensitive gene expression pattern has not been well characterised in literature. The few studies that have been published mainly focus on NFKBIA (Smallie et al., 2010, Castellucci et al., 2015, Murray, 2005) or DUSP1 (Hammer et al., 2005). In this thesis I have expanded this list of genes further and shown that these genes are upregulated by multiple pathways from LPS signalling (not all controlled by NF-κB) and that STAT3 is not required for their expression.

Previous work on the *NFKBIA* locus, in both Smallie et al. (2010) and Casetellucci et al. (2015) has shown that IL-10 is unable to inhibit the recruitment of Brd4 and CDK9 to the TSS of *NFKBIA*, unlike what is observed at the TNF locus. In fact at the *NFKBIA* locus, Brd4 recruitment to the locus is constant between unstimulated and stimulated cells. This signal independent recruitment of Brd4, may prove to be a discriminating factor between IL-10 sensitive and IL-10 insensitive genes.

6.3 – LPS and IL-10 Cooperative regulation of gene expression

Previously studies on LPS/IL-10's cooperative effect on gene expression have focused on a small number of key targets (such as SOCS3 and TNIP3). In this thesis, I have examined this gene expression behaviour genome wide and dissected some of the mechanisms that control this pattern of gene expression. Similar to the other gene expression patterns in response to IL-10 I observed, NF-κB was required for the LPS induction of many cooperatively induced genes. On further analysis of several select LPS/IL-10 cooperatively regulated genes, most were still able to retain their pattern of response to IL-10 despite inhibition of NF-κB. Only one gene (*TNFSF9*) of

the eight I investigated was found to lose all LPS/IL-10 cooperative regulation after treatment with the inhibitor MLN-4924.

Collaboration and cooperative binding of the transcription factors STAT1 and NF-κB have been shown previously to cooperatively-regulate *CXCL10* expression (Yeruva et al., 2008, Burke et al., 2013, Qiao et al., 2013). Given the similarities in protein structure between STAT1 and STAT3 there is the potential that STAT3 might also be act in concert with NF-κB in a similar manner. In order to test this theory STAT3 and NF-κB could be immunoprecipitated and assessed for cooperative binding or ChIP-seq performed on the samples to assess the level of local binding on DNA.

Due to the opposing signalling effects of LPS and IL-10, the idea that some genes would be synergised almost appears counter-intuitive. However, there are many situations these signals are likely to co-exist together. Many organs such as the lungs and gut are continually exposed to pathogens or microbes but are not in a continuous state of inflammation. One of the mechanisms to suppress the potentially harmful inflammatory response in these organ is a constant production and secretion of IL-10 into the tissue (Sellon et al., 1998, Hacham et al., 2004, Zigmond et al., 2014). IL-10 and pro-inflammatory signals co-exist would also co-exist during inflammation. As many studies have shown, after stimulation with cytokines or LPS, macrophages are able to produce IL-10 (Pattison et al., 2012, Sellon et al., 1998, Iyer et al., 2010). The induction of IL-10 on activation is delayed compared to other cytokines (such as TNFα) and could assist in pushing the macrophage to a pro-resolution phenotype. However, if exogenous inflammatory stimuli and IL-10 co-exist this may indicate a lack of pathogen clearance and it would be necessary to activate or enhance the adaptive immune response to clear the pathogen.

Using a STAT3 luciferase reporter, I observed a significant cooperative induction of luciferase when cells were co-stimulated with LPS and IL-10, which was sensitive to inhibition of the p38 MAPK and JAK1/2 cell signalling pathways. The sensitivity of the reporter to PI3K inhibition was inconclusive since the inhibitors LY29002 and Wortmanin produced different effects. This increase in STAT activity with the two stimuli, has not been reported previously. Physiologically, this mechanism may have evolved to increase IL-10's anti-inflammatory properties, by increasing STAT3's transcriptional ability at the peak of inflammation. If STAT3 activity was broadly enhanced by LPS then all IL-10 induced genes would be induced further when co-stimulated with LPS which I did not observe in my dataset.

6.4 – TNFSF9 expression

The protein TNFSF9 (also known as 4-1BBL) is a T cell co-stimulatory molecule which assist in the antigen-dependent activation of CD8/4+ T cells (Cannons et al., 2001). Mice which are deficient or unable to activate TNFSF9 induced signalling, display a diminished protection from severe viral infections due to reduced CD8+ expansion (DeBenedette et al., 1999, Wen et al., 2002).

In this thesis I have demonstrated that the synergistic expression of this gene in response to LPS/IL-10 requires STAT3, but is also heavily dependent on NF-κB and that this pattern of expression is mimicked by a luciferase reporter containing an evolutionary conserved element upstream of the gene. In order to confirm whether NF-κB and STAT3 bind the ECR in human macrophages future experiments should use ChIP to confirm the presence of these factors.

The protein itself is only upregulated on the cell surface after co-stimulation, for a short time, before returning to basal expression levels. With such a short window of expression, it is difficult to grasp, what the result of this upregulation would be physiologically. To test this LPS+IL-10 stimulated MDM could be cultured in the presence of TNFSF9 blocking antibody and autologous T cells. The T cells could then be assessed for phenotypic and gene expression changes due to the presence macrophages expressing TNFSF9.

There are a few potential hypotheses as to how this synergistic regulation might have developed. Firstly, if other PRRs cooperate similarly with IL-10 as LPS does, this indicates that viral derived PAMPs may cooperate in IL-10 rich tissues, such as the lungs or the gut mucosa. This would cause the rapid proliferation and activation of viral specific CD8+ T cells, in tissues where the immune response would otherwise be inhibited (so as to not disrupt organ function with an inflammatory response).

Another possible explanation is that IL-10 signalling and sustained activation of STAT3 can act in a pathogenic manner. IL-10 has been acquired by multiple extant viral lineages and its production by cells can be induced by bacterial species and utilized by tumour cells to evade immune surveillance. Therefore, dampening of the immune response by IL-10 would allow the pathogen or tumour to replicate or spread, with potentially lethal consequences. Therefore the ability of IL-10 to stimulate specific immunity (such as CD8+ T cells) would be advantageous to an organism. Viral IL-10 homologues of IL-10 have been shown to signal differently to human IL-10 (Liu et al., 1997, Ding et al., 2000, Kotenko et al., 2000) and it is not known if these cytokines will induce *TNFSF9* expression to a similar extent.

IL-10 deficient mice have been previously shown to have compromised CD8+ T cell memory generation and survival in response to infection (Foulds et al., 2006) as well as a deficient expansion of anti-tumour CD8+ T cells (Emmerich et al., 2012). This phenotype of compromised CD8+ immunity is very similar to IL10 knockout mice (Watts et al., 2011, Humphreys et al., 2010, Lin et al., 2009, Mogi et al., 2000, DeBenedette et al., 1999) and therefore could be linked. However, for this to be confirmed this would require experiments to confirm whether the exogenous addition of IL-10 is able to restore the ability of IL-10 knockout mice to produce CD8+ anti-tumour/viral memory cells and whether this restoration of protective memory is dependent on TNFSF9.

Finally, the synergy with IL-10 may only be coincidental. Multiple cell signalling pathways utilise STAT3 to effect gene expression changes and the duration of STAT3 activation is key to defining that response (Yasukawa et al., 2003b, Braun et al., 2013, Bang et al., 2015). Therefore, the upregulation of TNFSF9 by IL-10 and LPS may be unavoidable and therefore downregulated by a separate mechanism later. STAT3 is not the only STAT family member reporter to be activated by LPS or IL-10. STAT1 has been shown to be activated by LPS and IL-10 (Finbloom and Winestock, 1995, Ohmori and Hamilton, 2001) and the consensus binding motifs of these proteins are similar. To investigate whether STAT1 is driving the cooperative expression of TNFSF9 macrophages could be stimulated IFNγ (an activator of STAT1) and LPS and the expression of TNFSF9 could be monitored.

Recent studies on the function of TNFSF9 expressed by macrophages have shown that the intracellular domain interacts with TLR4 components to sustain the production of TNF in response to LPS (Kang et al., 2007, Ma et al., 2013). TNFSF9 is also able to induce cell signalling cascades on

binding to its cognate receptor TNFRSF9. The protein is able to induce the PI3K and Src cell signalling pathways to enhance macrophage activation and proliferation.

6.5 – Methodology

The use of primary human cells in this study makes the observations I have made more relevant to assessing the interaction of these signalling pathways in diseased systems. By characterising the patterns of gene expression and the mechanisms responsible in healthy cells, aberrant gene expression in diseased systems can be more easily identified and potential mechanisms for therapeutic intervention can be highlighted.

Primary cells however are not without drawbacks. For example, primary macrophages are notoriously difficult to efficiently transfect without viral vectors. Due to the time constraints of engineering an adenoviral construct of the TNFSF9-ECR reporter plasmid, I opted to utilise a macrophage like cell line (RAW 267.4), for which a transfection protocol was already optimised. This cell line has previously been used to demonstrate similar enhancer effects (Qiao et al., 2013), the level of luciferase induction was quite low (similar to the native gene induction), which may make further investigation of the mechanism with cell signalling inhibitors or plasmid mutations difficult.

It is clear that the effects of signalling on gene expression at later time points are obscured by the autocrine feedback of cytokines such as IL-10. Many of the one hour LPS-induced, IL-10 inhibited genes were no longer significantly induced by LPS at four hours or else equal in levels of expression to those co-stimulated with LPS and IL-10, potentially hinting that IL-10 autocrine

signalling was occurring at this timepoint. Therefore, I focused the bulk of this thesis to the dissection of the early induced LPS response. However, there is no guarantee other cytokines or chemokines are not also feeding back on the macrophages at this early stage. For instance, I was able to detect low levels of STAT3 phosphorylation at 60 minutes post LPS stimulation and this was dependent on *de novo* protein synthesis. To counteract IL-10 autocrine feedback, antibodies targeting IL-10 or its receptor complex can be used, but to prevent wider secretion of proteins, Brefeldin A or other Golgi transport inhibitors, would have to be used (however this would not prevent the shedding of proteins on the cell surface).

The investigation of cooperation between transcription factors is a topic that has garnered much interest in recent years. Many of these investigations utilise next-generation sequencing (NGS) to investigate the genome wide binding of transcription factors to demonstrate close proximal binding of the transcription factors. In future work to assess whether NF-κB cooperate STAT3, NF-κB and, the histone modification H3K27ac should be immunoprecipiated and sequenced to assess whether cooperative regulation between these two transcription factors occurs generally or at specific loci.

The comparison between the two microarray platforms in assessing macrophage gene expression is one of the shortcomings of this study. Due to differences in microarray probe structure, as well as the variation in the hybridisation procedures carried out by the different companies, made a direct integration of the two datasets problematic. A number of the transcripts that contribute to

the separation of the two datasets by PCA, are in fact detected by multiple probes. These probes also display differential sensitivity to the transcripts and finding a probe specific for a transcript that displays similar sensitivity is computationally difficult and intensive. Data integration between microarray platforms is a well-known problem in the literature. The decision to use Agilent rather than Affymetrix was made for financial and operational reasons, and the difficulties of data integration were not fully appreciated at the time.

More recent studies have overcome these problems of data integration by utilising NGS of RNA (RNA-seq). This allows the quantification of RNA transcripts, through assessing the number of sequencing reads that map to a sequence. However, at the start of this project the cost of RNA-seq was prohibitive to the initial experiment (4 donors x 11 conditions) and subsequently microarrays were selected to assess the gene expression of these cells.

6.6-Future work

Much of the work on IL-10's ability to modulate macrophage responses has been restricted by focusing on potent NF-κB activators, such as LPS, IL-1α/β and TNF. In the context of host-pathogen/ tissue damage response, many more signalling pathways are activated to induce gene expression and few studies have examined the effect of IL-10 on other PRR or cytokine induced gene expression pathways. Since IL-10 is able to cooperate with LPS to induce the CD8+ T cell co-stimulatory molecule TNFSF9, there may be other molecules derived from virus (such as dsRNA) that may more potently induce or sustain the expression of immunostimulatory molecules.

Work on TNFSF9 has highlighted some potential inflammatory mediators being co-operatively regulated by LPS and IL-10 which are later down regulated (such as CXCL10 and CCL8). It would be interesting to investigate if IL-6 signalling leads to a sustained cooperative regulation of these genes.

Assessing the promoters of the different groups of gene expression did not show a predominant occurrence of any single motif, very little of the upstream regions of the genes were used in this analysis, (2000 bp from the TSS). These analyses also work on the assumption that these upstream regions are permissible to TF binding. In order to ascertain which local regions of DNA to genes are available for transcription factor binding, I would employ DNase-seq or ATAC-seq. These techniques also allow for the interrogation of which TF's are binding chromatin and show as a unique "footprint" of variably accessible chromatin, in the centre of a nucleosome free region.

This technique could also be paired with RNA-seq and ChIP-seq (especially for: p65, STAT3 and Brd4) to further gain more specific insight into how the macrophage transcriptome is controlled.

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Chapter 8 - Appendix

8.1 – GO analysis

Gene Ontology term	Benjimani adjusted p-value
Epithelial cell signalling in Helicobacter pylori infection	5.16E-03
Transcriptional misregulation in cancer	1.34E-02
Neurotrophin signalling pathway	2.41E-02
Renin secretion	4.35E-02
Shigellosis	4.35E-02
Renal cell carcinoma	4.60E-02

Table 8.1 – 1 hour IL-10 inhibited cluster enriched GO terms

Gene Ontology term	Benjimani adjusted p-value
Leishmaniasis	1.10E-06
Malaria	3.50E-06
African trypanosomiasis	2.00E-05
Osteoclast differentiation	3.60E-05
HTLV-I infection	2.10E-04
Toxoplasmosis	2.50E-04
Asthma	4.00E-04
Allograft rejection	7.60E-04
T cell receptor signalling pathway	1.60E-03
Epstein-Barr virus infection	2.70E-03
Viral myocarditis	2.80E-03
FoxO signalling pathway	4.00E-03
Apoptosis	8.20E-03
Haematopoietic cell lineage	8.70E-03
Prion diseases	1.30E-02
Primary immunodeficiency	1.30E-02
MicroRNAs in cancer	1.40E-02
Graft-versus-host disease	1.70E-02
Type I diabetes mellitus	1.90E-02
Intestinal immune network for IgA production	2.20E-02
Type II diabetes mellitus	2.30E-02
Autoimmune thyroid disease	2.70E-02
Measles	2.80E-02
Systemic lupus erythaematosus	2.80E-02
Staphylococcus aureus infection	3.00E-02
Cell adhesion molecules (CAMs)	3.20E-02
Adipocytokine signalling pathway	4.60E-02
B cell receptor signalling pathway	4.90E-02

Table 8.2 – 1 hour IL-10 insensitive cluster enriched GO terms

Gene Ontology term	Benjimani adjusted p-value
Hepatitis C	3.28E-02
Primary bile acid biosynthesis	3.63E-02

Table 8.3 – 1 hour IL-10 cooperative cluster enriched GO terms

Gene Ontology term
Shared terms between IL-10-inhibited and IL-10-insensitive cluster
MAPK signalling pathway
NF-kappa B signalling pathway
NOD-like receptor signalling pathway
RIG-I-like receptor signalling pathway
Cytosolic DNA-sensing pathway
Salmonella infection
Pertussis
Legionellosis
Chagas disease (American trypanosomiasis)
Amoebiasis
Tuberculosis
Hepatitis B
Influenza A
Herpes simplex infection
Inflammatory bowel disease (IBD)
Rheumatoid arthritis
Shared terms between IL-10-inhibited and LPS/IL-10 cooperative cluster
Chemokine signalling pathway
Shared terms between IL-10-insensitive and LPS/IL-10 cooperative cluster
Prolactin signalling pathway
Shared terms between IL-10-inhibited , IL-10-insensitive and, LPS/IL-10 cooperative cluster
Cytokine-cytokine receptor interaction
TNF signalling pathway
TNF signalling pathway
Toll-like receptor signalling pathway
Toll-like receptor signalling pathway
Jak-STAT signalling pathway

Table 8.4 – Shared GO terms between the 1 hour cluster data

Gene Ontology term	Benjimani adjusted p-value
Notch signalling pathway	6.09E-03
Adherens junction	1.91E-02
Salmonella infection	2.84E-02

Table 8.5 – GO terms enriched in 4 hour IL-10 inhibited cluster

Gene Ontology term	Benjimani adjusted p-value
Influenza A	2.83E-13
Measles	1.97E-10
NF-kappa B signalling pathway	1.19E-09
Apoptosis	3.25E-08
Herpes simplex infection	2.71E-07
Allograft rejection	3.15E-07
Chagas disease (American trypanosomiasis)	4.41E-07
Hepatitis B	1.13E-06
African trypanosomiasis	1.93E-06
Leishmaniasis	2.22E-06
Inflammatory bowel disease (IBD)	6.11E-06
Graft-versus-host disease	8.68E-06
Toxoplasmosis	1.12E-05
Prolactin signalling pathway	1.56E-05
Pertussis	2.25E-05
Cytosolic DNA-sensing pathway	3.33E-05
Osteoclast differentiation	3.48E-05
Hepatitis C	4.09E-05
Natural killer cell mediated cytotoxicity	4.43E-05
Tuberculosis	5.63E-05
RIG-I-like receptor signalling pathway	7.83E-05
Legionellosis	8.08E-05
Rheumatoid arthritis	1.01E-04
MAPK signalling pathway	3.49E-04
Autoimmune thyroid disease	3.73E-04
Epstein-Barr virus infection	6.91E-04
T cell receptor signalling pathway	1.54E-03

Malaria	1.63E-03
HTLV-I infection	3.05E-03
Acute myeloid leukemia	3.57E-03
Intestinal immune network for IgA production	7.35E-03
Pathways in cancer	7.69E-03
Adipocytokine signalling pathway	9.75E-03
Prion diseases	1.27E-02
Non-alcoholic fatty liver disease (NAFLD)	1.48E-02
RNA degradation	1.52E-02
Viral myocarditis	1.75E-02
HIF-1 signalling pathway	1.81E-02
ErbB signalling pathway	2.61E-02
Signalling pathways regulating pluripotency of stem cells	3.26E-02
Type II diabetes mellitus	3.63E-02
B cell receptor signalling pathway	3.99E-02
Proteoglycans in cancer	4.02E-02
Chronic myeloid leukemia	4.19E-02
Asthma	4.28E-02

Table 8.6 – GO terms enriched in 4 hour IL-10 insensitive cluster

Gene Ontology term	Benjimani adjusted p-value
PPAR signalling pathway	1.66E-03
PI3K-Akt signalling pathway	2.97E-02
Fatty acid biosynthesis	4.43E-02

Table 8.7 – GO terms enriched in 4 hour LPS/IL-10 cooperative cluster

Gene Ontology term
Shared terms between IL-10-inhibited and IL-10-insensitive cluster
Toll-like receptor signalling pathway
Jak-STAT signalling pathway
TNF signalling pathway
Type I diabetes mellitus
Epithelial cell signalling in Helicobacter pylori infection
Amoebiasis
Transcriptional misregulation in cancer
Shared terms between IL-10-insensitive and LPS/IL-10 cooperative cluster
Chemokine signalling pathway
NOD-like receptor signalling pathway
Shared terms between IL-10-inhibited , IL-10-insensitive and, LPS/IL-10 cooperative cluster
Cytokine-cytokine receptor interaction

Table 8.8– Shared GO terms between the 4 hour cluster data

8.2 – CXCL10 and CCL8 expression in macrophages

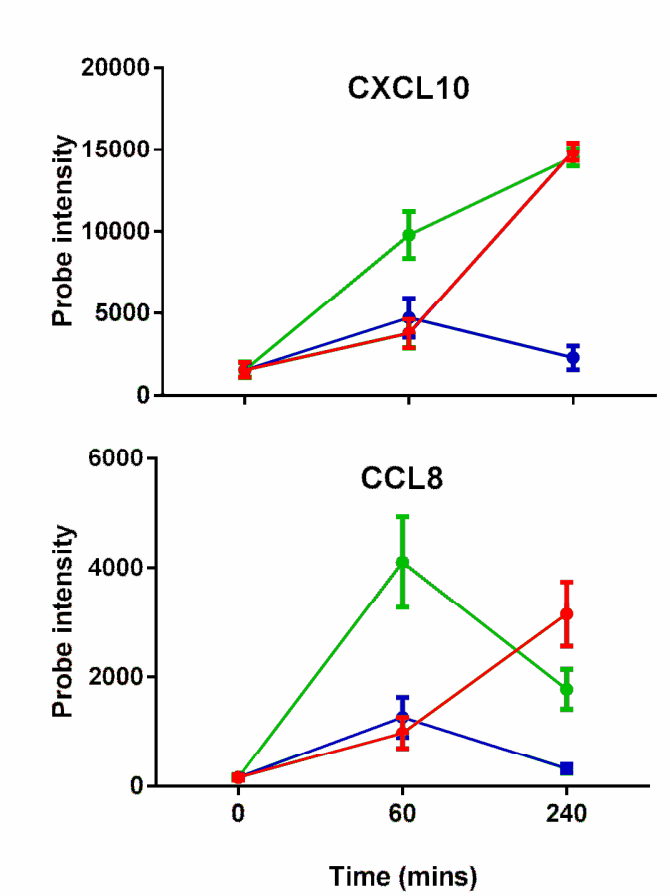

Figure 8.1 – Microarray expression profiles of CXCL10 and CCL8

Expression of CXCL10 and CCL8 expression in macrophages stimulated with LPS (red), IL-10 (blue) or in combination (green) extracted from the microarray data.

8.3 – Flow cytometry

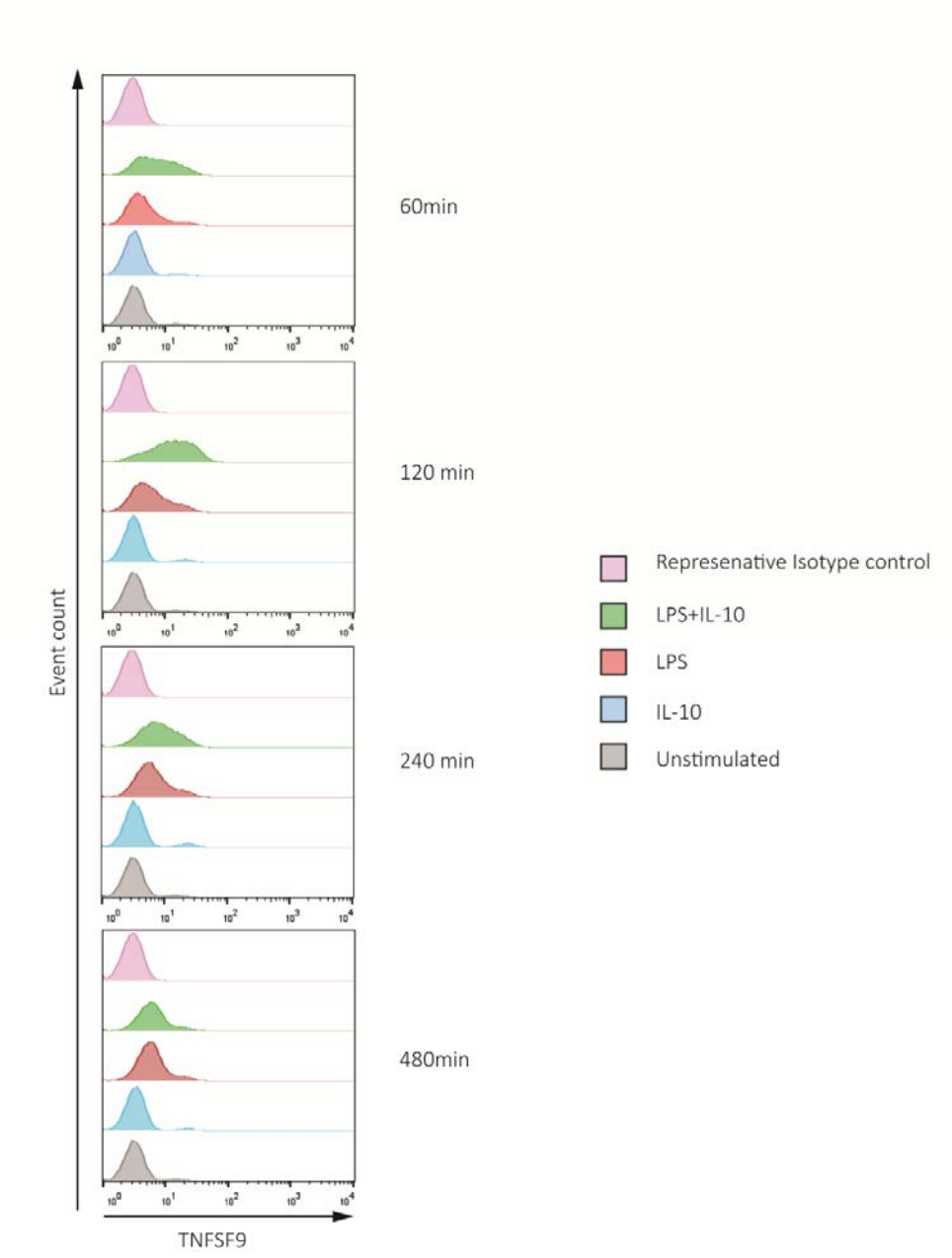

Figure 8.2 – Representative LPS/IL-10 time course of TNFSF9 expression

Macrophages stimulated with LPS, IL-10 or in combination for 60, 120, 240 or 480 minutes were assessed for surface expression of TNFSF9 expression by flow cytometry. Histograms of the surface expression show a representative experiment.

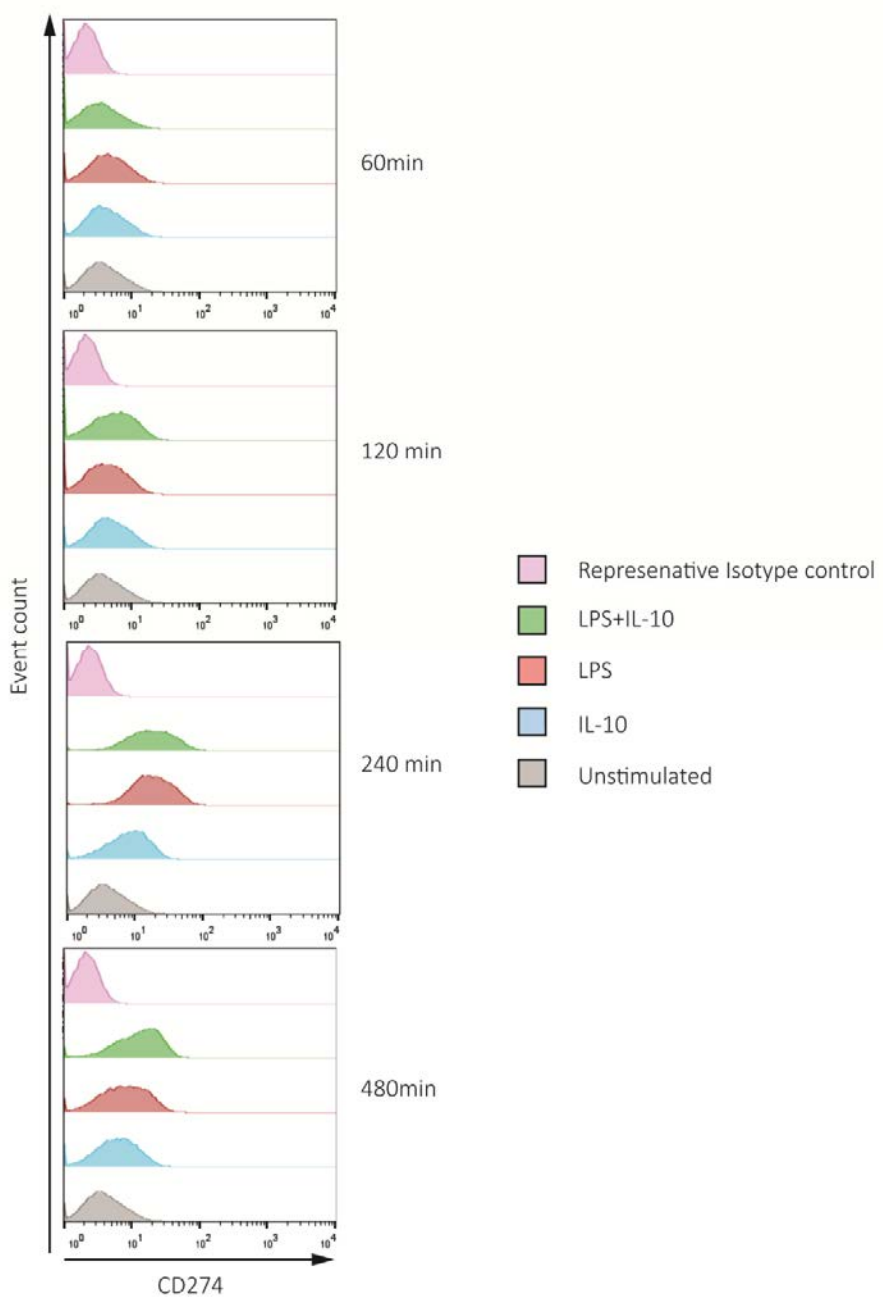

Figure 8.3 – Representative LPS/IL-10 time course of CD274 expression

Macrophages stimulated with LPS, IL-10 or in combination for 60, 120, 240 or 480 minutes were assessed for surface expression of CD274 expression by flow cytometry. Histograms of the surface expression show a representative experiment.