Transcriptional Regulation of the Anti-Inflammatory Protein Tristetraprolin (TTP)

Helen Elizabeth Cunliffe

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Centre for Translational Inflammation Research
School of Immunity and Infection
University of Birmingham
March 2015

Abstract

Feedback node genes (FNGs) are essential for negative feedback control of inflammatory responses. By definition, their expression is controlled by both pro- and anti-inflammatory stimuli, often in a cooperative manner. This thesis investigates three FNGs, namely Dual specificity phosphatase 1 (DUSP1), Tumour necrosis factor alpha inducible protein 3 (TNFAIP3) and Tristetraprolin (TTP, encoded by the *Zfp36* gene). DUSP1 is a negative feedback regulator of mitogen-activated protein kinases, TNFAIP3 negatively regulates the nuclear factor κB (NF-κB) signalling pathway and TTP is a destabiliser of pro-inflammatory mRNAs.

All three FNGs were induced by pro-inflammatory stimuli, with very different dependence on NF-κB signalling. *Tnfaip3* expression was strongly dependent on NF-κB, *Zfp36* was minimally affected by an NF-κB inhibitor, and *Dusp1* expression was actually increased. The anti-inflammatory agonists dexamethasone, prostaglandin E_2 (PGE_2) and transforming growth factor β (TGFβ) all impaired NF-κB activity, yet cooperated with pro-inflammatory agonists to increase expression of all three FNGs. Experiments in primary mouse knock-out macrophages suggested that DUSP1 may be necessary for some anti-inflammatory effects of PGE_2, and for the cooperative regulation of other FNGs by pro- and anti-inflammatory agonists.

To try to identify mechanisms of cooperative regulation of FNGs by pro- and anti-inflammatory agonists I concentrated on the relatively small *Zfp36* locus. Three putative regulatory elements were identified on the basis of evolutionary conservation and scanning chromatin immunoprecipitation across the locus, using an antibody against RNA polymerase II. Those elements were cloned into a luciferase reporter construct and demonstrated to mediate cooperative transcriptional regulation by various combinations of pro- and anti-inflammatory agonists. Chromatin immunoprecipitation experiments also demonstrated dynamic remodelling of the locus in response to a pro-inflammatory stimulus, RNA polymerase being "transferred" from an upstream enhancer region to the *Zfp36* gene itself.

Taken together, the data in this study identify novel mechanisms by which FNG expression is maintained and even augmented during treatment with anti-inflammatory stimuli. These findings can be applied to the development of anti-inflammatory agents as therapeutic compounds.

Acknowledgements

First and foremost, I owe gratitude to my supervisor, Professor Andy Clark, who has devoted so much of his time to my help and support. Andy has patiently guided me through this project with encouragement, ideas and advice, which I wholly appreciate. The commitment, enthusiasm and flair he has for his research has been motivational and truly inspiring.

The Clark lab are a source of merriment and strong scientific talent which I am lucky to have been a part of. I would like to thank Dr Tim Smallie. He has been a fantastic mentor and has provided technical support, enthusiasm and useful discussion. Dr. John O'Neil, Tim and Dr. Ewan Ross have contributed to both my professional and personal growth and were always too happy to acknowledge the 'work-life balance'. Tina Tang has been a joy to work with and has facilitated my work on this project as a friend and as a scientist.

Finally, I would like to thank my friends and family for their continuous support. Particularly my father, who's pillar of support has been unconditional, unequivocal and unsurmountable throughout this project and indeed, all of my life accomplishments and adventures.

Contents

Abbreviations

AP1/2	Activator protein 1/2
ARE	adenosine-uridine (AU)-rich element
AREBP	ARE binding protein
BMM	bone marrow derived macrophage (murine)
C/EBP	CCAAT enhancer binding protein
cAMP	cyclic adenosine 3',5'-monophosphate
CCCH	cysteine-cysteine-cysteine-histidine
ChIP	chromatin immunoprecipitation
COX2	cyclooxygenase-2
CREB	cAMP responsive element binding protein
DEX	dexamethasone
DUSP1	dual specificity phosphatase 1 (MKP1/ mitogen activated protein kinase 1)
ECR	evolutionarily conserved region
ERK1/2	extracellular signal-regulated kinase 1 and 2
FNG	feedback node gene
GC	glucocorticoid
GM-CSF	granulocyte-macrophage colony stimulatory factor
GR	glucocorticoid receptor
HuR	human RNA binding protein
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
IL1R	IL1 receptor
JNK	c-Jun N-terminal kinase
KO	knockout/ gene deletion

KSRP	K-homology domain type splicing regulatory protein
LPS	lipopolysaccharide
MAPK	p38 mitogen activated kinase p38
MK2	MAPK-activated protein kinase (MAPKAPK) 2
M-MDM	M-CSF monocyte derived macrophage (human)
NFκB	nuclear factor -κB
PGE_2	prostaglandin E_2
PKA/C	protein kinase A/C
PRR	pathogen recognition receptor
QPCR	quantitative PCR
RA	rheumatoid arthritis
RNAPII	RNA polymerase II
SEM	standard error of the mean
siRNA	small interfering RNA
smad	mediators of TGFβ family signals. The term is a unification of the *sma* from *C. elegans* and *Mad* (mother against decapentaplegic) in *D. melanogaster* (Derynck et al 1996)
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription
TGFβ	transforming growth factor –β
TLR	toll-like receptor
TNFAIP3	tumour necrosis factor –α inhibitor protein 3 (/A20)
TNFα	tumour necrosis factor -α
TTP	tristetraprolin (gene switch protein 24 (G0S24)/ TPA-induced sequence 11 (TIS11)/ (Nup475)/ zinc finger protein 36 (Zfp36)
UTR	untranslated region
WT	wild type

1.0 INTRODUCTION

1.1 Inflammation

Inflammation is a host protective response to invasion by an infectious agent, antigen challenge or simply physical, chemical or traumatic damage. The main function of the inflammatory response is to facilitate the delivery of proteins and cells of the immune system capable of eliminating the initiating agent and promoting the healing process (Newton & Dixit 2012). The classical symptoms of inflammation are heat, redness, swelling, pain and loss of tissue function. Each of the symptoms described can be related to the physiological changes that take place. Local vasodilation of the inflamed area increases blood flow, allows extravasion of fluid, cellular influx, elevated cellular metabolism and the subsequent release of the soluble mediators of inflammation. Thus inflammation is not ancillary to, but an active function of the immune response, effectively evolved to defend and heal host tissues and processes (Beutler 2004). The benefit of an evolved immune system comes at a cost since inflammation is essentially injurious to host tissues. Indeed, immune dysfunction can be detrimental, resulting in any number of chronic inflammatory syndromes that may be a primary pathology or the secondary outcome to another condition. Often, the treatments prescribed to alleviate symptoms of numerous conditions act to reduce inflammation and other immune processes. The link between cancer and inflammation is significant and bi-directional. Carcinogenesis is often contributory to or the corollary of deregulated inflammation. The treatment of cancer-related inflammation with anti-inflammatory agents is not only effective in modulating host-response mediated

symptoms but has an anti-tumour effect on several hallmarks of cancer, including; proliferative capacity; evasion from apoptosis; and cell cycle regulation (Park et al. 2014).

1.2 Dysregulation of inflammation

Inflammation is a host protective mechanism, intended to remove the injurious stimulus and promote healing. However, the progressive destruction of tissue in the absence of inflammation can lead to inflammatory disorders and chronic inflammatory diseases including rheumatoid arthritis (RA), inflammatory bowel disease (IBD), diabetes and cancer. Thus, a functioning immune response is both rapidly induced and transiently executed. To this end, powerfully cytotoxic pro-inflammatory factors are actively curtailed by equally potent anti-inflammatory mediators (Figure 1.1).

Whilst pro-inflammatory factors negatively feedback and activate anti-inflammatory mechanisms, they also positively feed-forward on themselves (Hu et al. 2008). That is, pro-inflammatory messengers directly increase their own expression at multiple levels of regulation in order to elicit a precipitous response. Equally, anti-inflammatory factors positively regulate themselves also. Thus the inflammatory response is a precise and acutely controlled balance between potent factors protecting the host from noxious stimuli and powerful watchdogs that harness that initial response.

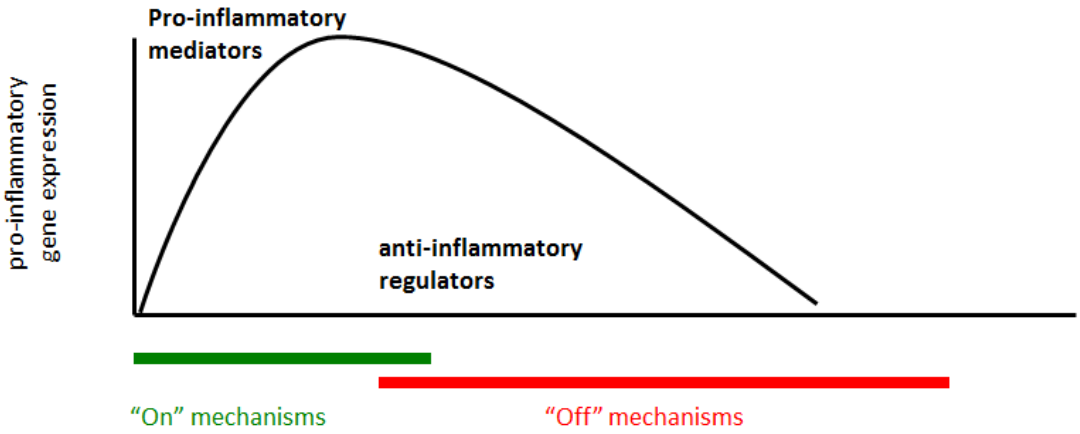


Figure 1.1 Negative regulation of inflammation

The rapid and powerful biosynthesis and secretion of pro-inflammatory mediators is efficiently curtailed by the succinct activation of regulatory factors with anti-inflammatory effects.

These "negative feedback" mechanisms are critical to limiting the inflammatory response and preventing excess tissue damage. Imbalances of 'on' and 'off' processes can lead to dangerous unchecked inflammatory responses that may be inadequate or excessive in magnitude or duration, becoming detrimental to the host. A disproportionate inflammatory response then results in debilitating chronic inflammatory conditions that cause severe and irreversible tissue destruction.

1.2.1 Rheumatoid arthritis (RA)

For example, in RA, spontaneously activated leukocytes infiltrate the joint and, unchecked secrete the inflammatory mediators responsible for the debilitating joint inflammation. The exact cause of erroneous leukocyte activation in the RA joint pannus is unknown; however the role of TNFα, a potent inflammatory cytokine, is pivotal. Feldman's lab (Williams et al. 1996; Feldmann) were able to show that blockade of TNFα in cells of activated synovium significantly reduced the level of pro-inflammatory cytokines. Additionally, synovial inflammation in collagen induced murine models of arthritis (CIA) was abolished in mice treated with anti-TNFα antibodies (Williams et al. 1992).

The endogenous TLR ligands of the RA joint drive excessive production of pro-inflammatory cytokines TNFα, IL-1, IL-6, IL-8, IFNγ and VEGF, amongst many more as a positive feed-forward process (Brennan & Feldmann 1996). A number of anti-inflammatory factors, including IL-10, TGFβ and TNFR are equally up-regulated in RA, though the pathways of inflammatory resolution are evidently debilitated (Feldmann et al. 2009). Indeed, microarray analysis by the Ivashkiv group revealed that macrophages isolated from RA synovium express significantly less IL10 responsive genes than control macrophages (Antoniv & Ivashkiv 2006).

1.2.2 Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) refers to inflammation of the colon, mucosal membrane and regions of the gastrointestinal tract (GI). This system of diseases includes ulcerative colitis (UC) and Crohn's disease (CD) and is characterised by an over-activation of transcription factor NF-κB and subsequent overexpression of pro-inflammatory cytokines (Beck & Wallace 1997; Rogler et al. 1998; Schreiber 2005). Indeed, neutralisation of TNFα in Crohn's effectively reduces inflammation and anti-TNFα therapy has proved successful

(Marini et al. 2003; Present et al. 1999). IBD is a complication of the failure to supress an immune response to luminal bacteria and the consequential exacerbated expression of pro-inflammatory molecules including TNFα (Korhonen et al. 2013). Here, inadequacy of anti-inflammatory feedback transforms miss-directed acute inflammation into a chronic inflammatory syndrome. Genome wide association studies and the use of IL10-/- mice has revealed a central role for the IL10 axis in patients with early onset UC and CD (Büchler et al. 2012; Barnett et al. 2010; Hansen et al. 2009). Polymorphisms in the IL10 locus and homozygous IL10R loss of function mutations impair ligand-associated signalling and cause severe intestinal inflammation and increased secretion of pro-inflammatory cytokines critically involved in the development of spontaneous colitis. Three recent publications in Immunity (Zigmond et al. 2014; Shouval et al. 2014; Mantovani & Marchesi 2014) highlight IL10R signalling in murine macrophages as key to driving their homeostatic tolerogenic functions in gut health and inflammation. They also outline that ability to respond to, rather than release of, IL10 is fundamental to macrophage anti-inflammatory phenotype in the GI tract.

1.2.3 Cancer

For decades, microbial infection, injury, inflammation and tissue repair have been linked to the development of cancer (Balkwill & Mantovani 2001). Bacterial infection and immune activation can negatively regulate cancer, primarily through tumour necrosis factor –α (TNFα) (Cullen & Martin 2015). A number of therapeutic compounds have been developed based on the actions of microbial-compounds that activate immune signalling (Vacchelli, Eggermont, et al. 2013; Vacchelli, Vitale, et al. 2013). Used for the treatment of cervical, gastric and oral squamous cell carcinomas, OK-432 (Picibanil) is a lyophilized mixture of

group A Streptococcus pyogenes with antineoplastic activity (Rebuffini et al. 2012) that stimulates receptor signalling upstream of TNFα release (Hovden et al. 2012). The BCG vaccine triggers similar pathways and has been therapeutically successful in the treatment of bladder cancer (Jinesh G & Kamat 2012). Additionally, the administration of purified bacterial compounds lipopolysaccharide and flagellin in phase II clinical trials of colorectal and lung cancer has yielded positive results (Burdelya et al. 2008; Garaude & Blander 2012). Evidently, carcinogenesis is negatively affected by a functional immune and anti-tumour response. However, aberrant activation of inflammatory signalling in the tumour microenvironment can lead to immune suppression, tumour angiogenesis, tumour progression and chemoresistance (Yuntao Zhang et al. 2012; Yan Zhang et al. 2012; Galluzzi et al. 2012). A number of immune functions are responsible for driving malignancy, particularly the anti-apoptotic and proliferative effects of NFκB, a transcription factor abundantly expressed during inflammation (Chen et al. 2013; Alexopoulou et al. 2001). Additionally, numerous inflammatory mediators cause oxidative damage, including iNOS and NADPH. Coupled with the tissue repair response, this system can promote the incorporation of DNA damage and cell survival. Neoplasms reach a threshold of $1mm^3$ before requiring their own vasculature. Pertinently, VEGF, another inflammatory target, is aberrantly expressed by cancer-associated fibroblasts and facilitates tumour angiogenesis (Riddell et al. 2012).

Inflammatory skin conditions such as lupus can predispose patients to squamous cell carcinomas (SCCs) by way of immune cell infiltration and stromal cell remodelling. Interestingly, by reducing the CD4+ T-cell population in SCC-susceptible mice, tumour emergence and progression is delayed (Nakao et al. 1995; Itoh et al. 1995). Inflammatory bowel disease is a well-recognised and formidable risk factor for colorectal cancer. There is

strong evidence to suggest that the endogenous intestinal microbiota is central to the development of IBD-driven colorectal carcinoma (Hale & Greer 2012; Ock et al. 2011). As mentioned previously, IL10 knock out mice present with IBD as well as rectal dysplasia, adenocarcinoma and colitis, induced by *Enterococcus faecalis* (Chichlowski et al. 2010). Pro-biotic modification of the enteric flora in mice null for IL10 associated with reduced mucosal inflammation and colon cancer (O'Mahony et al. 2001). Another potent inflammatory antagonist, TGFβ, inhibits inflammatory signalling and cell activation. Disruption to TGFβ signalling through ligand depletion or receptor knock out amplifies immune cell infiltration, metastasis and malignant growth potential in a number of SCCs (Gasparoto et al. 2012; Oshimori et al. 2015).

Here I will focus on understanding the processes of anti-inflammatory mechanisms and determining how the inflammatory response escapes limitation in chronic immunopathologies.

1.3 The immune system

1.3.1 Innate and adaptive immunity

Vertebrates have two components to their immune system: innate and adaptive. The innate response is evolutionarily ancient and present in all metazoans, whereas the adaptive system is an asset of vertebrates only (Iwasaki & Medzhitov 2010).

The innate immune response consists of two sequential parts. Initially, the invading pathogen is identified by host cell surface receptors with the ability to recognise pathogen or host-damage associated products. Subsequently, the recruitment of effector cells and release of inflammatory mediators results in the destruction and elimination of the

infectious agent (Iwasaki & Medzhitov 2015). The receptors of innate immunity are numerous in type but non-specific i.e. they do not distinguish between individual pathogens. The non-specific nature of innate immunity is necessary to assist a rapid inflammatory response and prevent the onset of infection. In fact, the efficiency of the mammalian innate immune system is such that everyday infections are cleared before painful and clinically relevant symptoms are experienced. Thus our knowledge of innate immunity is quite rudimentary, relative to what we understand of the adaptive immune system, which becomes activated when infection persists and the innate immune system 'calls' for help.

The adaptive response is activated when effectors of the innate immune system present antigen to lymphocytes with the appropriate pathogen-specific receptors (Vincenzo et al. 2015). Thus the adaptive immune system is antigen dependent and pathogen specific, since only cells expressing the cognate receptor become activated. This specificity results in an immunological memory that allows the host to inaugurate a much stronger attack each subsequent time the pathogen is encountered. Protective immunity has been manipulated in medicine for the use of vaccines that introduce live-attenuated organisms or inactivated toxins to the host, which cause long-lasting immunity without causing disease.

1.4 Cells of the immune system

The immune system incorporates numerous cell types and molecular mediators, accounting for its heterogeneity of actions and allowing agonists to deliver specific but also potentially innumerable cellular responses. Pluripotent haematopoietic stem cells (HSCs) are the progenitors to leukocytes; which is the umbrella term for cells of the immune system (Kanji

et al. 2011). A large class of cell types, Leukocytes may be further subcategorised into; erythroid, including red blood cells and the megakaryocytes that give rise to platelets; myeloid, including granulocytes, monocytes, macrophages and dendritic cells; and finally lymphoid, comprising the large and small cells that stem from the lymph. The lymphoid organs offer niche environments that cater for the maturation of HSCs but however, do not give rise to de novo leukocyte precursors. These tissues include, non-exclusively, the thymus and spleen.

1.4.1 Origins of myeloid cells

The myeloid lineage encompasses the largest population of leukocyte cells and includes granulocytes, monocytes, macrophages and dendritic cells. This subpopulation of leukocytes comprehends the first line of defence against pathogenic invasion and is key in coupling systems of innate and adaptive immunity.

1.4.1.1 Myelopoiesis and embryonic haematopoiesis

Myelopoiesis is the process by which early monocyte progenitors colonise tissues and organs and differentiate into resident macrophages that will self-maintain throughout adult life (Baron et al. 2012; Bellantuono 2004). Quite recently the early genesis of tissue macrophages has come under scrutiny and been reconsidered. Embryonic haematopoiesis begins at E8.5 in mice, when primitive macrophages migrate to the central nervous system and form a stable compartment in the yolk sac (Ginhoux et al. 2010; Kierdorf et al. 2013; Schulz et al. 2012). A number of adult tissue macrophages, including Kupfer cells of the liver (Schulz et al. 2012) originate from this early population of myeloid precursors. Subsequent to this stage, the first haematopoietic stem cells are formed in the aorta–gonad–mesonephros (AGM) region from cells of the ventral aortic wall (Medvinsky & Dzierzak 1996). From here, the foetal liver becomes the foremost site of haematopoietic maturation and expansion, giving rise to erythroid, lymphoid and myeloid cells (Medvinsky et al. 1996; Kumaravelu et al. 2002). Up-regulation of foetal PU.1 favours the maturation of monocyte, macrophage and dendritic cell precursors by enhancing the expression of myeloid specific factors including IRF8, ERG1 and KLF4 and inhibiting mediators of other differentiation pathways (Kierdorf et al. 2013). It is at this point, following colonisation of the foetal liver, that pro-monocytes enter the circulation and seed niches such as the thymus and spleen

and postnatally, the bone marrow. During adult life, cells of the bone marrow niche are responsible for maintenance and replenishment of HSCs (Godin & Cumano 2005).

1.4.1.2 Monocyte, macrophage and dendritic cell precursors

HSCs develop in the foetal liver through multipotent progenitor stages and under the influence of PU.1 specify monocyte/macrophage and dendritic cell precursors (MDPs) (Dahl et al. 2003). MDPs precede common monocyte progenitors and dendritic cell precursors but have lost the ability to enter the granulocyte lineage.

The balance of growth factors and cytokines involved in MDP differentiation and maturation is crucial to defining the fate and specific function of cells. Up-regulation of PU.1 enhances the expression of IRF8, ERG1, KLR4 and MCSFR (Kanji et al. 2011). Mice null for PU.1 exhibit reduced granulocyte and macrophage populations and die perinatally (Dahl et al. 2003). In addition, conditional deletion of MCSFR or its ligand in vivo results in severe osteoporosis, reproductive defects and abnormal organ development (Felix et al. 1994; Dai & Holland 2003). Adult, tissue resident macrophages derive from essentially three origins, the yolk sac macrophages, foetal liver monocytes and the bone marrow and most importantly, at different and multiple stages of development (Gekas et al. 2005; Ottersbach & Dzierzak 2005; Kumaravelu et al. 2002). Furthermore in response to infection or injury, classical monocytes may be recruited to target sites and differentiate into specialised macrophages.

Still, even in their mature form macrophages remain a highly heterogeneous group of cells and may adopt different phenotypes depending on the needs of the tissue and prevailing cytokine milieu. For example, two extreme macrophage polarisation states include classically (M1) and alternatively (M2) activated (Gordon 2003; Sica & Mantovani 2012). In response to IFNγ and TLR activation, type M1 macrophages release ‘pro-inflammatory’

cytokines associated with immune resolution. M2 macrophages on the other hand, respond to signalling through IL3 and IL4 and are associated with wound healing and tissue repair (Wynn et al. 2013). In vitro we can program monocytes to become enriched for classic or alternative macrophages using various stimuli, for example GM-CSF for 'more M1' and M-CSF for 'more M2' –like. These classifications of macrophages exist on a spectrum which, as aforementioned reflect cellular microenvironment and therefore serve tailored roles in homeostasis, immunity, tissue repair, fibrosis, scavenging and angiogenesis (Wynn et al. 2013; Gautier et al. 2012).

Dendritic cells are the major antigen processing and presenting cells of the immune system, forming the bridge that links innate and adaptive immunity (Banchereau et al. 2000; Mellman & Steinman 2001). Conventional DCs (cDCs) fall under two categories expressing either CD8α or CD103/CD11b cell surface markers in mice and, equivalently CD141/BDCA3+ or CD1c/BDCA1+ in humans (Ginhoux et al. 2009; Varol et al. 2009; Shortman & Naik 2007). CD8α+ cDCs present viral antigens to CD8+ T-Cells and may also be involved in driving Th1/Th1 cytokine synthesis and release. CD11b cDCs on the other hand present to CD4+ T-Cells and utilise transcription IRF4 to induce pro-inflammatory cytokine production (Schlitzer & Ginhoux 2014).

The common cDC lineage is selected by high concentrations of PU.1. idr specifies the differentiation of conventional DCs, whereas and inhibition of idr leads to the production of plasmacytoid DCs (pDCs) (Shortman & Naik 2007). Present in all adult lymph and non-lymphoid tissues, cDCs undergo constant replenishment from bone marrow common dendritic cell precursors (CDPs) expressing haematopoietic cytokine receptor Flt3 (Tahoori et al. 2015). The common DC lineage is driven by PU.1, Gfil and Cbfβ expression and gives rise to non-conventional plasmacytoid- and pre-cDCs also (Wynn et al. 2013). In the blood,

so called ‘monocyte derived’ or ‘inflammatory’ DCs may differentiate under activated conditions, on-site from pre-cDCs or monocyte infiltrates (Seillet et al. 2013; Seillet & Belz 2013). Plasmacytoid cells produce a large amount of interferon on activation and play a key role in protection against viral infection ((Kim et al. 2014; Kim & Lee 2014; Domínguez & Ardavín 2010).

1.4.1.3 Granulopoeisis

Finally, within the most abundant classification of the myeloid lineage are the granulocytes or polymorphonuclear leukocytes, so called because of their five-lobed, irregularly shaped nuclei. The granulocyte family includes neutrophils, eosinophils and basophils, all of which stem from granulocyte/monocyte progenitors (GMPs) that mature into eosinophil-committed progenitors in the foetal liver under conditions of C/EBPα, PU.1 and GATA2 (Panopoulos & Watowich 2008; Dunn et al. 1994). Basophils and mast cells mature from respective precursors after this step. Neutrophils comprise a large proportion of the granulocyte lineage, however a common progenitor is yet to be described.

1.4.2 The lymphoid system of cells

1.4.2.1 Natural killer cells

Large lymphoid cells include natural killer (NK) cells that are involved in the destruction of viral machinery and virally-infected host cells. Although NK cells are activated by DCs (amongst other effectors), the non-specific nature of NK cells places them within the innate immune system of cells (Moretta et al. 2002; Moretta 2002; Chauveau et al. 2010).

1.4.2.2 B- and T- lymphocytes

Small lymphoid cells however incorporate the highly evolved, antigen specific cells of the adaptive immune system. The effector cells (monocytes and dendritic cells) of innate immunity are recruited to and subsequently activate T- and B- lymphocytes, thus initiating the adaptive immune response. T-lymphocytes express T-cell receptors on their cell surface. Whereas Immunoglobulins (IgGs) are expressed either as B-cell surface receptors or may be secreted by plasma B-cells as soluble mediators of adaptive immunity known as antibodies (Vazquez et al. 2015). Antigens are molecules recognised by specific B- and T-cell receptors that in turn elicit a strong and precisely targeted immune response. The pathogen-specific B- and T- lymphocytes expressing antibodies proliferate and are retained long after the infection is cleared thus creating an immunological memory, which enables efficient clearing of a secondary infection. Both B- and T- lymphocytes originate as bone marrow precursors, however their maturation processes occur in the spleen and thymus respectively.

T-cells undergo a process of accelerated somatic hypermutation and VDJ recombination that allows a small number of genes to generate a vast number of different T-cell surface antigen receptors coupled to numerous effector functions (Kaech et al. 2002). T-cell Random hypermutation introduces a potentially injurious disadvantage in that the adaptive immune

system cannot distinguish self from non-self. In the thymus, self-antigen presenting cells strongly activate those potentially deleterious T-cells leading to apoptosis; a self-tolerising process known as negative selection (Germain 2002; Germain et al. 2002).

During maturation in the spleen, B-lymphocytes undergo random isotype switching of the IgG receptor heavy chain, which is relevant to the effector function (Lin & Chen 1993). The light chain however, carries the unique and specific antigen receptor and is not subject to random mutation (Esser & Radbruch 1990). Thus the cell types of adaptive immunity are few – but their variety is both infinite and random.

Following maturation and positive selection, T-lymphocytes migrate to the peripheral lymph organs as either CD8+ cytotoxic T-cells or CD4+ helper T-cells (Kaech & Ahmed 2001; Germain et al. 2002).

Cytotoxic T-cells are the adaptive equivalent of NK cells; they kill host cells infected with virus or bacteria. Helper T-cells (T_h) on the other hand act as mediators of adaptive immunity, interacting with both macrophages and B-cells and facilitating a more direct response against a specific antigen infection. Linked recognition allows B-cells to become activated by cognate T_h cells i.e. those that bear the same antigen receptors. T_{h2} cells are a subdivision of helper cells that primarily mediate the humoral response and support B-cell proliferation and class switching (Cantor & Boyse 1977; John et al. 2008). The T_{h2} response produces, namely IL4, IL5, IL6, IL10 and IL13. IL10 is inhibitory to IFNγ and IL12, which potentiate the T_{h1} response (Rissoan 1999). T_{h1} cells are associated with increasing macrophage killing potential by producing IFNγ, TGFβ, IL10 and increasing IL12 as a type of feed-forward response (Cherwinski et al. 1987; Santana & Rosenstein 2003). Additionally, T_h17 and T_{reg} have been identified and characterised as subsets of helper T cells. T_h17 cells characteristically release IL17 and IL22 in response to bacterial compounds (Infante-Duarte

et al. 2000). Whereas T_{reg} supress autoimmunity and release IL10 and TGFβ (Sakaguchi et al. 2009).

1.5 Soluble mediators of immunity

Macrophages and other cells of the immune system are potent producers of pro-and anti-inflammatory cytokines and chemokines. Chemokines are small cytokines able to induce chemotaxis, some of which are considered to be pro-inflammatory and are induced at the site of infection during the inflammatory response (Arango Duque & Descoteaux 2014). Cytokines are intercellular signalling proteins, peptides and glycoproteins including interferons (IFN) and interleukins (IL) that, generally, have immunomodulatory effects (Davoine & Lacy 2014). Most cytokines are pleiotropic and show redundancy, acting as part of a cascade where they are released in succession to one-another, can be synergistic and often counter-regulated by inhibitory cytokines and soluble receptors. Thus, through complex signalling cascades, cytokines are able to influence the regulation of genes, transcription factors, additional cytokines or cytokine receptors and also negative feedback regulators of inflammation (Cruvinel et al. 2010).

1.5.1 The role of TNFα in inflammation

TNFα was identified following observations of tumour regression in patients who had contracted bacterial infection (St-Pierre & Chadee 2014). Bacterial lipopolysaccharide (LPS) is a potent activator of the immune response and subsequently, TNFα biosynthesis. The biological roles of TNFα are somewhat double edged (Aggarwal 2003). Cytokines of the TNFα superfamily mediate basic cellular functions, including the intricate interplay between

cellular proliferation, differentiation and apoptosis in inflammation (Zelová & Hošek 2013; Sessler et al. 2013; Gaur & Aggarwal 2003). Accordingly, inappropriate expression of these potent yet crucial factors can be damaging and as such have been implicated in the pathogenesis of a number of inflammatory diseases and cancers (Landskron et al. 2014).

Currently, we recognise 19 TNF ligands and all except 1; VEGI, are expressed by cells of the immune system (Figgett et al. 2014; Cabal-Hierro & Lazo 2012). Major sub-types TNFα/ β share 30% sequence homology and bind to cell surface TNFα receptors (TNFRs) 1 and 2. Perhaps accounting for diversity of function and non-specific toxic potential, TNFR1 is expressed in every cell type, while TNFR2 can be found in immune and endothelial cells (Cabal-Hierro & Lazo 2012). Receptors of TNFα do not trigger a biological response themselves but recruit adaptor proteins including TRAFs or death domain (DD) containing TRADD/ FADD. TNF binding and DD interaction in turn activates TNFR1 accessory proteins RAF2, cIAP1, cIAP2 and RIP1 and signalling ultimately leads to the activation of AP1 and NFκB (Schneider-Brachert et al. 2013; Cabal-Hierro & Lazo 2012). Additionally, ligand bound TNFR1 may undergo endocytosis thus altering accessory protein binding –potential. This secondary pathway is associated with promoting cell death by apoptosis and necroptosis (Cabal-Hierro & Lazo 2012).

TNFα is a mediator of inflammation and tumour development, due to its pivotal roles in NFκB activation and promoting cell death. Accordingly, dysregulated expression of TNFα is, invariably central to the processes of systemic inflammation and autoimmune disease and in many instances responsible for tumour cell proliferation and survival (Landskron et al. 2014). TNFα is implicated in a number of cancers including B-cell chronic lymphoid leukaemia, acute leukaemia, ovarian cancer and glioblastoma (Yang et al. 2014; Ji et al. 2013; Eisele & Weller 2013; Zhu et al. 2012). TNFα biosynthesis and expression is also

fundamental to the development of inflammatory conditions such as type II diabetes, IBD, ankylosing spondylitis, glomerulonephritis and SLE and rheumatoid arthritis (Bradley 2008).

1.5.1.1 Regulation of TNFα expression

Due to the significant pathological consequences of either over-expression or under-expression of TNFα, biosynthesis is subject to multiple levels of complex regulation (Juhász et al. 2013). In response to activation by TLR stimulation, lipid or cytokine interactions, myeloid and T-cell populations transcribe TNFα rapidly and independently of *de novo* protein synthesis. Additionally, there are major mechanistic distinctions in TLR signalling which drive the expression of such rapidly expressed genes as TNFα. For example, prior to induction by pro-inflammatory pathways, the TNFα promoter, like many immediate early gene promoters, is pre-bound with TBP and exhibits RNAPII pausing directly upstream of the TSS. Sequences 'poised' in this way are subject to rapid synthesis following RNAPII release and activation of transcriptional elongation (Adamik et al. 2013). Productive elongation of nascent transcripts requires the dynamic posttranslational modification of RNAPII; a mechanism which is now recognised as a key phase in the regulation of transcription. In promoter-proximal pausing, RNAPII is associated with promoter regions at, downstream and occasionally even upstream of the transcription start site (TSS). Phosphorylation of the accessory protein DRB-sensitivity-inducing factore (DSIF) and the carboxy terminal of RNAPII by positive transcription elongation factor b (P-TEFb), leads to rapid activation of transcription. Thus promoter proximal pausing negates the lag-time that would be imposed by active recruitment of RNAPII (Jonkers & Lis 2015).

Depending on cell type and stimulus, TNFα may be regulated by an array of transcription factors and the promoter region is littered with binding motifs including CRE, ATF, C/EBP, AP1/2, Egr1 and NFκB (Pauli 1994; Kotlyarov et al. 1999). A considerable number of studies

have focused on NFκB dependent TNFα transcription, largely due to the significant abundance of inflammatory conditions in which NFκB function is perturbed and TNFα is characteristically overexpressed. Cell and tissue specific expression of TNFα may also be defined by epigenetic mechanisms of regulation. DNAse hypersensitivity generally confers genomic regions of relaxed chromatin and associated DNA that is accessible to transcription factors. Regions of DNAse hypersensitivity at the TNFα locus differs between cell type and implicate enhancer regions under epigenetic control (Skoog et al. 2006; Barthel & Goldfeld 2003; Chen et al. 2009). Additionally, glucose-rich microenvironments induce proximal histone acetylation in myeloid cells, increasing transcription factor binding and enhanced TNF expression. Cytokine biosynthesis is commonly regulated at the epigenetic level (Vanden Berghe et al. 2006; Csaba 2014).

1.5.2 Interleukins

The interleukins were coined as early as 1977 with the discovery of IL1, previously identified as human leukocytic pyrogen (Onozaki 2013). They are a large family of cytokines that are synthesised and expressed by leukocytes. Based on sequence homology, receptor chain similarities or functional properties, the interleukins are further categorised into a number of other sub-groups, for example the IL1, IL10, common γ-chain, Th2-like and a handful of other families. A functioning immune system depends on interleukin production, mostly by CD4 Th-cells, monocytes, macrophages and some endothelial cells.

1.5.2.1 Interleukin 1α (IL1 α)

Originally entitled human leukocytic pyrogen, IL1α is the archetypal member of the IL1 family of cytokines. Both IL1α and IL1β were identified almost simultaneously to exert

similar effects through the IL1 receptor I (IL1RI) (Garlanda et al. 2013). Inappropriate expression of IL1β is key to a number of auto-inflammatory or periodic fever syndromes, with which patients experience recurrent episodes of systemic and organ specific inflammation. Autoinflammatory syndromes differ from autoimmune diseases in that the latter involve misdirection of adaptive immunity as opposed to the former, which are resultant of innate immune dysfunction. IL1α is a mediator of the acute phase of inflammation and induces pain sensitivity, fever, vasodilation and hypotension and patients suffer fever, pain in the joints and abdomen, skin rashes and in severe cases, amyloidosis. Therefore synthesis of and exposure to IL1α is critically regulated. Precursor of IL1α is synthesised without a signal peptide fragment and may be processed into a mature form by calpain (Martinon et al. 2009). However cleavage of pro-IL1α is not a requirement for binding to IL1RI. Similarly, IL1β is cleaved post induction. The inflammasome is an NLR-multiprotein complex required for the biological activation of inflammatory proteins including IL1β and IL18. Upon stimulation, NLR subsets NALP1, 2 and 3 assemble into common cytoplasmic structures that recruit and activate caspases responsible for the cleavage and activation IL1β (Martinon et al. 2009).

As an additional means of regulatory control, IL1α and β may also bind to IL1RII; a decoy receptor that associates ligand without prompting a signalling cascade. Thus IL1RII acts as a natural inhibitor of the IL1α/ β pathway (Gabay et al. 2010).

Following activation of membrane bound TLRs, for example by endotoxin or LPS, active pro-IL1α is produced by a number of immune effector cells including macrophages, monocytes, lymphocytes, fibroblasts and neutrophils (Dinarello 2009; Feldmeyer et al. 2010). Cells responding to IL1α include T-cells, B-cells, fibroblasts, epithelial cells and endothelial cells to name a few, all of which co-express IL1RII with IL1RI (Akdis et al. 2011). Binding of IL1α to

IL1RI provokes signal transduction via the adaptor molecule MyD88, subsequent activation of IL1R-associated kinases (IRAKs) and induction of NFκB and MAPK- regulated factors such as p38 and JNK. By inducing the expression of pro-inflammatory genes such as COX2 and iNOS amongst many more, IL1α plays an important role in potentiating local and systemic inflammation (Weber et al. 2010). Therefore IL1α has a major role in numerous autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis as well as immune-associated conditions like atherosclerosis and Alzheimer's disease.

Somewhat complementary to IL1RII, IL1Ra shares sequence similarity to and is produced in response to the same stimuli as IL1α (Jesus & Goldbach-Mansky 2014)but lacks the necessary IL-1AcP accessory protein binding domain and therefore competitively binds to IL1RI without instigating signal transduction (Dinarello 2009; Akdis et al. 2011) . Mice lacking IL1Ra develop a spontaneous inflammatory syndrome similar to human rheumatoid arthritis, where overexpression of inflammatory cytokines IL1β, TNFα and IL6 is pathogenic (Fantuzzi & Dinarello 1996; Cartmell et al. 2001). Concurrently, therapies for some immune disorders involve administration of IL1Ra for effective neutralisation of IL1α (Bao et al. 2014; Hu et al. 2015).

1.5.2.2 Interleukin 6 (IL6)

Originally identified as a B-cell differentiation factor (BSF2), IL6 is a pleiotropic cytokine and myokine with a significant role in immune regulation (Scheller et al. 2011). Membrane bound or soluble, IL6 has functions in T-cell proliferation, B-Cell differentiation, survival and production of IgG, IgA and IgM antibodies and has effects on leukocyte trafficking, including recruitment of neutrophils and mononuclear cells. IL6 is also expressed in muscle and, independent of preceding TNFα or NFκB expression, is up-regulated by Ca2+/NFAT and

glycogen/ p38 MAPK signalling and is anti-inflammatory. As a cytokine, IL6 is released by monocytes, macrophages and fibroblasts during systemic inflammation (Scheller et al. 2011).

The IL6 cell surface receptor is found coupled to gp130 (/CD130) an adapter protein which is shared with other receptor molecules including leukaemia inhibitory factor (LIF), IL11, ciliary neurotrophic factor (CNTF), oncostatin-M and cardiotrophin-1 (CT-1) (Taga & Kishimoto 1997; P. Wang et al. 2010). Target cells lacking the membrane bound receptor can respond to IL6 through expressing the soluble IL6R (sIL6R), which, subsequent to ligand binding forms a complex with sgp130 (Boulanger et al. 2003; Xu et al. 2010). Downstream of ligand binding, phosphorylation of gp130 Y759 leads to Ras-ERK-MAPK signal transduction and activation of the transcription factor C/EBP. Posttranslational modification of gp130 Y767, Y814, Y904 or Y915 in response to IL6 is required for survival and instigates JAK activation and the induction of STAT3 target genes (Taga & Kishimoto 1997; Hemmann et al. 1996; Feng et al. 1997).

Signalling through sIL6R is considered to be important for T-cell function and survival in inflamed tissues whereas membrane bound IL6R is plays a key role in T_{reg} activity. By suppressing T_{reg} responses, through driving T_h17 and T_h2 differentiation, IL6 promotes a pro-inflammatory milieu and therefore may be a relevant target for the treatment of chronic inflammatory and autoimmune disease (Eulenfeld et al.; Babon et al. 2014). Evidence supporting the involvement of IL6 in autoimmune disease burgeoned from a study that identified that approximately one third of patients with cardiac myxoma also experience symptoms of autoimmune disease. Successively, cardiac myxoma cells and their supernatants were identified to contain elevated IL6 mRNA and protein (Kishimoto 1992). Latterly, substantial evidence has been obtained to support a role for IL6 in the

pathogenesis of numerous chronic inflammatory and autoimmune diseases including chronic inflammatory proliferative disease (CIPD), SLE, B-cell malignancy, RA and IBD (Scheller et al. 2011; Rossi et al. 2015).

Expression of IL6 is required for experimentally induced models of arthritis in which T-cells overproduce Th1 and Th17 cytokines. Furthermore, blockade of IL6 in these models reduces the level of Th17 synthesis and release as well as alleviates the clinical symptoms and histopathological signs of arthritis (Alonzi et al. 1998; Yen et al. 2006).

Serum levels of IL6 are substantially elevated in the synovial fluid of arthritis patients. Indeed, drugs that are clinically effective have been shown to suppress IL6 secretion, a function that correlates with patients' response to therapy (Kishimoto 1992; Rossi et al. 2015; Moghaddami et al. 2014). Patients with SLE often develop arthritis, which increases in severity proportionally with the level of IL6 overexpression. As a B-cell stimulatory factor, IL6 stimulates autoantibody production and immunoglobulin secretion. The involvement of IL6 in SLE pathogenesis is clear. Immunoglobulin anti-DNA antibodies are produced by the low density B-cells of SLE patients, and supressed by anti-IL6 antibodies. Anti-DNA antibody accumulation is also key to the development of glomerulonephritis and kidney dysfunction observed in SLE sufferers (Rönnelid et al. 2003; Ball et al. 2014; Zhu et al. 2013).

A number of studies have highlighted the positive correlation between IL6 expression and IBD severity in humans. Serum IL6 is higher in patients with CD when compared to those with UC. Mice lacking STAT3 expression in macrophage and gut-epithelia develop a much milder form of dextran induced colitis than those with full inducibility. Additionally, those mice overexpressing STAT3 suffer a much more severe disease (Yen et al. 2006). In a clinical study, 80% of CD patients receiving anti-IL6 antibody showed a positive response, compared

to 30% of a placebo group, highlighting the potential of anti-IL6 strategy for the treatment of IBDs (Allocca et al. 2013).

Further to this, IL6 appears to play a predominant role in linking chronic inflammation with tumour growth. The JAK/STAT3 pathway is linked to the development of solid tumours, including colorectal cancer. Through downstream STAT3 activation, IL6 generates an inflammatory microenvironment in which anti-tumour mechanisms are supressed and therefore promotes tumour proliferation, survival and invasion. Concurrently, SOCS3, a repressor of JAK/STAT3 signalling, is silenced in a number of malignancies. Additionally, antiviral infection of CRC cells with SOCS3 significantly inhibited proliferation (Wei et al. 2014).

1.5.2.3 Interleukin 10 (IL10)

Interleukin 10 (IL10) is produced by activated macrophages and dendritic cells and limits the expression of pro-inflammatory cytokines and chemokines (Smallie et al. 2010; de Waal Malefyt et al. 1991; Akdis et al. 2011).

In response to ligand binding, IL10R signals through the JAK pathway, activating STAT transcription factors, particularly STAT3 (Finbloom & Winestock 1995; Chen et al. 2014). In fact, STAT3 is essential for the anti-inflammatory actions of IL10 and *vice versa* (Takeda 1999; Lang 2002; Yasukawa 2003). Anti-inflammatory and pro-inflammatory targets of STAT3 may, respectively be activated or directly inhibited at the level of transcription (Schottelius et al. 1999; Murray 2005). Through up-regulation of soluble TNFR, tissue inhibitor of MMPs (TIMPs) IL1Ra and IL1RII, IL10 is able to down regulate a multitude of pro-inflammatory factors including TNF, IL6, IL1, MCP1/5, CCL5 and GMCSF (Kontoyiannis et al. 2001; Denys et al. 2002; Schaljo et al. 2009).

The central role of IL-10 in restraint of inflammation is demonstrated by the development of severe IBD in Il10-/- mice (Kühn et al. 1993). Production of IL10 by protective T_{reg} cells is central to suppression of the inflammatory response to commensal bacteria in the gut (Barnes & Powrie 2009a; Barnes & Powrie 2009b). Moreover, mice deficient in IL10 develop severe inflammatory bowel disease (Kühn et al. 1993; Kuhn & Stappenbeck 2013). Additionally, patients with early onset colitis are often homozygous for IL10R mutations and demonstrate reduced IL10 signalling and activation of the critical STAT3 (Glocker et al. 2009). It is also important that the immunosuppressive effects of IL-10 are limited. Aberrantly enhanced expression of IL-10 has been implicated in a number of conditions including SLE and multiple scleprosis (MS), where patients and family members alike have high numbers of cells that spontaneously produce and are less responsive to IL-10 (Beebe et

al. 2002; W. Yuan et al. 2011; Sun et al. 2012). Additionally, IL-10 is also known to enhance proliferation and survival of melanoma cells (Krüger-Krasagakes et al. 1994; Itakura et al. 2011).

1.5.3 Interferon γ (IFNγ)

Type I and II interferons were first identified as inhibitors of viral replication and categorised according to sequence similarity and receptor specificity. As the only type II member, IFNγ has a distinct structure and binds specifically to the interferon gamma receptor (IFNGR). Ligand binding catalyses a JAK-STAT auto-phosphorylation cascade that leads to nuclear translocation of STAT1 homodimers and altered IFNγ target gene expression (Rauch et al. 2013).

The production of IFN γ by CD4+ T-cells and NK cells is induced mostly by IL12 and its synergism with IL18, secreted by macrophages, dendritic cells and neutrophils. The immunostimulatory and immunomodulatory functions of IFNγ include lymphocyte recruitment and prolonged tissue activation, macrophage production of TNFα, iNOS and hydrogen peroxide and enhanced antigen presentation by MHC class I and II (Watford et al. 2003; Smith & Denning 2014). Prototypic of cell-mediated immunity, IFNγ and IL12 are the key cytokines driving the Th1 primary response. The humoral response is powered mainly by IL4, which is strongly antagonised by IFNγ. A robust positive feedback loop between IL12 signalling and IFNγ release, in addition to Th1 inhibition of IL4 ensures further polarisation towards a cell-mediated response.

Macrophage biosynthesis of a number of genes, including iNOS, in response to TLR agonism requires IFNγ 'pre-treatment'. This phenomenon, whereby IFNγ facilitates a more rapid and heightened response to inflammatory stimuli in previously exposed macrophages is called

priming (Schroder et al. 2004). The physiological significance of this mechanism is highlighted in IFNGR knock out mice which are highly resistant to LPS-induced toxicity (Vermeire et al. 1997; Manoury-Schwartz et al. 1997; De Klerck et al. 2004). In RAW264.5 cells, IFN pre-treatment improved DNA binding kinetics and enhanced degradation of IκBα to instigate a superior NFκB response in LPS-activated macrophages when compared to untreated cells.

The role of IFNγ in autoimmune disease is, like its function, pleiotropic and complex. Autoimmune diseases characteristically present with chronic activation of macrophages and/ or neutrophils at sites of inflammation. Th17-mediated disease, generally speaking, is affiliated with neutrophil infiltration, whereas Th1-immunity plays a large role in macrophage activation (Steinman 2008). In EAE, ablation of IFNγ or the IFNGR increases morbidity and mortality (Ferber et al. 1996). Similarly, in the CIA model of disease, IFNGR deficiency significantly reduced time of onset and increased the incidence of disease (Manoury-Schwartz et al. 1997; Vermeire et al. 1997). The apparent protective role of IFNγ in these two animal models of chronic inflammation is attributed to its ability to suppress the Th17 response (Seery et al. 1997). Indeed, the symptoms of arthritis in animal models that lack IFNγ are alleviated by treatment with Th17 antibodies. However, various other autoimmunities showcasing Th1-mediated pathogenesis and affiliated macrophage activation are exacerbated by IFNγ expression (Luger et al. 2008). For example, MS is a Th1 condition that is severely aggravated by administration of exogenous IFNγ (Kroenke et al. 2008), as is proteoglycan induced arthritis. The role of IFN in autoimmune pathogenesis is complicated. Various models of disease result from a combination of dysregulated Th1, Th17 (Steinman 2008) and, indeed Th2 and other 'unrelated' responses. Therefore it is

important to consider IFNγ-related pathogenesis in a context that is specific for each autoimmune disease

1.5.4 Transforming growth factor β (TGFβ)

Transforming growth factor –β (TGFβ) is an inhibitor of growth-related cytokines and additionally is critical to modulating the progression and resolution of inflammatory processes (Dennler et al. 2002; Massagué 2012). Importantly, TGFβ is a central mediator of wound healing where its transient expression activates fibroblasts and extracellular matrix (ECM) synthesis (Walraven et al. 2015). During the inflammatory and tissue formation phases of an immune response, TGF is required for the activation and recruitment of, amongst other cells, macrophages and fibroblasts. Transient expression of this growth inducing cytokine promotes ECM synthesis, keratinocyte migration and proliferation and re-epithelialisation (Hameedaldeen et al. 2014). However in episodes of fibrotic disease, TGFβ does not abate, ensuing chronic fibroblast activation and ECM expansion (Massagué 2012). TGF also promotes angiogenic factors. Taken together, these properties of TGFβ, when dysregulated, have high tumourogenic potential.

Translated as a pro-protein coupled to latency associated protein (LAP) and latent TGF binding protein (LTBP), TGFβ is sequestered on the extracellular matrix. The bioactive form of TGFβ is made available through cleavage by a number of constitutive and inducible proteases (Shi & Massagué 2003). Canonical TGFβ-receptor signalling leads to the phosphorylation of receptor associated smads -2 and 3 which may have direct effects on gene expression (Shi & Massagué 2003). The TGFβ receptors are unusual in that they are able to directly transduce extracellular signals to the nucleus via smad intracellular mediators. Binding of TGFβ to type II receptor (TβRII) homodimers on the surface of target

cells subsequently engages TβRI homodimers, forming a heterotetrameric complex. Autophosphorylation by TβRII facilitates transphosphorylation of the TβRI kinase domain, which instigates recruitment and phosphorylation of R-smads 2 and 3 (Zi et al. 2012). Activation of R-smads-2 and 3 promotes smad4 binding to form a complex, which may translocate to the nucleus and drive the transcription of numerous target genes (Kretzschmar & Massagué 1998; Kretzschmar et al. 1997; Macías-Silva et al. 1996). In a non-canonical, smad-independent pathway TGFβ can activate MAPKs via Ras, Rho and TAK1 upstream mediators (Kamato et al. 2013). Additional to DNA binding activity, smads may also facilitate chromatin remodelling as a form of gene expression regulation, by associating with accessory proteins that modify DNA accessibility (Weiss & Attisano).

Selective alterations to TGFβ signalling that promote survival, invasion and metastasis of malignant cells are implicated in the pathogenesis of many cancers including colorectal tumourogenesis (Zhu et al. 1998; Ogawa et al. 2003; Sambuelli et al. 2000). The consequence of TGFβ dysregulation in regards to inflammatory pathways may also be detrimental. Disruption of TGFβ and downstream smad signalling in mice results in a severe and multifocal inflammatory response (Ogawa et al. 2003; Shull et al. 1992; Yang et al. 1999). Mice lacking TGFβ develop multiple organ inflammatory response and lethal cachexia within 2 weeks (Christ et al. 1994). TGFβ has been implicated in SLE and glomerulonephritis (GN) following over expression of the downstream effector smad7, which resulted in the production of auto antibodies against the basement membrane (Kanamaru et al. 2001). Furthermore systemic administration of TGF alleviates autoinflammatory disease whilst anti-TGFβ antibodies drive pathogenesis (Kajdaniuk et al. 2013).

1.5.5 Prostaglandin E2 (PGE2)

The prostaglandins are homeostatic compounds derived from phospholipids via arachidonic acid (AA) which is converted to prostaglandin H_2 (PGH2) and PGE2 by cyclooxygenases (COX-1 and COX-2) and prostaglandin E synthase, respectively (Honda et al. 2006; Díaz-Muñoz et al. 2012). Aside from having significant roles in neuronal signalling, haematopoiesis, regulation of blood flow and pressure, vascular permeability and renal filtration, PGE2 is a mediator of immune responses (Kawahara et al. 2014; Gomez et al. 2005; Konya et al. 2013). Importantly, PGE2 regulates the activation, maturation, migration and cytokine secretion of innate immune cells, which are a primary source of PGE2 production during inflammation (Kalinski 2012).

In response to inflammatory agonists like LPS, IL1β and TNFα, PGE2 is released by mostly myeloid and stromal cells and binds to one of the four specific soluble and membrane-bound PGE2 receptors EP1-EP4 (Sugimoto & Narumiya 2007). Receptors EP1 and EP2 are low affinity and require an abundance of PGE2 to become activated, whereas EP3 and EP4 are high affinity and are subject to ligand-desensitisation. The variety of receptor functions available to PGE2 outlines its versatility and adaptability to differences in agonist, cell type, temporal expression and effector function (Yokoyama et al. 2013; Sugimoto & Narumiya 2007). Generally, prostaglandins are considered to be pro-inflammatory. Indeed, some of the most clinically relevant compounds; non-steroidal anti-inflammatory drugs (NSAIDs), reduce prostaglandin biosynthesis indirectly via COX inhibition. However, PGE2 also up-regulates anti-inflammatory factors such as IL10 and even dampens the phagocytic properties of macrophages (Shi et al. 2010; Hata & Breyer 2004).

The anti-inflammatory and suppressive activities of PGE2 are dependent on receptors EP2 and EP4; both of which are G_s coupled and instigate the cAMP/ PKA/ CREB pathway.

Additionally, EP4 can trigger signalling via PI3K and ERK1/2 (Fujino et al. 2003). On the other hand, EP1 and EP3 are not cAMP-dependent. Although splice variants of EP3 exist, signalling is primarily via G_i coupled receptors, which inhibit adenylate cyclase and, consequently cAMP.

Downstream of cAMP and PKA, cAMP response element binding (CREB) protein mediated the transcription of target genes and inhibits NFκB, thus limiting pro-inflammatory gene transcription.

1.5.6 Anti-inflammatory signalling by cAMP

Generated in response to neurotransmitters, lipid mediators, hormones and chemokines amongst other first messengers, increased intracellular cAMP has mostly anti-inflammatory effects (Aronoff et al. 2006). The cellular functions of cAMP elevation are ultimately mediated by the ability of effectors such as PKA and Epac I to regulate the activation of transcription factors and signalling molecules such as protein kinases, calcium and small GTPases. Inhibition of leukocyte activation by cAMP may be at the level of mediator generation, modulation of phagocytosis and inhibition of microbicidal activity (Serezani et al. 2008). Transcriptional suppression of pro-inflammatory mediators such as cytokines TNF, IL12 and chemokines MIF1α, MIF1β and LBβ4 alongside activation of anti-inflammatory IL10, SOCS3 decisively reduces the activation potential of macrophages, monocytes and neutrophils (Aronoff et al. 2006; Luo et al. 2004; Gasperini et al.). Secondly, through modulating cell surface expression of opsonin-dependent complement receptors (CR) and FcγR I, cAMP is able to modulate phagocytosis. In addition, cAMP also drives expression of inflammatory receptor FcγR 11β (Makranz et al. 2006). Leukocyte microbicidal activity is also suppressed by elevated cAMP expression (Lin et al. 2005). Reactive oxygen

intermediates (ROI), reactive nitrogen intermediates, phagosomal acidification and lysozyme enzyme are all targets of cAMP-mediated down-regulation (Won et al. 2004).

Generally, overexpression of cAMP associates with infectious disease, due to the primary effects of cAMP signalling on phagocytosis. The respiratory pathogen *Bordetella pertussis* is one such which overwhelms the innate immune response by overwhelming the system with cAMP. Two toxins produced by *B. pertussis*, pertussis toxin (PT) and AC-toxin CyaA augment cAMP in target cells by blocking activity of Gαi and catalysing the unregulated conversion of ATP to cAMP respectively (Kamanova et al. 2008). Two other well-known pathogens capable of compromising innate immunity through amplification of cAMP are *Vibrio cholera* and *Escherichia coli*. Both toxins cause constitutive activation of adenylate cyclase through Gαs ADP-ribosylation (Vanden Broeck et al. 2007).

Effectors of cAMP include PKA, EPAC and the cyclic-nucleotide gated ion channels (Sassone-Corsi 2012). Activation of PKA, through cAMP binding to two regulatory subunits, has the potential to affect a number of signalling pathways. Phosphorylation and activation of the metabolic enzymes glycogen synthase and phospholipase kinase reduces glycogen expression whilst acetyl CoA carboxylase inhibits lipid synthesis (Gasperini et al.; Fujino et al. 2005). Phospholipase C is phosphorylated but inactivated by PKA, whilst the MAPKs are activated by PKA-mediated dissociation of inhibitory tyrosine phosphatase. Direct phosphorylation of CREB, CREM and ATF1 by PKA in response to cAMP signalling is a crucial event which facilitates interactions with co-activators of transcription including CBP and p300 and enhancing CRE sequences in the promoters of target genes (Schindler et al. 2013). The cAMP pathway of gene activation is subject to negative feedback through up-regulation of the *CREM* gene, which encodes the powerful repressor ICER (Della Fazia et al. 1997; De Cesare & Sassone-Corsi 2000; Sassone-Corsi 1998).

1.6 Cell signalling and the immune response

Cell signalling is a complex system that allows cells to perceive and correctly and accurately respond to the microenvironment. It is essential for coordinating basic cellular activities including development, tissue repair, homeostasis and immunity.

In some instances, ligand binding and consequent receptor activation is able to elicit a direct cellular response. However, for many cellular actions an activated receptor must first interact with other cellular factors before the ligand has its ultimate physiological effect on the target cell. Signal transduction is the process by which a ligand sets off an intracellular cascade of events involving protein production, interaction and modulation, allowing precise yet reactive control of cell actions. An effective immune response is specifically targeted and stringently controlled in a multitude of cell types, tissue types and under temporal constraints. By layering levels of control in a signalling cascade, it is possible to elicit a direct, operational and efficient immune response.

1.6.1 Pattern Recognition receptors

The initial sensing of infection or injury is mediated by cell surface pattern recognition receptors (PRRs). There are four different classes of PRR; including transmembrane toll-like receptors (TLRs) and c-type lectin receptors (CLRs); as well as cytoplasmic proteins such as retinoic acid inducible gene (RIG)-1-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi & Akira 2010). Pathogen recognition receptors are expressed on non-professional innate-immune cells as well as on macrophages and dendritic cells. Ligands of PRRs are expressed as pathogen associated molecular patterns (PAMPS) on foreign microorganisms or macromolecules. Damage associated molecular patterns (DAMPS) produced by the host

in response to tissue insult or injury are also detected by PRRs. DAMPs may be heat shock proteins or those produced by the ECM in response to injury. Non-protin DAMPs include AT, uric acid and nucleic acids. Activation of PRR signalling pathways, whether by PAMPs or DAMPs, results in the transcriptional expression of inflammatory mediators, the elimination of pathogens or alien agents, repair processes and restoration of tissue homeostasis (Newton & Dixit 2012).

1.6.2 Toll-like receptor signalling and bacterial lipopolysaccharide

The TLRs are the best understood family of PRRs, which characteristically contain an evolutionarily conserved C-terminal Toll-IL-1R (TIR) domain (Bowie & O'Neill 2000; Takeuchi & Akira 2010) and an N-terminal leucine rich repeat (LRR). The TIR-domain is responsible for the recognition of various PAMPS/DAMPS and, as the name would suggest, is common between TLRs and the IL1R. To date ten different TLRs have been identified, each of which able to recognise different host and non-host molecular patterns and elicit unique signalling responses accordingly. Further sub-categorisation of TLRs includes those expressed on the plasma membrane; TLR1, TLR2, TLR4, TLR5 and TLR6 or the endosome; TLR3, TLR 7, TLR 9. The latter, along with TLR 8, recognise unmodified bacterial, viral and endogenous nucleic acids and lead to the production of type I interferons (IFNs) (O'Neill 2000; Medzhitov et al. 2011). Lipoproteins related to viruses, fungi, bacteria and mycoplasma activate surface TLR2 and TLR 1 or TLR6 heterodimer signalling and the production of various inflammatory cytokines (Barbalat et al. 2009).

Lipopolysaccharide (LPS) is a component of gram-negative bacteria and a potent inducer of Inflammatory signalling through TLR4. LPS activation of TLR4 is facilitated by two accessory proteins. LPS binding protein (LBP) is a shuttling molecule that catalyses transfer of LPS from

bacterial plasma membrane to CD14, which presents LPS to TLR4. TLR4 then forms a complex with myeloid differentiation factor 2 (MD2) (Miyake 2006; Takeuchi & Akira 2010). In the presence of LPS, two homodimers of this complex interact to initiate signalling (Park et al. 2009). TLR4-MD2-LPS homodimer formation instigates two different signalling pathways. On the one hand, translocation of TLR4 to the endosome activates TLR3 and TRIF-dependent signalling; activating inflammatory gene transcription via NF-κB and IRF3. Alternatively, homodimers of TLR4-MD2-LPS signal through the MyD88-dependent pathway, common to TLR2-TLR1/6 signalling also. This pathway relies on TIRAP (/MAL) to facilitate the recruitment of adaptor protein MyD88 and TIR domain containing adaptor inducing IFNβ (TRIF) to the TLR. MyD88 engages IL-1 receptor associated kinase (IRAK) -4, which in turn activates IRAK1 and IRAK2 and their interaction with TNFR associated factor (TRAF) -6. Self-ubiquitination by E3 ubiquitin ligase TRAF6 in turn establishes TGFβ activated kinase (TAK) -1, TAK1 binding protein (TAB) -1 and TAB2/3 complex formation. This TAK1-TAB1-TAB2-TAB3 complex subsequently phosphorylates and activates MAP kinase (MKK) -6 and IκB kinase (IKK). Consequently, degradation of IκB releases NFκB for nuclear translocation and inflammatory gene transcription (Medzhitov 2001).

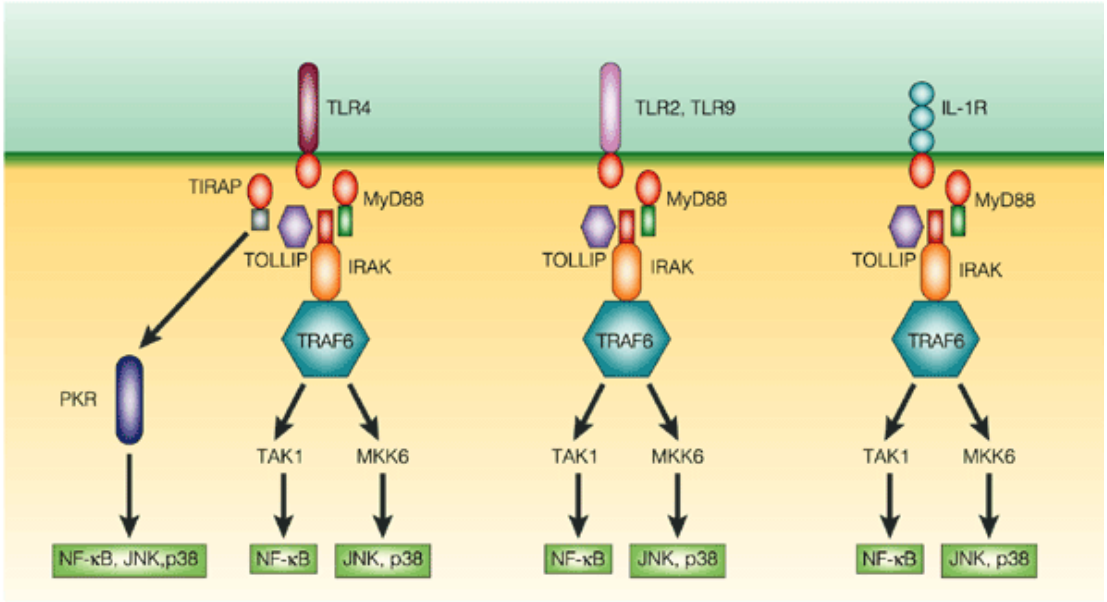


Figure 1.2 TLR4 and IL1R signalling pathways

Members of the IL-1/TLR superfamily activate a common signalling pathway. Ligand-activation of IL1 or TLR transmembrane receptors by IL1 or LPS respectively, causes the recruitment of MyD88, IRAK and its phosphorylation followed by activation of TRAF6/TAB/TAK1 complex. Subsequent phosphorylation of IKK and MAPKK leads to the activation of NFκB and MAPK pathways. Independently of MyD88, TLR4 also signals through TIRAP. *Figure adapted from Nature reviews Immunology* (Medzhitov et al. 2011)

1.7 MAPKS and inflammatory gene regulation.

The MAPK signal transduction cascade is a three tiered phosphorelay system that ultimately has the power to directly affect transcription factors and regulate gene transcription. Additionally, MAPKs are also involved in post-transcriptional regulation of gene expression through modulating the mechanisms of mRNA stability and decay (Wilusz et al. 2001; Garneau et al. 2007). Downstream of TIR stimulation, activated MAP3Ks (/MKKK) phosphorylate MAP2Ks (/MKK). In turn, MAP2Ks transfer phosphate groups to MAPK specific 'TXY' (T = threonine, X = glutamic acid/ glycine/ proline and Y = tyrosine) motifs. These pathways are highly conserved and responsible for the regulation of a vast array of cellular processes, including the immune modulation. The three conventional MAPK families described below are most relevant to this study. However, there are a number of atypical or minor MAPKs, including extracellular signal related kinase (ERK) -3/4, ERK5, ERK7/8 and NLK (Moens et al. 2013).

1.7.1 ERK1/2

The first mammalian MAPKs to be identified were the extracellular signal related kinases (ERKs), encoded by the ERK1 and ERK2 genes. Insulin and mitogen activation of ERK1/2 begins with the upstream engagement of RAS proto-oncogene, followed by recruitment of RAS MAP3K and ERK specific MAP2Ks MEK -1 and -2. ERK-1 and -2 require dual phosphorylation of Thr203-Glu-Tyr205 and Thr185-Glu-Tyr187 for activation respectively (Raman et al. 2007). ERK MAPKs may also be activated independently of RAS by pro-inflammatory stimuli, through PRR signalling (McKay & Morrison 2007).

1.7.2 JNK

Acutely responsive to environmental stressors, the c-Jun NH2 terminal kinases (JNKs) are key facilitators of the immune response. MAP2K7 (MKK7) specifically phosphorylates the JNK activation motif Thr-Pro-Tyr. MAPK kinase 4 (MKK4) has a high affinity for JNK but will also phosphorylate p38 MAPK (Jiang & Gong 2000).

JNKs are encoded by three genes, JNK-1, JNK-2 and JNK-3 (MAPKs 8, 9 and 10), each of which may be differentially spliced to produce numerous JNK isoforms, which are largely similar to one-another within their catalytic cores. JNK is essential for the activation of AP-1, a heterodimeric transcription factor consisting of proteins belonging to the c-Fos, JDP, c-Jun and ATF families, of which the latter two are directly phosphorylated by JNK. Through JNK activation, AP1 up-regulates the transcription of genes containing a specific DNA response element and in turn controls a number of cellular processes including differentiation, proliferation, and apoptosis (Davies & Tournier 2012; Raman et al. 2007).

1.7.3 MAPK p38

Also activated by growth factors and immune agonists via TLRs, MAPK p38 is pivotal to initiating, and restraining the inflammatory response. MKK3 and MKK6 are specific and selectively activate p38 MAPK (Cuenda et al. 1996; Cuenda et al. 1997; Raingeaud et al. 1996; Dérijard et al. 1995). Two MKK3/6 independent pathways of p38 MAPK activation have been identified and involve autophosphorylation through direct interaction of TAB1 and TRAF6 or Zap70 in T-lymphocytes. However the fundamental roles of these activating mechanisms are unclear (Salvador, Mittelstadt, Guszczynski, et al. 2005; Salvador, Mittelstadt, Belova, et al. 2005).

The p38 family of MAPKs consists of four members, p38α, β, γ, δ (MAPK 14, 11, 12, 13 respectively). Of the four p38 kinase isotypes, α and β share the most sequence homology and have a similar function central to immune modulation. A group of anti-inflammatory pyridinyl compounds known to specifically block $p38^{\alpha\beta}$ phosphorylation activity and subsequently IL1 and TNFα expression downstream of p38 MAPK, have been invaluable to the investigation of this pathway (Clark et al. 2003).

Downstream substrates of p38 MAPK include protein kinases, transcription factors and chromatin remodelling agents, all with a variety of effector functions in protein degradation, mRNA stability, endocytosis, apoptosis and cell migration. Through activation of MNK1/2 and subsequent phosphorylation of eukaryotic initiation factor (eIF4E), p38 may regulate gene expression at the level of protein synthesis (Clark et al. 2003; Moens et al. 2013). The MAPK-activated protein kinases 2 and 3 (MK2/3) primarily phosphorylate mRNA binding protein tristetraprolin (TTP), mediating p38 posttranscriptional effects on gene expression (Clement et al. 2011; Brook et al. 2006; Marchese et al. 2010; MacKenzie et al. 2013). MK2 can also recruit and initiate CREB interaction with DNA. Mitogen and stress activated kinases (MSKs) 1 and 2 modify gene expression by histone modification and chromatin remodelling as well as direct activation of transcription factors CREB, ATF1, p65 (NFκB) and STATs 1 and 3 (McKay & Morrison 2007).

Additionally, p38 MAPK may also act on gene transcription directly by recruiting and phosphorylating transcription factors or facilitating their binding to DNA through histone modification. In response to immune stimuli, a number of pro-inflammatory genes are enriched for p38-dependent H3 phosphorylation at their promoter regions, thus enabling interaction of DNA and transcription factors; particularly NFκB (Healy et al. 2012; Kikuchi et al. 2014; Arthur 2008).

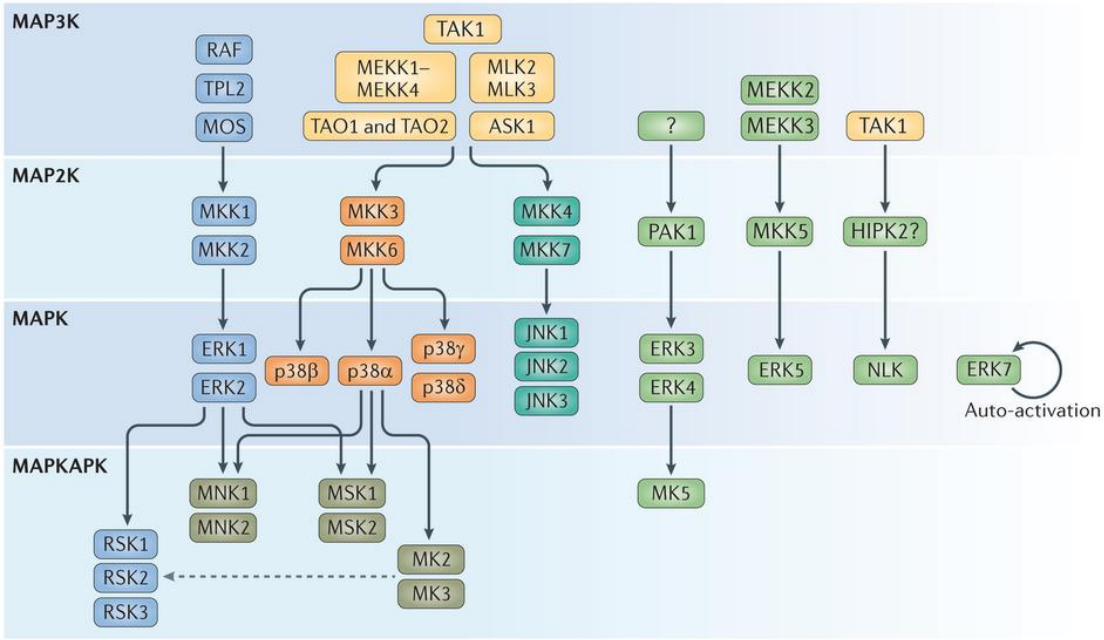


Figure 1.3 Overview of classic MAPK signalling cascades

MAPK activation is preceded by a phosphorylation cascade involving at least two other kinases; The MAP3Ks at the top of the cascade activate MAP2Ks, which in turn activate MAPKs via phosphorylation of the Thr-x-Tyr motif. ERK1/2 MAPKs are regulated by the MAP2Ks MKK1 and MKK2, which themselves are substrates for RAF and TPL2 in response to growth factor/ antigen receptor activation and in innate immunity following stimulation of TLR, TNFR or IL1R subtypes. MOS is involved in activation of the ERK1/2 MAP2Ks in fertilised oocytes. The four isotypes of p38 MAPK lie downstream of MKK3 and MKK6 and whilst the three JNK subtypes are activated by MKK4 and MKK7, the four MAP2Ks of these two pathways share common MAP3Ks. Tertiary MAPKs including MEKKs 1-4, TAO1/2, ASK1 and MLK2/3 and TAK1 all activate JNK/P38 associated MAP2Ks through mechanisms dependent on cell type and stimulus. *Figure adapted from Nature Reviews Immunology* (Arthur & Ley 2013)

1.8 The role of NF-κB in inflammation

For a subset of genes and transcription factors, prior activation of MAPK p38 is required, the most notable of which being NFκB. Consisting of five related proteins, the NFκB family exist as either homo- or hetero-dimers or both, each with the ability to initiate transcription, they are Rel (c-rel), Rel A (p65), Rel B, NFκB-1 (p50 and its precursor p105) and NF-κB-2 (p52 and its precursor p100) (Hayden & Ghosh 2014). By far the most common dimer is the p65/p50 heterodimer and, as all of these dimers, is retained in the cell cytoplasm bound to inhibitor of NF-κB (IκB) (Hinz et al. 2012). The IκB kinase (IKK) is an enzyme complex composed of catalytic subunits IKKα, IKKβ and IKKγ and is essential for the activation of NFκB by pro-inflammatory agents. Specific phosphorylation of the IκBα subunit by IKK causes dissociation of the inhibitory complex, unmasking the nuclear localisation signals (NLS) of NFκB proteins and consequentially allowing translocation of the latter to the nucleus (Ghosh & Baltimore 1990; Lin et al. 1995; Henkel et al. 1993). In the nucleus, NFκB binds to variants of a consensus motif (GGGGACTTCCC) on target genes, enabling their transcription. Targets of NFκB are many and often associated with immune modulation; they include pro-inflammatory TNFα, COX2, IL6, IL1α, IFNγ and IL8 and anti-inflammatory IL10, TGFβ, A20, TTP, DUSP1 to name just a few (Hayden & Ghosh 2014).

1.8.1 A20 & ubiquitin editing in the NFκB pathway

Central to so many cellular processes, NFκB is subject to tight regulatory controls and transient expression that depends on continuous pro-inflammatory stimulation (Catrysse et al. 2014). Ubiquitination is one such mechanism by which NFκB signalling is controlled in order to maintain tissue homeostasis. Ubiquitin editing proteins may be deubiquitinating (deubiquitinases), polyubiquitinating (ubiquitin ligases) or both. An important ubiquitin editing enzyme involved in the NFκB signalling cascade is A20, which contains an amino-terminal deubiquitinating activity and a carboxy-terminal zinc finger domain that facilitates E3 ubiquitin ligase activity (Wertz, O'Rourke, Zhou, et al. 2004). The first substrate of A20 to be recognised was receptor interacting protein 1 (RIP1). TNFR-mediated lysine 63 (K63) polyubiquitination of RIP1 by cellular inhibitor of apoptosis proteins (cAIPs) 1 and 2 is promptly counteracted by the deubiquitinating activity of A20 (Verstrepen et al. 2010). Deubiquitination renders RIP1 unable to interact with NFκB essential modulator (NEMO) and consequently stunts the NFκB activation cascade. In addition, A20 targets RIP1 for degradation by the proteasomal pathway through E3 ubiquitin ligase-mediated addition of chains to lysine 48 (K48), further constraining this key signalling module (Bertrand et al. 2008). In addition to blocking TNF-dependent mechanisms, A20 also protects cells from IL1, CD40, T-/ B- cell antigen activation and PRR driven NFκB signalling pathways. The TLR4, IL17 and NOD2 pathways are also subject to A20 mediated deubiquitination of TRAF6 and RIP2 respectively (Shembade & Harhaj 2012). Furthermore, A20 utilises a number of non-catalytic mechanisms. For example, through preventing the E3 ligase interaction of TRAF6 and two key ubiquitin conjugating enzymes Ubc13 and UbcH5c, subsequently targeting both of which for degradation by polyubiquitination of K48. In addition, A20 directly blocks interactions between Ubc13 and TRAF2/5 and cIAP1/2 (Shembade et al. 2010).

More recent evidence provides a role for A20 in restraining TNF-induced apoptosis. Experimental overexpression of A20 revealed its ability to inhibit apoptotic signalling through deubiquitination and subsequent inactivation of caspase-8 (Jin et al. 2009; Bellail et al. 2012). Indeed, within the death-inducing signalling complex (DISC), A20 is positioned proximal to said activator of TNF-induced apoptosis. Physical interaction of A20 with caspase 8 prevents ubiquitination of caspase-8 by cullin3-associated E3 ligase and opposes apoptotic signalling. Following activation of the TNF-related apoptosis-inducing ligand (TRAIL), A20 has been shown to block apoptotic pathways through polyubiquitination of RIP1 K63 – via a mechanism dependent on the E3 ligase domain. This contrasting action of A20 on RIP1 in an alternate pathway prevents cleavage of caspase-8 and limits cell death signalling (Won et al. 2010; Jin et al. 2009).

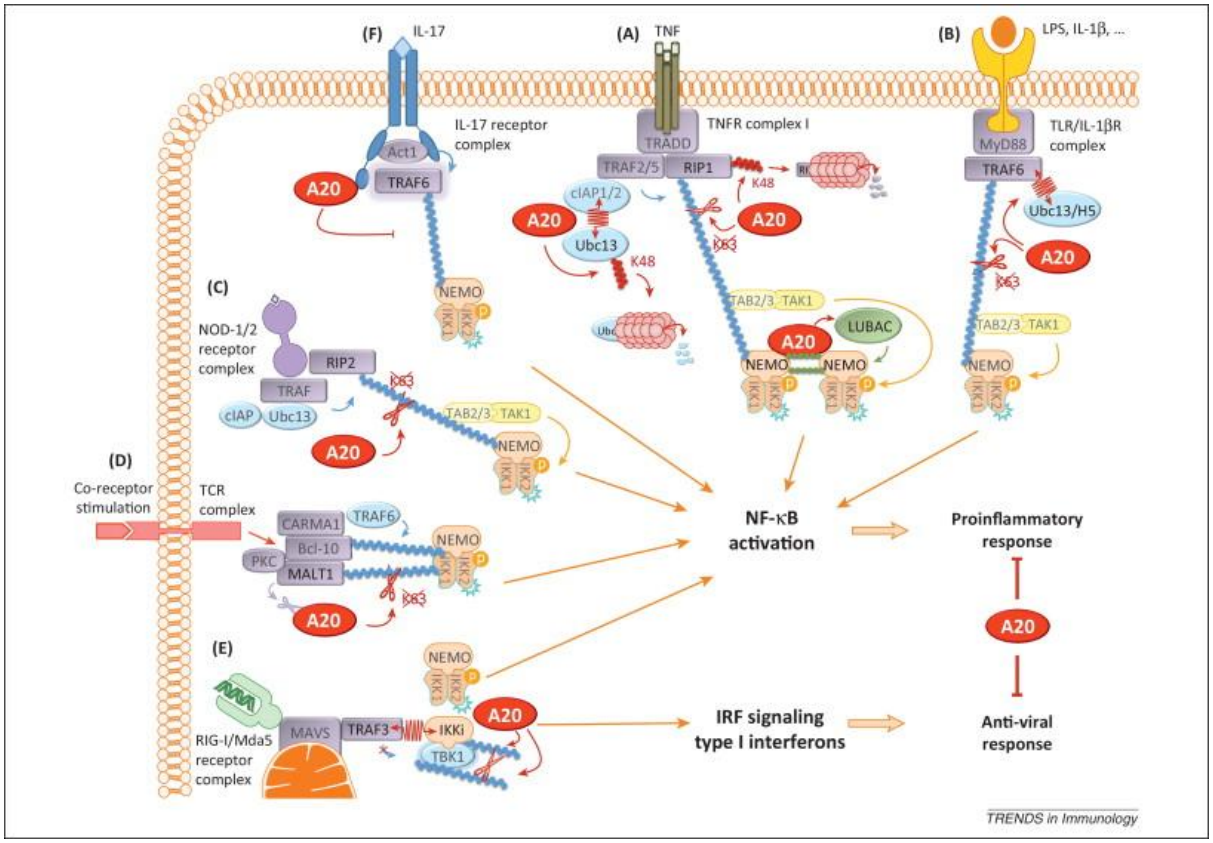


Figure 1.4 Regulatory activities of A20 in multiple signalling pathways

(A) During TNFR signalling, A20 removes K63 ubiquitin chains from RIP1 and subsequently polyubiquitinates K48, targeting this crucial activator of NEMO for degradation and therefore blocking NFκB activation. Furthermore, A20-mediated K48 ubiquitination of Ubc13 prevents cAIP1/2 ubiquitination of TRAF2/5. **(B)** In response to TLR activation by, for example LPS or IL1β, A20 inhibits the MyD88 signalling pathway of NFκB activation by removing TRAF6 K63 ubiquitin chains, both catalytically and through additional mechanisms that prevent physical interaction between TRAF6 and Ubc13/H5. Similarly for IL17 signalling **(F)**, A20 removes TRAF6 K63, preventing direct interaction with NEMO. Deubiquitination of RIP2 and MALT1 K63 by A20 **(C&D)** curbs signalling via NOD1/2 and TCR complexes respectively. Finally, IRF signalling and NFκB activation instigated by the RIG1/Mda5 receptor complex **(E)** are inhibited by interaction of A20 with and removal of K63 from TBK1.*Figure adapted from Trends in Immunology* (Catrysse et al. 2014)

It was the use of overexpression studies that first determined a role for A20 in immune signalling. Mice deficient in A20 suffered TNFα hypersensitivity and died prematurely because cells lacked the ability to prevent NFκB induced TNFα production and consequent apoptosis (Turer et al. 2008; E. G. Lee et al. 2000). Several large GWAS studies have identified TNFAIP3 as a major susceptibility locus for many chronic inflammatory pathologies including RA, Junior Idiopathic Arthritis, SLE, IBD, Type I Diabetes, psoriasis, coronary artery disease and systemic sclerosis (Mele et al. 2014; Tejasvi et al. 2012; Fan et al. 2011; Zammit & Grey 2014; Kawasaki et al. 2010; Trynka et al. 2009; Wang et al. 2013; Vereecke et al. 2014). Most of the SNPs identified in these studies are located within intragenic regions and therefore are likely linked with regulatory enhancer elements. For example the TT>A transition associated with SLE results in reduced NFκB binding to the TNFAIP3 promoter region and consequently decreased gene expression (Li et al. 2012). Two nonsynonymous mutations in exon 3 affect the DUB activity of A20 and therefore its ability to regulate NFκB signalling (Musone et al. 2011). Additionally, polymorphisms linked with psoriasis have also been implicated in patient responsiveness to anti-TNF therapy (Tejasvi et al. 2012). Conceivably because of its anti-apoptotic propensities, aberrant regulation of A20 is also implicated in glioma stem cell survival, oestrogen-resistant and aggressive breast carcinomas (Verstrepen et al. 2010). In addition, un-abated NFκB signalling owing to reduced A20 activity can also contribute to multiple B-cell lymphomas (Wang et al. 2014).

In contrast to most cell types, where basal concentrations of A20 are low and rapidly increase following inflammatory activation, leukocytes express high initial levels of A20. A20 is a primary response gene and harbours two NFκB binding motifs within its promoter region (De Valck et al. 1999; Opipari et al. 1990). Following the initial inflammatory stimulus, protein concentrations of A20 rapidly deplete to facilitate optimal NFκB signalling, before a

rapid resurgence in response to NFκB-mediated transcriptional up-regulation and elevated protein synthesis and stability. The primary fall in leukocyte A20 concentrations is a consequence of proteasomal degradation and MALT-1 driven cleavage (Düwel et al. 2009). Posttranslational modification of A20 is key to its functional potential, purportedly by affecting stability. For example, phosphorylation by IKK2 augments the ability of A20 to inhibit NFκB by incompletely understood mechanisms (Heyninck et al.; Wagner et al. 2008; Iha et al. 2008; Shembade et al. 2007). Similarly, A20 binding inhibitor of NFκB (ABIN) and TAX1 binding protein 1 (TAX1BP1) associate with A20 and not only increase its stability but also relocate A20 proximal to its substrate (Shembade et al. 2007). In addition, the stability of Tnfaip3 mRNA is subject to regulation via consensus binding sequences for miRNA-29a and miRNA-125 within its 3'UTR (Kim et al. 2012; Gantier et al. 2012; Wang et al. 2011). miRNA-29a protects the *Tnfaip3* message by interacting with mRNA binding protein HuR, whereas miRNA-125 promotes instability of Tnfaip3 (Balkhi et al. 2013).

1.9 Regulation of MAPK signalling; DUSP1

The spatiotemporal regulation of MAPKs is critical to the physiological outcome of their activation. Stringent control the magnitude, duration and spatial distribution of activated MAPKs serves for an efficient and precise response (Ebisuya et al. 2005; Owens & Keyse 2007)

The transient activation of MAPKs is delimited most efficiently, by phosphorylation. A number of phosphatases exist, each with different catalytic actions and substrate specificities.

MAPK phosphatases (MKPs) are a family of negative regulators that catalyse the removal of phosphate groups from MAPKs and terminate their signalling processes. MKPs can be separated into three subgroups depending on the MAPK residue(s) that they dephosphorylate; tyrosine phosphatases; serine/threonine phosphatases; and dual specificity phosphatases (DUSPs). Most specific to this system are Protein phosphatase 2-A (PP2A), a broad spectrum Ser-Thr phosphatase and the DUSPs, which are also known as MAPK phosphatases (MKPs). At least 13 DUSP family members show substrate specificity for MAPKs. Since all MAPKs are substrates for a number of MKPs, there is a level of functional redundancy within the cluster; however this provides selectivity through sensitive expression kinetics and sub-cellular compartmentalisation (Salojin & Oravecz 2007). Although generally widely expressed, there is some cell-lineage specificity of DUSP expression, as well as the subcellular localisation of their activity.

The archetypal MKP and perhaps most important to this study, because of its negative regulatory role in p38 MAPK signalling, is DUSP1/MKP1. DUSP1 is a nuclear protein, containing the fundamental dual specificity region as well as an N-terminal MAPK binding sequence.

With specificity for p38 MAPK > JNK ≫ ERK (Chu et al. 1996; Wu & Bennett 2005), DUSP1 is widely distributed within the immune system. Basal expression levels of DUSP1 are low but strongly and transiently up-regulated in response to pro-inflammatory stimuli, downstream of the MAPKs it inactivates (Chi et al. 2006). The temporal expression of DUSP1 is so that it correlates with the off phase of p38 MAPK (Clark & Dean 2012).

DUSP1 knockout (*Dusp1-/-*) mice are viable, have no observable developmental defects and appear normal under standard conditions. However, in response to bacterial - LPS challenge, *Dusp1-/-* mice show significantly increased serum cytokines and related factors when

compared to wild type. Susceptible to gram negative and positive sepsis (Frazier et al. 2009), LPS induced bone loss (Sartori et al. 2009; Valerio et al. 2015), cardiac dysfunction (T. Zhang et al. 2012) and lethal endotoxemia (Salojin et al. 2006), the DUSP1-/- phenotype results from a lack of negative feedback control and subsequent exaggerated MAPK activation (Salojin et al. 2006). A number of chronic inflammatory conditions are exacerbated by an absence of DUSP1-/- including experimental colitis (Matta et al. 2012), contact hypersensitivity, anaphylaxis (Maier et al. 2007), TNF-induced systemic inflammatory disease (Vandevyver et al. 2012) and experimental models of RA, in which the severity, penetrance and time to onset of disease are all increased (Vattakuzhi et al. 2012). These observations, along with the greatly increased rate of DUSP1-/- mortality due to endotoxin shock outline the significance of DUSP1 in negative feedback regulation of MAPK during the inflammatory response (Zhao et al. 2005; Chen et al. 2002; Wu & Bennett 2005; Nimah et al. 2005; Shepherd et al. 2004).

A classic negative feedback regulator, DUSP1 effectively limits its own expression profile. In a p38 MAPK dependent manner, DUSP1 is induced by pro-inflammatory agonists and therefore, rationally, negatively controlled by anti-inflammatory inhibition of the p38 MAPK pathway. However a wide variety of inflammatory mediators act upon DUSP1 and a number of anti-inflammatory factors powerfully enhance its expression. For example, LPS-driven transient expression of DUSP1 is enhanced and extended by immunosuppressive cytokine IL-10 (Hammer et al. 2005; Hammer et al. 2006) and correlates with more rapid MAPK down-regulation. Additionally, MKP1 deficiency has been shown to enhance IL-10 production, due to increased activity of p38 MAPK. On the other hand, pro-inflammatory IFNγ attenuates MKP1 expression and prolongs MAPK activity (Zhao et al. 2006).

1.10 Glucocorticoids

Glucocorticoids are endogenous steroid hormones produced by the adrenal cortex in response to a neuro-endocrine cascade involving the hypothalamic-pituitary-adrenal axis (Newton 2000; Buckingham 2006; Whitehouse 2011). They are fundamental to a vast number of physiological processes, including growth, metabolism, apoptosis and importantly, modulation of the inflammatory response (Buckingham 2006; Clark & Belvisi 2012; Taves et al. 2011). Inflammatory cytokines cause excitation of the hypothalamus, which responds by secreting corticotrophin releasing hormone (CRH). In turn, the anterior pituitary is prompted to generate and release adrenocorticotropic hormone (ACTH), which acts on the adrenal cortex, in turn releasing glucocorticoids like cortisol. In this way, GCs are antagonistic to pro-inflammatory signalling and act systemically as a negative feedback mechanism to prevent a potentially irrepressible immune response.

For decades, synthetic glucocorticoids have been prescribed for and remain the cornerstone of treatment in numerous chronic inflammatory conditions (Hillier 2007). They are fundamental in the treatment of asthma and rheumatoid arthritis and exert genuine disease modifying effects (Kirwan et al. 2007; Gorter et al. 2010; Hoes et al. 2007; Clark & Belvisi 2012). However, prolonged use of glucocorticoids can give rise to serious, often life-threatening side effects such as osteoporosis, diabetes or hypotension and often patients experience GC insensitivity (Kanis et al. 2007; Hofbauer & Rauner 2009; Weinstein et al. 2011). The therapeutic effectiveness of GCs has conventionally been associated with their ability to reduce inflammatory gene expression.

1.10.1 Glucocorticoid receptor and GRE

Glucocorticoid actions are mediated entirely by the glucocorticoid receptor (GR); a ligand activated nuclear receptor with the ability to bind directly to DNA. Inactive GR resides in the cytoplasm bound to a multiprotein complex containing numerous heat shock proteins, including Hsp90. On ligand binding, GR undergoes a conformational change and dissociates from the inhibitory multiplex, unmasking dimerization, nuclear localisation and DNA binding domains (Grad & Picard 2007). Once in the nucleus, the GC-bound receptor complex is able to modify target gene expression either directly or through co-activator/repressor interaction (Lefstin et al. 1994; Meijsing et al. 2009).

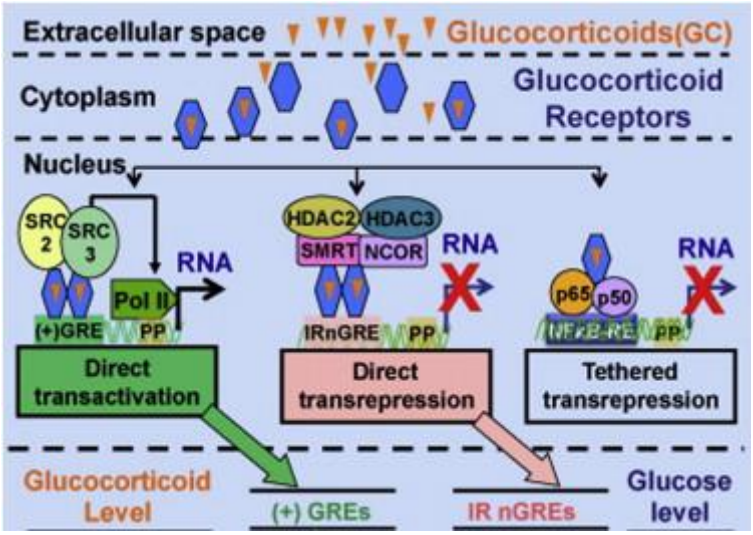


Figure 1.5 Glucocorticoid receptor mediated regulation of target-gene expression

Ligand-activated glucocorticoid receptor (GR) translocates to the nucleus and transcriptionally regulates target genes through discrete mechanisms. GR may interact with (+) GRE or nGRE DNA binding sequences and directly have either positive or negative effects on gene transcription respectively. Engagement of (+) GRE leads to recruitment of co-activators of transcription including SRC2/3 and RNAPII, whereas dimeric GR-complex activation of (IR) nGRE amasses HDAC2/3, SMRT and NCoR repressors of gene activation. Ligand-associated GR may also affect transcript production indirectly via tethering to previously bound transcription factors such as NFκB (p65/p50 heterodimer). Similarly, activated GR may directly bind to other sites that lack specific GREs but accept synergistic binding of GR and additional coactivators and repressors of transcription. *Figure adapted from Cell* (Surjit et al. 2011)

The glucocorticoid response element (GRE) is a conserved palindromic DNA sequence that directly interacts with ligand-bound GR at target genes (So et al. 2008). At 'simple' (+) GREs, GR homodimers bind directly to DNA and confer transcriptional transactivation through association with RNA polymerase II (RNAPII) and steroid receptor co-activator (SRC) 2 & 3 (Lonard & O'malley 2007; Gronemeyer & Bourguet 2009; Meijsing et al. 2009)). 'Simple' nGREs contain evolutionarily conserved palindromic GR-binding sequences different to those observed at (+) GRE sites. Through recruitment of transcriptional repressors including SMRT, NCoR and HDACs 2/3 dimeric GR may directly repress transcription through binding nGREs local to target genes (Surjit et al. 2011). The GR-nGRE complex has been linked to the anti-inflammatory actions of glucocorticoids (Newton 2000; Dostert & Heinzel 2004). However the immunomodulatory effects of GCs are diverse and very few genes are regulated in this way. Monomeric GR on the other hand, has the power to interact with cognate DNA motifs and sites relating to other factors that act synergistically to mediate transactivation (CBP/p300, p60) or transrepression (TIF2/GRIP1) 'composite' GREs (Surjit et al. 2011). Additionally, glucocorticoids have the ability to induce indirect transrepression through 'tethering' GREs, which do not contain specific GR DNA binding sequences but recruit GR indirectly through other sequence bound factors including NFκB and AP1 (Karin 1998; Kassel & Herrlich 2007).

1.10.2 Glucocorticoid-mediated transrepression

As aforementioned, glucocorticoids are powerful anti-inflammatory steroids that inhibit the production of countless pro-inflammatory cytokines, including TNFα, IL-1β, GM-CSF and IL-6, chemokines such as IL-8 and MIP1α, inducible enzymes iNOS and COX-2 and adhesion molecules ICAM-1 and E-selectin (Mukaida et al. 1994; Kleinert et al. 1996; van de Stolpe et

al. 1994; Brostjan et al. 1997). However, none of the above genes possess nGREs proximally located within their promoters, nor does the underwhelming selection of genes that do account for GC-mediated systemic down-regulation of pro-inflammatory mediators. Fittingly, GCs repress inflammatory gene expression via additional mechanisms at the level of transcription and also post-transcriptionally. Transrepression refers to a mechanism in which ligand-bound monomeric GR is "tethered" indirectly to DNA via protein-protein interaction with interferon response factor (IFN), NFκB or AP-1 (Schäcke et al. 2005; Newton & Holden 2007; De Bosscher 2010; Glass & Saijo 2010). In this context, GR blocks transcriptional activation by NFkB or AP-1 either by preventing co-activator recruitment or engaging co-repressors, or both (Auphan et al. 1995; R I Scheinman et al. 1995; Reily et al. 2006; Flammer & Rogatsky 2011).

Transcription activating protein AP-1 is involved in the induction of several growth factors and pro-inflammatory cytokines, to which GCs are repressors. Using co-precipitation experiments, a number of groups were able to detect low-affinity interactions between AP-1 and GR and subsequently EMSA experiments revealed a mutual inhibition of one another (Bogdarina et al. 2009). It was later shown that AP1- remained bound to its site within the promoter during GR repression and that the latter was likely to prevent the former from interacting with transcription initiation machinery or an essential co-activator complex (Biddie et al. 2011; Dobrovolna et al. 2012; Karmakar et al. 2013). Scores of pro-inflammatory moderators contain NFκB binding sites within their promoters, including TNFα, IL-1α, IL-6, COX2, IL8 and many more. Numerous anti-inflammatory genes are also known to contain NFκB binding sites, for example A20, TTP and DUSP1.

In order to act rapidly and in a direct manner, GR-mediated transrepression is downstream of DNA binding and is mediated by pre-existing factors (Clark & Belvisi 2012; Newton & Holden 2007; King et al. 2013). This was highlighted in a study by King et al. (2013) where treatment of A549 cells with cycloheximide was unable to diminish the repressive effects of DEX on IL6, ICAM1 and COX2 gene expression. Furthermore, the 50-100% reduction in gene expression did not alter NFκB activation or DNA binding (King et al. 2013). Thus transrepression is mediated by pre-existing factors, allowing a rapid and direct mode of action.

1.10.3 Separating transactivation and transrepression

Conventionally, transcriptional activation has been connected with the unpredictable side effects associated with glucocorticoid therapy and little else. To this end, intensive research is being focused on finding GR ligands with inferior transactivating properties that lead preferentially to GR-mediated transrepression. These selective glucocorticoid receptor modulators (SGRMs) are receiving a lot of attention; however there have been few positive results and this simple dualistic theory of GC action is not supported by substantial scientific evidence (Joanny et al. 2012). It is apparent that transrepression is not solely responsible for the therapeutic effects of GCs and transactivation plays a partial if not significant role (Clark 2007; Newton & Holden 2007). In fact, glucocorticoids up-regulate transcription of numerous anti-inflammatory molecules, including IL10, IL1Ra, annexin1, GC-inducible leucine zipper (GILZ) and DUSP1. Interestingly, a considerable number of glucocorticoid anti-inflammatory targets contain upstream NFκB and AP1 binding motifs and yet are not affected by transrepression.

The inducibility of a GR-target gene is increased by the number and proximity of GREs within its promoter region. GR binding to GRE causes confined changes in chromatin structure that facilitate transcription factor binding and the formation of a stable transcription initiation complex. Simultaneous local unwinding of DNA and recruitment of RNA polymerase II (RNAPII) instigates transcription of the target gene. There are a number of co-activators that associate with GR/GRE, including CREB binding protein (CBP), Glutamate receptor-interacting protein 1 (GRIP-1), transcriptional mediators/intermediary factor 2 (TIF2), p300/CBP and steroid receptor coactivator-1 (SRC-1). When combined, these co-activators enhance transcription by increasing histone acetyltransferase (HAT) activity, which increases DNA accessibility and stabilises the RNAP II initiation complex.

1.10.4 Post-transcriptional effects of glucocorticoids

It is widely documented that GCs have powerful posttranscriptional effects on pro-inflammatory genes (Stellato 2004; Clark 2007; Newton & Holden 2007). In order to resolve inflammation and down-regulate pro-inflammatory cytokines, GCs must be able to suppress an on-going response, i.e. using a mechanism that acts on pre-existing mRNA. Many pro-inflammatory genes, including IL-1, IL-6, TNFα and COX-2 are inhibited by GCs at the posttranscriptional level, which is unaccountable for by transrepression (Lee et al. 1988; Amano et al. 1993; Swantek et al. 1997; Gille et al. 2001; Lasa et al. 2001). Transrepression can only be effective if GCs are present during the period of active transcription, thus there must exist, suppressive mechanisms at the posttranscriptional level (Clark 2007). Newton and colleagues showed that DEX could down-regulate cyclooxygenase-2 (COX-2) even when administered long after the inducing stimulus, whereas the transcription inhibitor actinomycin D was ineffective at this time (Newton, Seybold et al. 1998). Dexamethasone

was shown to destabilise COX-2 mRNA, acting via adenosine/uridine rich elements (AREs) in its 3' un-translated region (UTR) (Newton et al. 1998; Lasa et al. 2001). The majority of pro-inflammatory mRNAs targeted by GCs in this manner contain AREs in their 3' UTR (Saklatvala et al. 2003) and associate with trans-acting ARE-binding proteins, which regulate mRNA stability (Garneau, Wilusz et al. 2007).

1.11 Post-transcriptional regulation of gene expression

Transcription is responsible for just half of the changes in gene expression and protein biosynthesis during the inflammatory response (Hao & Baltimore 2009; Raghavan & Bohjanen 2004; Cheadle et al. 2005; Clark & Dean 2012). Posttranscriptional control of gene expression is especially valuable in the precise regulation of highly active proteins like cytokines, growth factors and cell-cycle regulators due to context-dependent, rapid and reversible modes of action (Gingerich et al. 2004). Indeed the aberrant control of mRNA processing has been implicated in a number of inflammatory disease states and likely contributes to a number of disease processes (López de Silanes et al. 2007; Hitti & Khabar 2012; Schott & Stoecklin). Posttranscriptional methods of limiting cytokine expression are more rapid than de novo gene synthesis and therefore the regulation of RNA stability and rate of decay is an especially efficient system of delimiting the inflammatory response.

1.11.1 Pathways of mRNA decay

RNA is an adaptable intermediate of gene transcription and protein production that, in response to cellular signals, may be targeted in a way that facilitates rapid modification of gene expression profiles. Therefore RNA exists in a dynamic state of stability and responds

to specific signals that may initiate or protect the transcript either by removal or stabilisation of protective terminal structures. Further to this, the pioneer round of translation is a surveillance mechanism that initiates on nuclear export of mature transcripts to the cytoplasm (Brooks & Blackshear 2013). Should any RNA message contain aberrant splicing, interruptive sequences or stop codons, either non-sense (Silva & Romão 2009; Neu-Yilik & Kulozik 2008), non-stop (Frischmeyer et al. 2002) or no-go mediated (Harigaya et al. 2010)mechanisms ensure rapid degradation and removal.

All eukaryotic mRNAs are co-transcriptionally fitted with, a 3' stretch of ~25-200 adenine nucleotides known as the Poly (A) tail and a 5' 7-methylguanosine cap. These protective structures associate with Poly (A) binding protein (PABP) and eIF4E in the cytoplasm and form a complex which safeguards the transcript from exonuclease-mediated decay. The canonical mechanism of mRNA degradation is known as deadenylation dependent mRNA decay and involves removal of the Poly (A) tail followed by hydrolysis of the 5'-cap. However, a number of other, 'atypical' mechanisms of decay also exist. Shortening of the poly (A) is reversible and those mRNAs specifically marked may be readenylated as a unique form of regulation and returned to the polysome for further processing. In mammals, the poly (A) nuclease (PAN) 2 – PAN3 complex catalyses initial shortening of the Pol (A) tail and further removal is carried out by the carbon catabolite repressor protein (CCR) 4 – CCR4 associated factor (CAF) 1 complex. As an initiator of decay, PAN2-PAN3 is dependent on the presence of PABP, whereas CCR4-CAF1 is inhibited by it (Tucker et al. 2002). Poly (A)-specific ribonuclease (PARN) is a cap-dependent deadenylase.

Following deadenylation, to about 30-60 nucleotides in mammals, the message may be degraded by two non-mutually exclusive pathways; decapping and 5' to 3' digestion; or 3' to

5’ decay. Several enzymes stimulate decapping activity (Dcp1, DCP2 and Hedls) and are associated with various accessory factors (Lsm proteins, pb1) (Steiger et al. 2003; Fenger-Grøn et al. 2005; Yu et al. 2005). Once deadenylated and decapped, the mRNA body can be degraded 5’ to 3’ by exoribonuclease Xm1. Without the need for initial decapping, the 3’ to 5’ pathway employs a 10-12 subunit complex known as the exosome to digest the transcript using 3’ to 5’ exoribonuclease activity. Decapping of the remaining oligomer is catalysed by the scavenger enzyme Dcps (Liu et al. 2002).

P-bodies are transitory cytoplasmic structures formed in the presence of mRNA. Enriched for the components necessary (Anderson & Kedersha 2006), it is generally accepted that p-bodies are sites of messenger transcript decay (Sheth & Parker 2006; Parker & Sheth 2007). It is also probable that these granular foci provide compartmentalisation of those mRNAs tagged for degradation (Garneau et al. 2007).

Other transcript degrading processes include the aptly named deadenylation-independent decapping whereby transcripts bypass poly (A) removal and undergo direct decapping. Although this process does not require the catalytic activity of a deadenylase, study has shown that some subunits of the CCR4-CAF1 complex are required (Muhlrad & Parker 2005). Endoribonucleolytic decay uses endonuclease activity to cleave the transcript and produce two fragments of mRNA which are susceptible to exonuclease attack; a highly efficient and specific process (Hollien & Weissman 2006; Yang & Schoenberg 2004)

1.11.2 Regulation of messenger transcript stability; mRNA binding proteins and AU-rich sequence characteristics

Trans-acting factors known as mRNA binding proteins have an affinity for regulatory sequences, predominantly located in the 3' UTR of target transcripts. These factors may be constitutive or induced and, via association with cis-acting motifs, regulate transcript stability either by facilitating or inhibiting mRNA decay. By far the most well understood cis-acting 3'UTR sequence that modulates mRNA stability is the adenosine-uridine rich element or ARE. Located in the 3' UTR of up to 15% of all transcripts (Bakheet 2001; Bakheet et al. 2006), ARE motifs exist on a spectrum from indefinite U-/AU-rich sequences to distinct repeats of the signature AUUUA pentameter and everywhere in-between (Beisang & Bohjanen; Khabar 2010). The UUAUUUAUU nonomer is minimally required for ARE functionality.

Transcript binding proteins may be stabilising or destabilising, although these functions are not mutually exclusive and depending on varying conditions, some mRNA binding proteins may be capable of either process. Perhaps one of the most recognised AREBPs, HuR typically stabilises the RNA message and is thought to enhance associations between transcript and terminal protective structures through interaction with PABP and eIF4 (Nagakoa et al. 2006). In addition, HuR has been shown to compete with AUF1, KSRP and TTP for transcript binding, displacing their destabilising effects and relocating the transcript to the polysome for translation initiation (Bhattacharyya et al. 2006).

Destabilising AREBPs on the other hand rapidly eliminate transcripts by facilitating associations with deadenylases, decapping enzymes and the exosome. High turnover

transcripts like cytokines, growth factors and cell cycle regulators that have a potentially deleterious effect if not properly regulated are quite often degraded by mRNA destabilising proteins. Examples of destabilising proteins include AUF1, KSRP and BRF1/2 however in the context of inflammation, tristetraprolin (TTP) is perhaps the most studied AREBP.

1.12 Tristetraprolin (TTP)

1.12.1 TTP & the *ZFP36* locus

The earliest descriptions of TTP recognise Nup475 from serum-stimulated fibroblasts (DuBois et al. 1990); G0S24, a human lymphocyte G0/G1 switch gene (14); TPA-induced sequence (TIS) -11 (Varnum et al. 1989; Ma & Herschman 1991); and Zinc finger protein 36 (*Zfp36*) as the same factor. Originally, TTP was cloned from the mouse cDNA of 3T3-L1 murine fibroblasts simulated with insulin (Lai et al. 1990). The TIS11 family of nucleocytoplasmic shuttling proteins consists of three members, TTP (TISS11/ ZFP36), BRF1 (TIS11b/ ZFP36L1) and BRF2 (TIS11d/ ZFP36L2) (Sanduja et al. 2012), all of which are characterised by a highly conserved cysteine-cysteine-cysteine-histidine (CCCH) Zinc finger domain (Phillips et al. 2002)(Varnum, Ma et al. 1991). TTP was initially identified as an immediate-response gene whose expression was rapidly and transiently induced in response to numerous cellular stimuli including (12-O-tetradecanoylphorbol-13 acetate) TPA (hence TPA-induced sequence), insulin, serum and other mitogenic agents (Sanduja et al. 2012; DuBois et al. 1990; Lai et al. 1990; Varnum et al. 1989; Gomperts et al. 1990). Characteristic of an immediate-early gene, induction of *Zfp36* is independent of protein synthesis and in fact cycloheximide treatment enhances the level of transcript expression.

The *Zfp36* locus is small, occupying approximately 12kb on human chromosome 19q13.1. Murine *Zfp36* lies within a linkage group at the proximal end of chromosome 7 that is conserved within a segment of human chromosome 19 (Taylor et al. 1991; Saunders & Seldin 1990). The *ZFP36* gene-region its self is correspondingly small, comprising just two

exons and one intron. The entire sequence of *ZFP36* is just ~2.5kb, which comprises the 5'UTR ~49/ 28bp, exon1 ~35/ 27bp, intron1 ~381/ 681bp, exon2 ~962/ 932bp and the 3'UTR 711/763bp (human/mouse data from UCSC). The upstream and downstream genes that flank the *ZFP36* sequence are mediator complex subunit 29 (*MED29*) and plekstrin homology domain containing family-G member 2 (*PLEKHG2*), both of which are transcribed in the same direction as TTP. *MED29* is a multiprotein coactivator of transcription and forms interactions with factors including RNAPII and other general initiators of gene synthesis (Sato et al. 2003). *PLEKHG2* is involved in the signalling pathways leading to actin-cytoskeletal reorganisation and is activated by G-protein Gβγ subunits (Sato et al. 2003). The newly identified micro RNA 4530 (MIR4530) is also located within the human *ZFP36* locus and lies downstream of the 3'UTR, in a 3' to 5' direction (Jima et al. 2010). Micro RNAs are known for their role in posttranscriptional regulation of gene expression through modification of target transcript stability and translation efficiency (Pritchard et al. 2012).

1.12.2 TTP gene regulation

The full serum inducibility of TTP is dependent on a number of elements identified within the 5' proximal promoter as well as the intron of TTP. These sequences were found to contain essential binding motifs for factors including SP1, EGR1, AP2 and NFκB (Lai et al. 1990). In addition, a TGFβ-dependent smad responsive binding region (known as 'TRR') has been identified approximately 500bp upstream of the Zfp36 promoter (Rounbehler et al. 2012; Ogawa et al. 2003; Sohn et al. 2010).

Evidence suggests that a varied selection of endogenous factors and natural compounds stimulate the transcription of *Zfp36*. Green tea, cinnamon, β-adrenergic receptor agonists,

nicotinic acetylcholine receptor ligands, interferons and glucocorticoids have all been shown to enhance TTP expression.

Investigation of glucocorticoid-mediated up regulation of TTP has provided mixed results, however there are three putative glucocorticoid receptor binding elements surrounding the *Zfp36* sequence. This intensity of regulatory input requires complex sequence organisation and therefore the comparatively small *Zfp36* locus comprises a highly functional domain. Interestingly, the nucleotide base pairs surrounding the *ZFP36* region are highly conserved through the mammalian lineage and this might indicate the presence of 'distal' functional regulatory elements. As aforementioned, there have been a handful of studies that focused on the 5' proximal promoter region of *Zfp36* and also the single intron, however little work has been carried out to identify regulatory elements in the more distal upstream and downstream elements regardless of the evident evolutionary conservation.

Following processing, the mature *Zfp36* transcript is approximately 1.7kb in length and subject to posttranscriptional regulation by mRNA-binding factors. Indeed, TTP has been shown to directly interact with its own mRNA in an ARE-dependent manner (Brooks et al. 2004; Tchen et al. 2004).

1.12.3 TTP protein function & regulation

As the nomenclature might suggest, the amino acid sequence of TTP is rich in proline residues, which are arranged in three motifs containing four proline residue repeats i.e. PPPP in Figure 1.12.1 (Lai et al. 1990) Characteristically, TTP expression is low in quiescent cells and rapidly upregulated in response to mitogenic stimulation (Sanduja et al. 2009; Gomperts et al. 1990). When active, TTP is found located in the cytoplasm, where it can target mature mRNA transcripts. The integrity of the two conserved CCCH zinc finger

domains is essential for the ARE-binding mechanism of TTP function as well as nuclear-to-cytoplasmic shuttling (Taylor et al. 1996; DuBois et al. 1990; Varnum et al. 1991; Phillips et al. 2002; Carballo et al. 1998). However the residues involved in TTP function and shuttling are distinct and the latter requires a nuclear export sequence (NES) (Phillips et al. 2002). TTP has an affinity for the minimal AREBP nonamer 'UUAUUUAUU' however may also bind to UAUUUAU (Brooks & Blackshear 2013). Whereas HuR and most HuR-related AREBPs will recognise a U-rich motif, TTP on the other hand requires the presence of adenylate residues within the core element. The competition and exchange between HuR and TTP for target transcript AREs is well documented and part of the functional mechanism for each AREBP. In fact, TTP and HuR have been shown to compete for the same ARE sequences within the TNFα transcript (Tiedge 2012).

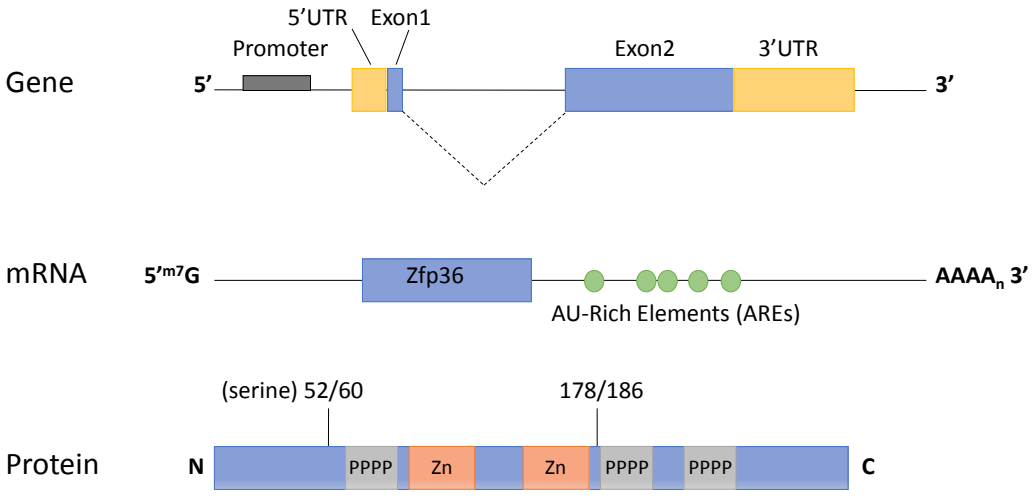


Figure 1.6 TTP gene, mRNA and protein structure configuration

Cartoon schematic detailing the arrangement of the *Zfp36* gene, mRNA and TTP protein. MAPK p38 /MK2 target sites for phosphorylation of mouse/human TTP protein are illustrated. *Figure adapted from Sanduja Blanco et al 2012*

TTP is expressed constitutively, at very low levels, in an active form. Upon binding to its ARE-containing mRNA target, TTP relies on C-terminal associations with Not-1 that facilitate recruitment of the CCR4-CAF1 complex (Sandler et al. 2011) in order to initiate deadenylation (Carballo et al. 2001; Sawaoka et al. 2003; Clement et al. 2011; Marchese et al. 2010). TTP also interacts with decapping enzymes, PARN deadenylase, Xrn1 and specifically recruits several components of the exosome (Fabian et al. 2013; Marchese et al. 2010; Sandler et al. 2011; Clement et al. 2011). In response to TGFβ, TTP is also critical to the induction of p-bodies, which comprise the structures necessary for mRNA degradation (Blanco et al. 2014).

TTP-interaction with the CAF1/CCR4 complex however, is critical and in fact depletion of either component results in ARE-transcript stabilisation (Brooks & Blackshear 2013; Clement et al. 2011; Marchese et al. 2010). This mechanism is strongly influenced by TTP phosphorylation and inactivation downstream of p38 MAPK. MAPK p38 and its substrate MK2 are both capable of TTP phosphorylation at two specific sites, they are serine 52 and 178 in mouse and serine 60 and 186 in human protein (Cao et al. 2003; Carballo et al. 2001; Mahtani et al. 2001; Chrestensen et al. 2004). This specific posttranscriptional modification event prevents recruitment of CAF-1; the main catalytic unit of deadenylation dependent mRNA decay (Marchese et al. 2010) and therefore renders TTP in reserve and promotes stress-dependent mRNA stabilisation. Cessation of p38 MAPK signalling and consequential dephosphorylation of TTP by PP2A (Sun et al. 2007) unleashes its degradation potential upon target mRNAs and effectively restrains inflammation.

MAPK p38 is necessary but not sufficient for inhibition of TTP function and it must be noted that posttranslational modification of TTP is extensive. It is therefore likely that additional signalling pathways also contribute deadenylase recruitment and TTP-mediated mRNA

destabilisation. For example a mutant form of TTP, wherein serines 52 and 178 are mutated to alanine residues, is un-phosphorylatable by MK2 and acts as a potent mRNA destabilising factor regardless of its reduced affinity for deadenylase (Clement et al. 2011). Fittingly, evidence suggests that both the ERK and p38 kinase pathways work in concert to inhibit TTP mediated mRNA decay (Deleault et al. 2008; Essafi-Benkhadir et al. 2010; Brook et al. 2006). As a target for ERK2, p38 MAPK, JNK, MK2, glycogen synthase kinase and protein kinases A, B and C (Cao et al. 2003; Stoecklin et al. 2004; Cao & Lin 2009), it is perhaps unsurprising that TTP is found phosphorylated to a remarkable extent in vivo and in vitro. At least 14 sites of phosphorylation exist in mice (Cao et al. 2006; Cao et al. 2014) and most correspond with a human counterpart.

With regards to serine 52 & 178, the TTP protein persists in a constant equilibrium between the unphosphoryltaed (active) and phosphorylated (inactive) form. Basally, TTP is mostly unphosphorylated and expressed at low levels in the nucleus. In response to pro-inflammatory stimuli and MAPK signalling, TTP undergoes rapid NFκB-dependent transcriptional up-regulation and P38-dependent mRNA and protein stabilisation. Moreover, studies in *Mk2-/-* mice and transient transfection of mutant forms of TTP into a macrophage cell line suggest that the inactivating ser-52 and ser-178 (Ser-60 and Ser-186 in humans) phosphorylation is critical to protecting TTP from proteasomal degradation (Rigby et al. 2005). A mutant form of murine TTP, in which the MK2 specific phosphorylation sites Ser-52 and Ser-178 are replaced by un-phosphorylatable alanine residues, has recently been investigated. Mice homozygous for the mutant form of TTP are healthy and fertile and show a strongly attenuated response to endotoxin. In addition, the mutation had little to no effect on acquired immunity in *Zfp36aa/aa* mice inoculated with *S. typhimurium*. Surprisingly,

heterozygous mutants show equally as potent TTP-mediated mRNA destabilisation and inhibition of pro-inflammatory cytokine expression (unpublished data - manuscript in review), highlighting the dominant nature of this mutation. Although potently effective however, the "TTP-AA" mutant is highly unstable and constitutively degraded by the proteasome, as is the active, Ser52/178 unphosphorylated protein. Shifting the phosphorylation equilibrium of murine TTP Ser-52/-178 towards a constitutively active state successfully augmented the mRNA destabilising and anti-inflammatory capacities of TTP without inducing any observed adverse effects. Critically, manipulation of the TTP Ser-60/186 in humans could be useful for the treatment of immune pathology.

1.12.4 The role of TTP in inflammation

The role of TTP in inflammation was originally established by studies in knockout mice (Taylor, Carballo et al. 1996), which initially appeared normal but soon developed a severe complex inflammatory syndrome including cachexia, dermatitis, destructive arthritis, myeloid hyperplasia and autoimmunity (Taylor et al. 1995). Treatment of young TTP-deficient mice with antibodies to TNFα prevented development of essentially all aspects of the phenotype and so the syndrome observed was put down to the overexpression of TNFα, owing to an absence of TTP (Taylor, Carballo et al. 1996). Mice subject to myeloid-specific disruption of *Zfp36* on the other hand are hypersensitive to low-dose LPS challenge but do not develop a spontaneous inflammatory syndrome and are otherwise phenotypically normal and fertile (Qiu et al. 2012). These data might suggest that the temporal and qualitative control of TNFα mRNA decay by TTP in myeloid cells is required for the re-instalment rather than maintenance of immune homeostasis (Kratochvill et al. 2011).

Subsequent confirmation about the role of TNFα in TTP-deficiency syndrome pathogenesis was made when *TTP-/-* mice lacking functional TNFRs appeared normal (Carballo, Cao et al. 2001). In concurrence, transgenic mice overexpressing TNFα were shown to develop an analogous phenotype to the TTP knockout mice (Keffer, Probert et al. 1991). Carballo et al (1997) demonstrated that macrophages derived from *TTP-/-* mice exhibited increased stabilisation of TNFα mRNA when compared to wild type animals. It was suggested that TTP had a crucial negative feedback role in restraining TNFα synthesis and stability during the immune response (Carballo, Lai et al. 1998). Kontoyiannis et al. (1999) then showed that deletion of the TTP binding region from the TNFα 3' UTR caused inflammatory arthritis. Further study revealed that TTP was able to bind directly to the TNFα 3’ UTR ARE and induce destabilisation of the mRNA transcript (Carballo, Lai et al. 1998; Lai, Carballo et al. 1999; Smoak and Cidlowski 2006).

Critically, as a form of positive feedback, TNFα enhances pro-inflammatory mRNA stability through MAPKs and activation and inhibition of stabilising and degrading mRNA binding proteins respectively. Responsible for the myeloid hyperplasia phenotype of TTP knockout mice, GM-CSF is another target of TTP that has been instrumental to uncovering the mechanisms of ARE-containing mRNA decay (Carballo et al. 2000). By introducing the GM-CSF AT-rich sequence to the 3’UTR of rabbit β-globin mRNA, Shaw and Kamen demonstrated the ability of AREs to confer instability of otherwise stable transcripts (Shaw & Kamen 1986). Furthermore, TTP deficiency was responsible for the greatly enhanced half-life of GM-CSF message in TTP knockout versus wild type bone marrow derived stromal cells (BMSCs) treated with TNFα and actinomycin D. In the same study, northern blot analysis confirmed that TTP deficiency prevents the initial deadenylation of transcript, rather than facilitating stabilisation (Carballo et al. 2000).

Knockdown studies conducted by Tiedje et al. (2012) showed that an absence of TTP resulted in increased association of TNFα mRNA with the polysome. On the other hand, removal of HuR reduced TNFα transcript localisation within translational processing fractions. The dynamic of this competition is strongly shifted in favour of HuR in the presence of active p38 MAPK/ MK2, even with TTP concentrations four-times that of HuR. In fact, whilst MK2 greatly reduces the affinity of TTP for a synthetic ARE, HuR remains unaffected (Tiedje et al. 2012). Interestingly the TTP/HuR double knockdown phenotype paralleled that of the HuR single knockdown; whereby TNFα transcripts were excluded from and reduced in polysomal and monosomal cytoplasmic fractions. These results suggest that not only is HuR required for the translational processing of TNFα and putatively a number of other pro-inflammatory mRNAs but also is a constitutive activator of TNFα protein expression (Tiedje et al. 2012).

A number of therapeutically invaluable inflammatory agents have been shown to induce TTP expression. Glucocorticoids have been used for management of chronic inflammatory syndromes for decades. However in truth, their exact mechanism of action is incompletely understood (Clark & Belvisi 2012; Newton 2013). Glucocorticoids have, however been shown to increase the expression of TTP at the level of mRNA and protein in A549 cells, pulmonary bronchial epithelial cells and MEFs (Smoak & Cidlowski 2006; Ishmael et al. 2008). Glucocorticoid-mediated up-regulation of TTP has a strong anti-inflammatory outcome. Ishmael et al. (2008) observed a striking reduction in the antagonistic effects of glucocorticoid on the TNFα-response in *Zfp36 -/-* MEFs. The post-transcriptional effects of glucocorticoids are well documented (Stellato 2004). Indeed, glucocorticoids have been shown to increase rates of decay for numerous inflammatory cytokines and chemokines. In

addition, the absence of TTP in MEFs reduced GC-mediated gene expression by up to 85% and therefore TTP may also influence anti-inflammatory transcriptional regulation by glucocorticoids (Ishmael et al. 2008). Glucocorticoids also strongly induce the expression of DUSP1, downstream of which lies the un-phosphorylation and activation of TTP protein (Kassel et al. 2001; Lasa et al. 2002; Issa et al. 2007; King, Holden, et al. 2009a; Holden et al. 2010; Abraham & Clark 2006). The anti-inflammatory effects of glucocorticoids are augmented by supplementation of Long-acting β_2-Adrenoceptor agonists. Numerous agonists of cAMP signalling pathways have powerful anti-inflammatory consequences and are often used for the treatment of asthma and conditions of chronic airway inflammation. Several of these cAMP-activating compounds have been shown to positively regulate TTP expression as a critical mechanism of their function. In addition to these two striking examples, a number of other anti-inflammatory acting agents have the capacity to up regulate the expression or activation of TTP or both. The question is, how this achieved and how depended are is the anti-inflammatory effects dependent on TTP expression.

NFκB is a key factor of inflammatory signalling, positively regulated by TNFα and hence inhibited by TTP. Additional to its AREBP properties, TTP also regulates inflammatory signal transduction in the nucleus. Nuclear translocation of the NFκB catalytic subunit p65 is enhanced in TTP knockout MEFs and knockdown HUVECs (Schichl et al. 2009). The same study showed that number of non-ARE genes were upregulated in TTP deficient cells. The TTP-p65 association was echoed by Liang's study, which also identified TTP-mediated suppression of NFκB at the corepressor level via an interaction with HDAC-1, -3 and -7 *in vivo*. TTP-dependent inhibition of an NFκB reporter was partly abrogated when cells were treated with HDAC inhibitors or HDAC siRNAs. Consistent with this finding, chromatin

immunoprecipitation after LPS treatment of TTP deficient cells showed a decreased accumulation of HDAC1 as well as an increase in CBP at the MCP-1 promoter (Liang et al. 2009). Together these findings coincide with previous suggestions that TTP may have inhibitory effects on pro-inflammatory gene expression at the transcriptional level by negatively regulating NFκB mediated gene expression (Carballo et al. 1998).

1.12.5 The role of TTP in cancer

Central to early events in tumourogenesis is the overexpression of factors that promote cell growth, inflammation and angiogenesis; often accompanied by an under-expression of genes orchestrating cellular senescence. As a putative tumour suppressor, TTP is evidently down regulated in a number of cancerous tissues and cell lines and in a number of cases, expression level restoration has anti-oncogenic effects. Hyperphosphorylation of TTP by MAPK p38/MK2 in malignant glioma may contribute to the aberrant stabilising effects of HuR and the observed overexpression of VEGF and IL8. Reestablishment of TTP expression and its ARE-mediated effects on VEGF and IL-8 mRNA inhibited disease progression in gliomal cells. Aberrant HuR-dependent stabilisation of VEGF has also been detected in colorectal cancer, alongside increased COX-2 production. Substrates of TTP, VEGF and COX-2 cause tumour vascularisation and cell proliferation; restoration of TTP abrogates these effects.

Hypermethylation of a single CpG in the Zfp36 promoter smad-binding sequence causes TTP down-regulation in hepatocellular carcinoma (HCC). CMyC is a TTP target that is upregulated in HCC. By reactivating TTP biosynthesis, DNA methylation inhibitor 5azadeoxycitidine inhibits tumourogenesis caused by cMyC up-regulation.

Cervical cancer is attributed to the oncogenic actions of HPV oncogenes E6/7, which inhibit the tumour suppressive functions of master cell regulator p53 and pRB. In non-carcinogenic cervical tissue TTP is abundant at the level of mRNA and protein; however cancerous cervical biopsies and cell lines are deficient in TTP. The E6AP message is AU-rich; up-regulation of TTP destabilises E6 RNA, prevents p53 ubiquitination and inhibits hTERT and leads to cellular senescence in HPV18-HeLa cells. TTP has also been shown to sensitise HeLa to proapoptotic stimuli such as TNF and staurosporine.

The reduced synthesis of TTP observed in breast cancers is often an indicator of high tumour grade and a poor prognostic outcome. An inverse relationship between MiR29a and TTP may be key to breast cancer pathogenesis since increased MiR29a supressed TTP expression malignant cell lines. The anti-oncogenic effects of TTP re-expression in these cells subsequently reduced metastasis and proliferation. A number of ARE-mRNAs are stabilised in breast cancer identifying them as novel targets of TTP. Also deficient in lung, ovary, prostate and thyroid tissues, TTP has clear tumour suppressive effects and a lot more research is becoming focused on these novel properties of TTP.

1.13 Project aims

This project is concerned with the regulation of negative feedback genes by pro- and anti-inflammatory pathways. We are interested in how these two opposing systems converge and cooperatively resolve inflammation. We will investigate the involvement of NFκB in the induction of three anti-inflammatory feedback node genes (FNGs). Further to this we would like to characterise the expression of these genes in response to a number of anti-inflammatory agents. I want to identify how pro-inflammatory stimuli and anti-inflammatory agents cooperate to control the expression and activity of DUSP1, A20 and TTP.

1.13.1 Endogenous mRNA and protein expression of FNGs in response to pro-inflammatory stimuli and anti-inflammatory agonists

DUSP1, TNFAIP3 and ZFP36 are rapidly and transiently up-regulated in response to pro-inflammatory agonists such as LPS and IL1. However the extent of NFκB involvement in these mechanisms however is not well characterised. Furthermore, for the purpose of this study, where we aim to elucidate some of the cooperative mechanisms between pro-inflammatory up-regulation of anti-inflammatory genes it will be necessary to have a clear understanding of NFκB mediated induction of these genes.

Initially, I will investigate the LPS-induced expression profile of all three FNGs in primary human and murine derived macrophages and RAW cells. Following this, I will utilise MLN4924, a powerful and specific inhibitor of NFκB-mediated nuclear processes, to explore the involvement of NFκB in the induction of cytokine and FNG expression. Additionally, I will

focus attention on the mechanisms pro-inflammatory mediated up-regulation of *Zfp36*, with the aim of identifying important and novel regulatory regions and enhancer sequences.

In order to begin to understand how pro-inflammatory and anti-inflammatory mechanisms converge, I will characterise cytokine and feedback node gene expression in several cell lines and primary macrophages of human and murine origin in response to a pro-inflammatory stimulus alone (IL-1/LPS), the anti-inflammatory agent alone and a combination of both. The effects of pro-inflammatory and/or anti-inflammatory stimulation at the mRNA level will be quantified by real-time quantitative PCR (QPCR) whereas the effects at the level of protein will be quantified by western blot or enzyme linked immunosorbent assay (ELISA). With the intention of elucidating the importance of DUSP1 as a central regulator of anti-inflammatory mechanisms, I will carry out parallel experiments in *Dusp1-/-* macrophages.

1.13.2 Elucidating the transcriptional regulation of Zfp36; novel regulatory elements and transcription factor binding sites

The rest of the study will focus on transcriptional regulation of *Zfp36*.

I will identify and characterise putative *Zfp36* regulatory elements capable of mediating transcriptional responses to antiinflammatory stimuli and LPS or IL1 in macrophage epithelial cell lines respectively. Initially, I will do this by transcriptional reporter assay. The regions involved in gene regulation often show evolutionary conservation, DNAseI hypersensitivity, or histone (H3K4) methylation and much of this information is accessible via public genome browsers like UCSC. I will select a number of putative regulatory sequences from the TTP locus and individually clone them upstream of a minimal promoter

and luciferase reporter gene. Responses of these constructs to IL-1/LPS or anti-inflammatory stimuli will be tested following transient transfection into HeLa or RAW264.7 cells.

This method is a valuable tool for identifying putative enhancer sequences. However describes the function of only a few transcription factor binding sites in isolation and does not reflect the true interaction of multiple responsive regions that are likely to surround a highly inducible gene such as *Zfp36*. In reality, it is likely that enhancer sequences act in concert as opposed to individually and in addition Lai et al 1998 showed that the intron is essential for full serum inducibility of *Zfp36*. Therefore, as a tool to investigate transcriptional responsiveness of the entire Zfp36 gene locus, I will synthesise a *Zfp36* mimic-gene, based on the *Zfp36* locus but containing a luciferase cDNA in place of the two endogenous *Zfp36* exons. I will measure the response of this mimic gene to LPS and, or an anti-inflammatory stimulus. If strong transcriptional responses are shown and given the time and materials, I will carry out further deletion and mutation analysis of this construct so as to identify important transcription factor binding sites.

Chromatin immunoprecipitation (ChIP) will be performed to identify sites of DNA-transcription factor (RNAPII, NFkB, and GR) association with the *Zfp36* sequence in primary macrophages and RAW cells treated with LPS & anti-inflammatory stimuli. I will use systematic approach to identifying important sites of protein-DNA interaction by designing primers that span the entire *Zfp36* locus.

2.0 MATERIALS AND METHODS

2.1 Materials

2.1.1 Cytokines and inflammatory Stimulants

The cytokines and other signalling proteins used in this study are listed in Table 2.1.

Table 2.1 Recombinant Cytokines

Cytokine/ Signalling molecule	**Application**	**Concentration**	**Source**
M-CSF	Cell culture	100ng/ml	Genetics Inst. MA, USA
GM-CSF	Cell culture	100ng/ml	PeproTech EC UK
IL-10	Cell stimulation	10ng/ml	Schering-Plough, USA
TNFα	Cell stimulation/ ELISA		PeproTech EC UK
IL-1	Cell stimulation	1ng/ml	PeproTech EC UK
IFNγ	Cell stimulation	10ng/ml	R&D Systems UK
TGFβ	Cell stimulation	10ng/ml	PeproTech EC LTD. UK
PGE2	Cell stimulation	10ng/ml	Torcis Bioscience UK
LPS (*E. Coli*)	Cell stimulation	10ng/ml	Sigma, Dorset UK

2.1.2 Antibodies

2.1.2.1 Antibodies used for western blotting

The antibodies used for western blotting are detailed in Table 2.2.

Table 2.2 Antibodies used for western blot analyses

Specificity	Species	Dilution	Source
α-Tubulin H	Mouse	1:5000	Sigma, Dorset, UK
β-actin M, H	Mouse	1:5000	
Sac-21, M	Rabbit	1:1000	
H-120, M, H	Mouse	1:1000	
RelA, M, H	Rabbit	1:1000	
TNFAIP3 M, H	Mouse	1:1000	Cell Signalling, USA

2.1.2.2 Antibodies used for chromatin immunoprecipitation

The antibodies used for ChIP and their dilutions are shown in table 2.3.

Table 2.3 Antibodies used for ChIP

Specificity	Concentration	Source	Species
RNAPII (sc-9001-X)	2mg/ml	Santa Cruz biotechnology, USA	Rabbit
RNAPII CTD phospho-ser2 (ab5095)	1mg/ml	Abcam, Cambridge, UK	Rabbit
ChIP isotype control (ab46540-1)	1mg/ml	Abcam, Cambridge, UK	Rabbit
RelA (p65) (ab7970-1)	2mg/ml	Abcam, Cambridge, UK	Rabbit
GR (H-300) (sc-8992)	2mg/ml	Santa Cruz biotechnology, USA	Rabbit
PU.1			

2.1.3 Inhibitors

Complete mini protease inhibitor tablets were purchased from Roche Applied Science as well as PhosSTOP phosphotase inhibitors both were used according to manufacturers' instructions.

2.1.4 PCR Primers

2.1.4.1 Taqman primers

All assay-on-demand premixed TaqMan probe master mixes were purchased form Applies Biosystems, Warrinton UK.

Table 2.4 TaqMan probes used for q-PCR analysis of mRNA and cDNA

Gene	Catalogue number	Species
Gapdh/ GAPDH	Mm99999915_g1/ Hs99999905_m1	Mouse/Human
Tnfα/ TNFα	Mm99999068_m1/ Hs00174128_m1	Mouse/Human
Zfp36/ ZFP36	Mm00457144_m1/ Hs00185658_m1	Mouse/Human
Dusp1/ DUSP1	Mm00457274_g1/ Hs00610256_g1	Mouse/Human
Tnfaip3/ TNFAIP3	Mm00437121_m1/ Hs00234713_m1	Mouse/Human
Med29	Mm00786410_s1/ Hs00378316_m1	Mouse/Human
PlekHg2	Mm00507178_m1/ Hs00293943_m1	Mouse/Human

2.1.4.2 Custom designed oligonucleptide primers

The oligonucleptide primers used in this study are listed in the following tables. They we all purchased from MWG, Germany.

Table 2.5 Oligonucleotide primers used for SYBER green q-PCR of cDNA.

Name	Sequence	Species
ActB For/ActB Rev	CGGCATCGTCACCAACTG AACATGATCTGGGTCATCTTCTC	Human
B2M For/B2M Rev	GTATGCCTGCCGTGTGAAC AAAGCAAGCAAGCAGAATTTGG	Human
hGAPDH_F1/hGAPDH_R1	GTCAGCCGCATCTTCTTTTGC AATCCGTTGACTCCGACCTTCC	Human
hIFNβ1_F1/hIFNβ1_R1	TGGCACAACAGGTAGTAGGC AGTGGAGAAGCACAACAGGAG	Human
hIL-10_F1/hIL-10_R1	GCCTAACATGCTTCGAGATC TGATGTCTGGGTCTTGGTTC	Human
hMPK1_F1/hMKP1_R1	ACAACCACAAGGCAGACATCA CAGTGGACAAACACCCTTCCT	Human
hRELA_F1/hRELA_R1	GCTATCAGTCAGCGCATCCA TCCCCACGCTGCTCTTCTAT	Human
hSTAT3_F1/hSTAT3_R1	GTCGCAGCCGAGGGAACAA CCATTGGGCCATCCTGCTAAAA	Human
TBP For/TBP Rev	TGCCCGAAACGCCGAATATAATC GTCTGGACTGTTCTTCACTCTTGG	Human
hTNFα_F1/hTNFα_R1	CCCCAGGGACCTCTCTCTAAT TCTCTCAGCTCCACGCCATT	Human
hTNFAIP3_F1/hTNFAIP3_R1	CCCTTGGAAGCACCATGTTTG GGTTGGGATGCTGACACTCC	Human
hZFP36_F1/hZFP36_R1	TCCACAACCCTAGCGAAGAC GAGAAGGCAGAGGGTGACAG	Human
mGapdh_f1/mGapdh_r1	CATCATCTCCGCCCCTTCTG CATCACGCCACAGCTTTCC	Mouse
mIfnβ1_f1/mIfnβ1_r1	AGTTTCTGGTAAGTCTTC TGC AGA GTT ACA CTG CC	Mouse
mMkp1_f1/mMkp1_r1	ACAACCACAAGGCAGACATCA CAATGAACAAACACTCTCCCT	Mouse
mRelA_f1/mRelA_r1	GCTTCTGGGCCTTATGTGG GTC TGG GCA GAG GTC AGC C	Mouse
mTnfα_f1/mTnfα_r1	TTCTATGGCCCAGACCCTCA ACAAGGTACAACCCATCGGC	Mouse
mTnfaip3_f1/mTnfaip3_r1	CAGGCCGCCGAAAGACG TCACAGCTTTCCGCATATTGCT	Mouse
mZfp36_f1/mZfp36_r1	TGGATCTCTCTGCCATCTACG ATTCGGTTCCTCCGTGGTC	Mouse

Table 2.6 Oligonucleotide primers used for amplification of Zfp36 sequences

Name	Species	Sequence	Location
mZfp36_ECR2 F1	mouse		
mZfp36_ECR2 R1			
mZfp36_ECR3 F1	mouse	GCGCTCGAGGGATCTAG	
mZfp36_ECR3 R1		GCGCTCGAGCACACGTG	
hZfp36_ECR3 F1	human	GCGCTCGAGGATCCAGG	
hZFP36_ECR3 R1		GCGCTCGAGCACACGTG	
mZfp36_ECR4 F1	mouse	GCGCTCGAGTGGGGGTT	
mZfp36_ECR4 R1		GCGCTCGAGGGATTTGT	
hZFP36_ECR4 F1	human	GCGCTCGAGTGGGGGTA	
hZFP36_ECR4 R1		GCGCTCGAGGGATTTGG	

Table 2.7 Oligonucleotide primers used for Chromatin Immunoprecipitation.

Name	Sequence	Location (UCSC format)	Species
Zfp36_1F1/ Zfp36_1R1	ACAGGAACCACCTGTTTTGC CATCAAAGCCCAGATCACCT	>chr7:29171741+29171915 175bp	Mouse
Zfp36_2F1/ Zfp36_2R1	TAACCCAGGCATTTTGGTTC TGAGCCCTTTAGACCTTCCTC	>chr7:29171369+29171527 159bp	Mouse
Zfp36_3F1/ Zfp36_3R1	CCGGCTATGGAGTGAGAGAC TCCCAGAGACTGTGGAGACC	>chr7:29170657+29170884 228bp	Mouse
Zfp36_4F1/ Zfp36_4R1	GCTGCGGTTTGTTAGTAGCC TGTGGTTCTTTCCCCTACCC	>chr7:29170177+29170347 171bp	Mouse
Zfp36_5F1/ Zfp36_5R1	TGAATGAAGGCTTGGCTTTT CTTCTGGCTCCCAAGTCCTA	>chr7:29169657+29169828 172bp	Mouse
Zfp36_6F1/ Zfp36_6R1	AGCTACCTCAACAGCCCAGA TTTCACAAGCCCACACCATA	>chr7:29169059+29169290 232bp	Mouse
Zfp36_7F1/ Zfp36_7R1	AGTGTGGGGTCCCTAGTGTG GGTCTTCTGGACACACAGCA	>chr7:29168570+29168788 219bp	Mouse
Zfp36_8F1/ Zfp36_8R1	GACTTGCACACACTGGCATC ATGACCAAGTGCCACAAACA	>chr7:29168133+29168338 206bp	Mouse
Zfp36_9F1/ Zfp36_9R1	CCGTGGGATCTGTGCTAAGT ACTGTCAGGAACAGGCAACC	>chr7:29167817+29167984 168bp	Mouse
Zfp36_10F1/Zfp36_10R1	GGAATCCCCTCTGGAATGAC GTCCCTGCTTGTTTTTCTGG	>chr7:29167065+29167262 198bp	Mouse
Zfp36_11F1/Zfp36_11R1	TGCCTGGTATTGGGCTACTC CCACTGGACAAGGAAAGGAA	>chr7:29166596+29166801 206bp	Mouse
Zfp36_12F1/Zfp36_12R1	GAACTTGCGATCCTCCAGTC TCCCTACCTCGATACGCAAC	>chr7:29165851+29166047 197bp	Mouse
Zfp36_13F1/Zfp36_13R1	CATGCAAAATGTGCCTGAAC CCTCAGTCTCTGCCCTTGTC	>chr7:29164406+29164607 202bp	Mouse
Zfp36_14F1/Zfp36_14R1	CAAACTCCAGGGAGAACCTG GGAGTCCTAGAGGCCCAAAG	>chr7:29163644+29163802 159bp	Mouse
Zfp36_15F1/Zfp36_15R1	GTGGCAGAGTTCCGTTTTGT CTGAGCTGTCACCCTCACCT	>chr7:29163117+29163307 191bp	Mouse
Zfp36_16F1/Zfp36_16R1	CTCTATCAAGTCCGCCCAAG GTAAGGGGTGCTCTGGTCCT	>chr7:29161385+29161611 227bp	Mouse
Zfp36_17F1/Zfp36_17R1	TGGCTTTGGCTATTTGCTTT CCCTCTGCAACTCTGGTCTC	>chr7:29162039+29162204 166bp	Mouse
Zfp36_18F1/Zfp36_18R1	TTGTGGAAGAGACCCGATTC CTTGGGCGGACTTGATAGAG	>chr7:29161230+29161404 175bp	Mouse
Zfp36_19F1/Zfp36_19R1	TCGCGTCCTTTTTCCTTGTA GATATGAACCCTCCCGGTTT	>chr7:29160789+29161033 245bp	Mouse
Zfp36_20F1/Zfp36_20R1	AGCCCCCGAGAGACTTTTTA TGGGTGAGGGAAACAAACTC	>chr7:29160213+29160373 161bp	Mouse
Zfp36_21F1/Zfp36_21R1	GCTAGACCAGGGTTTTGCAG GCCAGACTGAGTTCCAGGAC	>chr7:29159754+29159972 219bp	Mouse
Zfp36_22F1/Zfp36_22R1	GTGGAGCAGGTGAGAAGAGG TCATCGAAAGCCCACCTTAC	>chr7:29159169+29159332 164bp	Mouse
Zfp36_23F1/Zfp36_23R1	CCCAGACTGGCCTAAACTCC GGCCAAACACCCATACACAT	>chr7:29158739+29158895 157bp	Mouse
Zfp36_25F1/Zfp36_25R1	TCAATCAAATGTTTTGGCTTGT CCAGTACTAGGGAGGCAGAGG	>chr7:29158186+29158335 150bp	Mouse
hZFP36_1F1/hZFP36_1R1	GCCTTCTCACAGGAAACTGG GTCATGTGTACCCGGTTGTG	>chr19:39893046+39893195 149bp	Human
hZFP36_2F1/hZFP36_2R1	CGTCATTCCAGAGGGGATT TCGATCAGATCCAGGAGACC	>chr19:39893554+39893712 158bp	Human
hZFP36_3F1/hZFP36_3R1	CAGCAGATGGGAGAGAGGAG CGCAGCTTAAAGGATTGAGG	>chr19:39894260+39894439 179bp	Human
hZFP36_4F1/hZFP36_4R1	GCAGCCTGCAAGAATGAACT ATCAAAGGGTCGCCCTAAGT	>chr19:39894807+39894964 157bp	Human
hZFP36_5F1/hZFP36_5R1	CAGCTTGGTGATTTGGAGGT CTGAGACTTCAGCCCCAGAG	>chr19:39897732+39897942 210bp	Human
hZFP36_6F1/hZFP36_6R1	ATATCCGGGGAGGACAAGAG AGGAACCCAGAGTTGGAGGT	>chr19:39898081+39898238 157bp	Human
hZFP36_7F1/hZFP36_7R1	CGCGGTCTAGAGACAGGAAT TCTGGTGACCTCACCTGGTC	>chr19:39900067+39900227 160bp	Human
hZFP36_8F1/hZFP36_8R1	CGCGTCTTCAGACCATACAA GGTGAGGGTAGGGAAAGGAG	>chr19:39900769+39900967 198bp	Human

2.1.5 Solutions

2.1.5.1 General buffers

Phosphate buffered saline (PBS)	4.3 mM Na_2HPO_4
	1.4 mM KH_2CO_3
	1.4 mM KCl
	137 mM NaCl
	pH 7.2
Tris buffered saline (TBS)	20 mM
	137 mM NaCl
	pH 8.0
Triton lysis buffer	1% (v/v) Triton X100
	10 mM Tris HCl pH 7.6
	150 mM NaCl
	1 mM EDTA
	0.1 mM Na3VO4
	5 mM NaF
	1 x protease inhibitor cocktail

Western blotting reagents

Lysis buffer (protein)	
SDS-PAGE running buffer	25 mM Tris-base
	192 mM Glycine
	0.1% (w/v) SDS
5 X protein gel sample buffer	250 mM Tris HCL pH 8
(laemmli buffer)	10% (w/v) SDS
	50% (v/v) Glycerol
	12.5% (v/v) β-mercaptoethanol
Blocking buffer	TBS/Tween-20 (0.1%)
	5% (w/v) Marvel skimmed milk powder

2.1.5.3 ELISA reagents

2.1.5.4 ChIP solutions

Lysis buffer 1 (LB 1)	50 mM Hepes-KOH pH 7.5
- Cell membrane lysis	140 mM NaCl
	1 mM EDTA
	50% Glycerol
	10% NP40*
	10% Triton X-100*
	ddH_20
Lysis buffer 2 (LB2)	10 mM Tris HCL pH 8
- Detergent removal	200 mM NaCl
	1 mM EDTA
	0.5 mM EGTA
	ddH_2O
Lysis buffer 3 (LB3)	10 mM Tris HCL pH8

-Nucleic disruption	100 mM NaCl
	1 mM EDTA
	0.5 mM EGTA
	0.1% Na-deoxycholate*
	0.5% N-Lauroylsarcosine*
	ddH_2O
RIPA buffer	50 mM Hepes-KOH pH 7.6
	500 mM LiCl
	1 mM EDTA
	1% NP40*
	10% N-Deoxycholate*
	ddH_2O

*add after autoclave

2.1.6 Molecular Biology Reagents

2.1.6.1 Molecular biology solutions

Lennox L broth base (LB)	20g LB, Invitrogen Paisley UK 1L dH2O (Autoclave before use)
Lennox L agar	32g LB agar, Invitrogen Paisley UK 1L dH_2O (Autoclave and add antibiotics once cool before pouring plates)
Ampicillin	100mg/ml, Sigma Poole UK
Kanamycin	50mg/ml
DNA Hyperladders	5µl loaded per lane Bioline London UK
Ethidium Bromide	Final concentration 0.1µg/ml

2.1.6.2 Bacterial strains

One Shot® TOP10 Chemically Competent *E. coli*

Genotype: DH5α; F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1

2.1.6.3 Cloning and reporter vectors

The vectors used in this study, for the cloning of Zfp36 enhancer sequences and luciferase reporter construction are listed in table 2.10

Table 2.8 Vectors

Vector	Application	Antibiotic resistance	Details
pCR®-Blunt (Invitrogen)	Cloning	Kanamycin	Blunt end ligation
pGL3-P (Promega)	Luciferase reporter	Ampicillin	Luc+ SV40 minP
pGL4.1 (Promega)	Luciferase reporter	Ampicillin	Luc2 no promoter
pGL4.28 (Promega)	Luciferase reporter	Ampicillin	Luc2CP minP Hygro

2.1.7 Cell Culture Reagents

2.1.7.1 Cell lines

The cell lines used in this study are described briefly in Table 2.10

Table 2.9 Cell lines

Cell line	Species	Details	Application
RAW264.7	Mouse	leukaemic macrophage-like	Endogenous mRNA/ protein expression; reporter assays; ChIP.
HeLa	Human	Henrietta Lacks epithelial carcinoma	Endogenous mRNA/ protein expression; reporter assays.
A549	Human	Lung adenocarcinoma	Endogenous mRNA/ protein expression.
293/TLR4-MD2-CD14	Human	Fibroblast	*Zfp36* mini gene reporter assay.

2.1.7.2 Media and supplements

Media and supplements were sourced from Sigma (Dorset, UK) Including: Dulbecco's modified Eagle's medium (DMEM) (with L-glutamine and L-glucose); Roswell park memorial institute (RPMI) 1640 culture medium; heat inactivated foetal calf serum (HIFCS); penicillin (100U/ml)/ streptomycin (100ug/ml) mixture; trypsin-EDTA; and cell dissociation solution. Optimem-1 with glutamax was obtained from Invitrogen (Paisley, UK).

Cell culture was performed using Corning/ Falcon plastic and disposable scrapers were purchased from Greiner BioOne Stonehouse UK.

2.1.8 Western blot reagents

SDS-PAGE and western blot procedures were carried out using the Bio-Rad Trans-Blot® Turbo™ Transfer System and therefore Mini-PROTEAN® Precast Gels and Trans-Blot® Turbo™ PVDF Transfer Packs were also purchased from Bio-Rad.

A full-range molecular weight rainbow marker from Amersham Biosciences (UK) and 10 x SDS-PAGE running buffer, acquired from ** were used for electrophoresis.

2.2 Methods

2.2.1 Tissue Culture

All cell cultures were maintained at 37˚C; 5% CO_2; and 95% humidity.

2.2.1.1 Maintenance of cell lines

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2mM L-glutamine and 4.5g/L L-glucose and supplemented with 10% heat inactivated Foetal calf serum (FCS). Blasticidin and ** were also added to the medium for culture of HEK-293 cells. Being adhesive cell lines, RAW264.7 cells were detached by scraping whereas HeLa and A549 cell lines were lifted by trypsin-EDTA incubation for 10 min at 37°C. Cells were passaged at 80% confluency and used for experiments until passage 15-20.

Cryopreservation of cell lines was in a solution of 10% dimethylsuphoxide (DMSO) and HIFCS. Freezing serum/cell aliquots were frozen initially to -70˚C before being transferred to liquid nitrogen for storage at -198˚C. For cell thawing, a 37°C water-bath was used and the DMSO removed by washing with the appropriate culture media before seeding the cells at high density in medium sized flasks for overnight recovery.

2.2.1.2 Isolation of bone marrow derived macrophages BMDMs

Generated from mammalian bone marrow, BMDM are commonly used in immunology research. Following extraction, day 1 undifferentiated bone monocytes are cultured in lineage specific growth factor for 3 to 5 days.

Murine primary macrophages were isolated from the bone marrow of the femur and tibia. Severed mouse legs were sterilised with ethanol and all of the skin and tissue removed by scalpel. The bone must be cleaned carefully without breaking or scratching to avoid contamination of the marrow. The meniscuses were removed and the ends of the bones cut, to expose the marrow. The cut bones were then placed in 150µl tubes pierced at the bottom, within 1.5ml microcentrifuge tubes and centrifuged at 5000g for 4 minutes. The bone marrow collected was then resuspended in, per mouse, 50 ml of RPMI; 10% HIFCS; 1% Pen/Strep and 100ng/ml of M-CSF and seeded onto 5 X 10cm tissue culture dishes. Inoculation of the culture media with M-CSF directs an M2-macrophage differentiation. After 5 days the macrophages were mature and adherent and ready to seed for experimentation. For removal, cells were lifted by dissociation solution, scraped, washed and reseeded at an appropriate density in RPMI containing 5%HIFCS and 1%Pen/Strep.

2.2.1.3 Isolation and separation of peripheral blood monocytes (PBMCs)

Human monocyte derived macrophages represent a human model for studying immunological signalling pathways. Furthermore, medium supplementation with either GM-CSF or M-CSF allows isolation of M1 or M2 macrophage populations, respectively.

PBMCs were isolated from single donor plateletpheresis residues. The heparinised residues were diluted with an equal volume of HBSS media and layered over an equal volume of Ficoll-Hypaque lymphoprep in sterile 50ml tubes and centrifuged at 3500 g for 20 minutes,

with no brake. After centrifugation the PBMC-containing interface layer was isolated, washed 3 X in HBSS and resuspended in 20 ml 10% HIFCS/RPMI media. 20 X 10^6 cells/ml were layered on an equal volume of Percoll solution before centrifugation at 400 g from 30 minutes minimum acceleration and no brake. The monocyte-fraction or interphase was collected, washed by centrifugation at 3000 g for 5 minutes, resuspended in RPMI and plated at a density of 20 x 10^6 per 10cm tissue culture dish. Cells were left to adhere for 1 hour contaminating cells remain in suspension and were removed by washing. RPMI media was replaced with 5%HIFCS, 1%Pen/Strap and 50ng/ml of GM-CSF or 100ng/ml M-CSF to direct M1- or M2- type macrophage differentiation respectively. Cells were left to differentiate for 3 to 5 days. Once mature, cells were then washed, gently lifted with cell dissociation solution, scraped and resuspended in appropriate media for experimentation.

2.2.2 Molecular biology methods

2.2.2.1 Preparation of total RNA from cells

Total RNA was extracted from primary cells and cell lines alike using the Qiagen RNeasy extraction kit. Briefly, $1x10^6$ cells were placed on ice and washed with PBS, lysed in the provided buffer containing 10µg/ml β-mercaptoethanol and further homogenised using shredder columns, also provided by Qiagen UK.

Homogenised lysates were then applied to a second column to which the total RNA bound and impurities washed away. Total RNA was on-column DNase treated before elution in RNase free dH2O and stored at -80˚C. The quantity and quality of RNA was measured using a nanodrop.

2.2.2.2 Reverse transcription of total RNA to cDNA

Reverse transcription of total RNA was performed at 25˚C for 5 minutes followed by 42˚C for 30 minutes and 5 minutes at 85˚C. The iScript™ cDNA Synthesis Kit was purchased from Bio-Rad UK. The 5µl total reaction mixture was as follows:

RNA template (100fg - 1µg)	2.5µl
iScript reverse transcriptase	0.25µl
5X iScript reaction mix	1µl
Nuclease-free water	1.25µl

2.2.2.3 Real-time PCR

Quantitative PCR (qPCR) is a method of quantifying gene expression at the mRNA level by measuring the incorporation of a TaqMan fluorogenic probe or SYBR green-fluorescent dye. As the fluorescence intercalates with dsDNA during amplification a signal, directly proportional to the amount of DNA, is produced and detected using the qPCR instrument.

The threshold cycle (Ct) is the term used for the number of PCR cycles necessary for the fluorescence associated with the reaction to be greater than baseline. All readings are normalised to a housekeeping gene, like GAPDH that is constitutively expressed, unrelated to the gene(s) of interest and not altered by any conditions of the experiment. The method for calculating relative abundances are described in figure 2.1

TaqMan probes were used alongside LightCycler® 480 Probes Master whilst customised oligonucleotides were combined with Takara SYBR®Premix Ex Taq™ in the following reactions:

Table 2.10 Real-time PCR reaction components

Reaction component	**LightCycler® 480 Probes Master**	**Takara SYBR®Premix Ex Taq™**
cDNA	2.5µl	2.4 µl
Master mix	(5 X) 2µl	(2 X) 3µl
Probe	0.01µl	-
Forward primer	-	0.1µl
Reverse primer	-	0.1µl
H_2O	5.49µl	0.4µl
Total	**10µl**	**6µl**

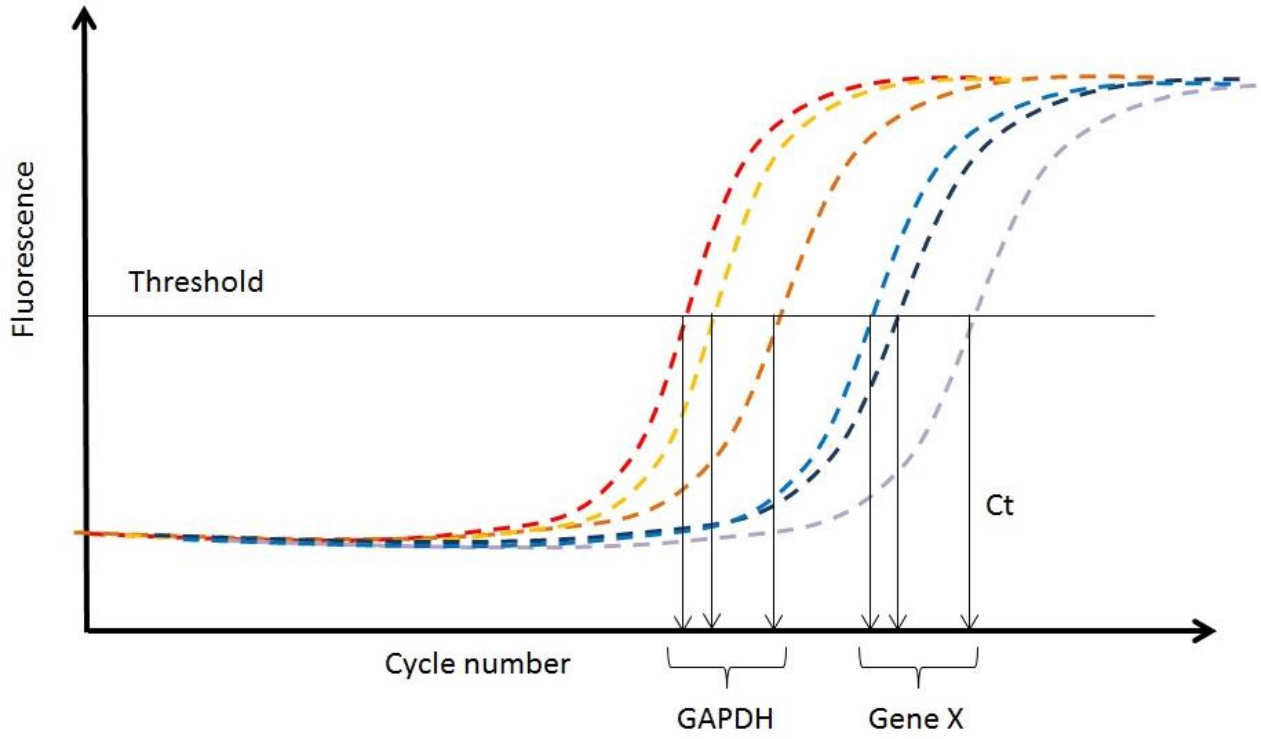


Figure 2.1 The C_t comparative method for Real-Time PCR

Above is a schematic of the typical Real-Time PCR profile. The calculation for measuring the relative amount of DNA is referred to as the comparative C_t, or $\Delta\Delta C_t$ method. The ΔC_t is the difference between the average threshold cycles for the housekeeping gene and the gene of interest. Whereas, $\Delta\Delta C_t$ provides an arbitrary constant to which other values from the test-group are normalised.

$$\Delta\Delta C_t = \Delta C_{t\ sample} - \Delta C_{t\ reference}$$

The $\Delta C_{t\ sample}$ refers to, for example an unstimulated, control (F in the schematic) value normalised to the housekeeping gene whereas $\Delta C_{t\ reference}$ corresponds to the value of a treated sample (E) being measured:

$$F - C = \Delta C_t F$$

$$E - B = \Delta C_t E$$

In order to ascertain the fold difference between sample mRNA contents the $2^{-\Delta\Delta C_t}$ value is used. The target can thus be normalised to the endogenous reference and to a relative calibrator included in the experiment, usually an un-stimulated control, for which the $2^{-\Delta\Delta C_t}$ value equals 1:

$$\Delta C_t D - \Delta C_t D = \Delta\Delta C_t D \qquad 2^{-\Delta\Delta CtD} = 1$$

$$\Delta C_t E - \Delta C_t D = \Delta\Delta C_t E \qquad 2^{-\Delta\Delta CtE} = x$$

$$\Delta C_t F - \Delta C_t D = \Delta\Delta C_t F \qquad 2^{-\Delta\Delta CtF} = y$$

2.2.2.4 Real-time primary transcript PCR

Transcriptional effects on genes can be measured by comparing the populations of primary and mature transcript at a given time. By designing oligonucleotide primers across the intron-exon boundary of a gene such as Z*fp36* or *Tnfα*, the level of primary transcript i.e. unspliced and unprocessed mRNA, can be measured.

Bone marrow derived murine macrophages were plated at a density of 1x 10^6 cells/ml. RNA was extracted from the cells, purified and DNase treated as described in section 2.2.2.1. Complementary DNA was synthesised from RNA as in section 2.2.2.2, including a control reaction for each sample that included all of the same components except the reverse transcriptase. Primary transcript directed oligonucleotides are designed such that they will also amplify trace amounts of contaminating genomic DNA. Thus by excluding the reverse transcriptase from the cDNA synthesis reaction, one can control for any interfering genomic DNA contamination.

To make a valid comparison, real-time PCR of the respective mature mRNA transcript was measured simultaneously. As is standard, PCR of a housekeeping gene was also simultaneously carried out and all samples were measured in triplicate.

2.2.2.5 Polymerase chain reaction

Targeted amplification of DNA by PCR was performed using Thermus aquaticus (Taq) DNA polymerase and made-to order oligonucleotides described in Table 2.6, unless otherwise stated. Generally, a total reaction volume of 50µl was combined of the following:

DNA template	0.5µg
10 X PCR buffer	5µl
$MgSO_4$ (20 mM)	1.5µl
dNTPs (1mM)	1µl
Forward primer (100 pmol/µl)	1µl
Reverse primer (100 pmol/µl)	1µl
Taq DNA polymerase	0.5µl
dH_2O	x µl (make up to volume)

Typically, the thermocycler lid temperature was set to 95˚C to prevent sample-lid condensation. Initial denaturation at 95˚C for 5 minutes proceeded 30 cycles of: denaturation at 95˚C for 30 seconds; 1 minute annealing at 55˚C; and 1 minute at 72˚C for elongation. The final 10 minute elongation step was performed at 72˚C before an infinite hold at 4˚C.

PCR products were resolved by agarose gel electrophoresis. Using 1 x Tris-borate EDTA (TBE) and agarose from *Ambion gels were made up to the appropriate percentage for the

fragment sizes to be analysed Table 2.11. Products used for cloning were mixed with 10% (v/v) SYBR Green in addition to 10% (v/v) DNA loading buffer before loading. After electrophoresis (approximately 120V for 45 minutes), a dark reader transilluminator was used to visualise the DNA fragments.

Table 2.11 Recommended % agarose for optimum resolution of linear DNA fragments

Fragment size	% (w/v) Agarose/ TBE
1000 – 30,000 bp	0.5 %
800 – 12,000 bp	0.7 %
500 – 10,000 bp	1.0 %
400 – 7,000 bp	1.2 %
200 – 3,000 bp	1.5 %
50 – 2,000 bp	2.0 %

2.2.2.6 DNA fragment purification from a preparative agarose gel

Fragments of DNA separated by electrophoresis were retrieved by extraction from the preparative gel, using Qiagen gel extraction kit, according to manufacturer's instructions. The fragment of interest was cut from the gel using a scalpel, weighed and three volumes of buffer QG added before incubation at 55°C to dissolve the gel completely. The resulting yellow solution was then applied to a QIAquick spin column and spun for 30 seconds at 10,000 g, this step was repeated for any remaining gel-mix and the flow-through discarded each time. An additional 500µl of buffer QG was added and the column spun, again for 30 seconds at 10,000 g. The columns were then washed with 750µl of buffer PE and centrifuged. To remove any residual ethanol, columns were placed in new collection tubes

and spun for 2 minutes at 18000 g. DNA was recovered by addition of 30µl of elution buffer and centrifugation for 2 minutes at 10,000 g. DNA was stored at 4°C until use. An analytical gel was used to verify the product size.

2.2.2.7 Restriction endonuclease digestion of DNA

The restriction enzymes and corresponding buffers used in this study were obtained from New England Biolabs, Hitchin UK or Promega Madison USA. Enzymes were, generally, used in a 20µl reaction volume with the following components:

DNA	2µg
NEB 10 X buffer	2µl
BSA	2mg
Enzyme	1µl
H_2O	x µl (make up to volume)

The reaction was incubated at 37°C for a standard time of 3 hours, unless otherwise specified by the manufacturer's instructions. Restriction fragments were resolved for cloning or analysis by preparative or analytical agarose gel electrophoresis respectively.

2.2.2.8 De-phosphorylation of DNA ends

Once linearised by the appropriate restriction endonuclease(s), dephosphorylation of DNA termini is necessary to prevent vector recircularisation in the absence of insert. Shrimp alkaline phosphatase (SAP) was purchased from promega, Madison USA, and the reaction set up as follows:

DNA	2µg
SAP	2µl
10 X SAP buffer	1µl
H_2O	x µl (make up to 20µl volume)

The reaction was incubated at 37°C for up to 30 min and heat inactivated at 65°C for a further 15 min. DNA was generally purified after SAP treatment using the Qiagen Gel and PCR clean up system.

2.2.2.9 Polishing of cohesive DNA termini

Pyrococcus furiosis (Pfu) polymerase calalyses the incorporation of free nucleotides onto ssDNA in a 3' to 5' direction (polymerase activity) and exhibits a 3' to 5' exonuclease activity. DNA 5' overhangs are therefore filled in while 3' overhangs are polished (care must be taken when cloning fragments in frame); the outcome of both is blunt ended DNA fragments. The enzyme and buffers were purchased from Promega (Madison USA) while the dNTP's were from Invitrogen (USA). The reaction below was incubated at 72°C for 30 min and placed on ice:

DNA	20µl
10 X buffer	5µl
10 mM dNTP	4µl
Pfu polymerase	4µl
dH_2O	21µl

2.2.2.10 Precipitation of DNA using sodium acetate and ethanol

3 volumes of ice-cols 100% ethanol and ¼ volume of 3M sodium acetate pH 5.2 was added to the solution of DNA. The sample was kept on ice or at -20°C for 1 hour and then centrifuged at 18, 000 g for 1 minutes and the supernatant was aspirated, taking care not to dislodge the fragile DNA pellet. The pellet was washed with 500µl of 70% ice-cold ethanol, dried and resuspended in either dH_2O or the required buffer.

2.2.2.11 Ligation of DNA

Ligation of DNA termini was carried out at 16°C overnight, in the following reaction:

Insert DNA	2µl
Vector DNA	1 µl
10 x T4 DNA ligase buffer	1µl
T4 DNA ligase	1µl
H_2O	5µl

In addition, reactions containing either vector or insert DNA alone were carried out to control for contamination by circularised DNA plasmid DNA that can cause high background levels of colonies on transformation plates. The T4 DNA ligase and accompanying buffer were purchased from New England Biolabs (Hitchin UK).

2.2.2.12 Transformation of chemically competent E.Coli

One Shot® TOP10 Chemically Competent *E. coli* were purchased from Invitrogen (USA). For transformation, 1-4µl of ligation product was added to a 50µl aliquot of chemically competent bacteria. The mixture was incubated on ice for 30 minutes before heat shock at 42°C for 45 seconds and transferred immediately back onto ice for 2 minutes. Cells were then incubated for 30 minutes at 37°C in 500µl of SOC medium, to allow recovery and the expression of antibiotic resistance. Pre-dried and warmed LB agar plates containing the appropriate antibiotic were inoculated with 200µl of transformation product, inverted and stored overnight at 37°C. Colonies were picked for LB suspension culture the next day.

2.2.2.13 Miniprep isolation of plasmid DNA from E.Coli

Purification of DNA from transformed plates was performed using kits from Qiagen. The technique is performed on columns with a silica matrix and uses approximately 1.5ml of LB broth culture. The technique is based on the alkaline lysis method of plasmid DNA purification. Plasmid DNA was eluted in 50µl of dH_2O and yielded approximately 0.2µg/µl of DNA.

2.2.2.14 Maxiprep isolation of plasmid DNA from E.Coli

Purification of endotoxin free DNA from 100ml LB broth bacteria cultures was performed using kits purchased from Qiagen. The procedure is scaled up method based on the miniprep procedure. The kit contains a buffer used for the removal of bacterial endotoxin. The preparation yielded approximately 0.6 – 1.0 µg/µl plasmid DNA.

2.2.2.15 Preparation of bacterial glycerol stocks

To prepare glycerol stocks, 20ml of LB bacterial culture was centrifuged at 3000 g for 10 min, most of the media was removed, leaving 2ml on the pellet. The bacteria were resuspended in the remaining media and split between two cryovials, each containing 0.5ml glycerol. The bacterial/glycerol cultures were mixed thoroughly and snap frozen in liquid nitrogen and stored at -70°C.

To grow bacteria from stocks, the glycerol was partly defrosted on dry ice. The defrosted top layer was scraped off and mixed with 200µl of LB and incubated over-night on a plate containing the appropriate antibiotic. Small/large scale preparation was carried out, as required.

2.2.3 Cloning of Zfp36 ECR luciferase reporters

The evolutionary conserved regions (ECRs) for luciferase reporter constructs were amplified by PCR from human and murine template DNA (Promega) using the primers detailed in Table 2.6. An *XhoI* site was designed into both the forward and reverse primers as part of the cloning strategy. The PCR product was cloned into the pCR™-Blunt vector purchased from Invitrogen.

Following miniprep purification (2.2.2.14), double restriction digests were carried out to identify positive clones and ensure the correct orientation of inserts. Maxipreps were prepared (2.2.2.15) for the appropriate clones. *Zfp36* fragments were recovered from pCR®-Blunt via restriction enzyme digestion (2.2.2.6) with *XhoI* and confirmed by agarose gel electrophoresis and sequencing. Inserts were subcloned, via *XhoI* into Luciferase containing minimal-promoter vectors, pgl3P and pGL4 (Promega) upstream of the reporter sequence.

2.2.4 *Zfp36* mimic gene construction

The Zfp36 mimic gene, Zfp-Luc was generated as follows. Upstream (Chr) and downstream (Chr) sequences were amplified from murine genomic DNA using primers described in table 2.6. In addition the Luc2CP secuence for replacement of the Zfp36 Exon II was amplified from the pGL4.28 vector purchased from Promega, using the primers detailed in table 2.6. The endogenous first exon of Zfp36 was replaced with an 18bp luc2cp sequence derived from directly upstream of the luc2cp region amplified from pGL4.28 in house. The last two base pairs in the 'synthetic' luc2cp were complementary to the first of the 'amplified' luc2cp and formed a splice-able motif. Firstly, the endogenous upstream (US) region of Zfp36 was cloned into the PEX-lucZfp36 containing vector. In the next step, the PEX-Zfp36US-lucZfp36 containing vector was ligated to the luc2cp fragment amplified from pGL4.28 to produce

PEX-Zfp36US-lucZfp36-Luc. Finally, the endogenous downstream (DS) region of Zfp36 was ligated as appropriate to form the final mimic gene PEX-Zfp36US-lucZfp36-Luc-Zfp36DS or "Zfp-luc".

2.2.4 Transient transfection of immortal cell lines

Transfection is the process of delivering nucleic acids into eukaryotic cells by non-viral methods and is used to study gene function and protein expression in the context of a cell. Transfection was used in this study to transiently introduce reporter constructs to cell lines for Zfp36 promoter analysis.

The transfection protocol specifics for each cell type/ reagent used are outlined in Table 2.12. Generally, cells were seeded in 6 well plates, as appropriate to achieve 40-60% confluency the next day.

Table 2.12 Transfection of immortal cell lines

Cell type	Cell density	Reagent	Incubation
RAW264.7	1.5 X 10^6/ 3ml	FuGene	5-10 min RT
HeLa	2 X 10^5/ 3ml	Superfect	10-15 min 4°C
HEK	5 X 10^5/ 3ml	FuGene	5-10 min RT

The transfection mixtures were made up as detailed below in Table 2.13 and incubated according to the provided instructions, allowing DNA complexes to form.

Table 2.13 Transfection mixture components (per well of a 6 well plate)

	FuGene	Superfect
DNA	3µg	1µg
Transfection reagent	11µl	5µl
Serum free media	x µl (make up to volume)	x µl (make up to volume)
Total	**150µl**	**105µl**

Superfect transfection reagent requires that cells be washed twice in PBS and 500µl of serum free media (SFM) replaced before treatment with 105µl of transfection mix per well. The FuGene transfection mix is sufficient to transfect cells in 3ml of complete media and thus 150µl can be added straight to the well. Transfection was carried out for 3 hours under general tissue culture conditions. After transfection, cells washed with warm PBS, supplemented with 3ml complete media and left to recover for 24 hours before experimentation.

2.2.5 Primary cell transfection of RelA siRNA

Human primary macrophages were derived from plateletpheresis residues by culturing the cells with macrophage-colony stimulating factor as described in 2.2.1.3. Human primary macrophages were plated in 10cm plates (10^{10} cells per plate) in RPMI 1640 for 24 hours and transfected for 48 hours with siRNA (Thermo Fisher Scientific) targeted to RelA (200 nM) or a double stranded non-targeting siRNA control using Dharmafect 1 as a transfection reagent in OPTIMEM medium.

2.2.6 Luciferase reporter assay

The Dual-Luciferase® Reporter Assay System (Promega) was used to quantify reporter luminescence. Following treatment, cells were washed with PBS and harvested with 200µl

of passive lysis buffer (Promega), scraped and transferred to 1.5ml microcentrifuge tubes. Cells were subjected to active lysis by incubation on dry ice and 2 X freeze thaw cycles. Lysates were centrifuged for 1 min at 18,000 g at 4°C and 20µl of each cleared lysate loaded onto an opaque 96-well luminometer plate (Costar). Luciferase Assay Reagent II was added to each sample and the Firefly luminescence measured. Once quantified, this reaction was quenched and the Renilla luciferase reaction initiated simultaneously by addition of Stop & Glo® Reagent.

2.2.7 Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE)

SDS page allows separation of proteins by their individual molecular weights. SDS is an amphipathis molecule that coats the denatured protein in a uniform charge; thus negating the individual charges that would alter the electrophoretic mobility of the proteins.

For protein isolation, $1x10^6$ cells/ml were treated with 60-70µl ice-cold protein lysis buffer containing, as appropriate phosphatase/ protease inhibitors and incubated on ice for 10 min. Cells were harvested by scraping and transferred to a cold shredder column (Qiagen) for homogenisation by centrifuge; 5 minutes, 18000 g at 4°C. At this stage a protein assay (Bio-Rad) was performed to ensure an equal amount of protein is loaded for each sample. Protein extracts were then mixed with an appropriate volume of 5 x SDS-PAGE sample buffer and boiled for 5 min at 95°C to denature the proteins. Proteins were then loaded on pre-cast gels purchased from Bio-Rad and run alongside a full-range molecular weight rainbow marker at approximately 120V for 90 min.

2.2.8 Western blotting

Proteins separated by SDS-PAGE may be transferred from gel to a membrane which allows for protein identification by antibodies. The antibodies used for western blotting are described in Table 2.1.

2.2.8.1 Protein transfer

Pre-assembled polyvinylidene difluoride (PVDF) membranes were bought from Bio-Rad. Assembled in the Trans-blot turbo system-cassette, the gel was placed directly onto the activated PVDF membrane, between the bottom and top layers of the transfer membrane sandwich. The complete cassette was then transferred to the Trans-blot Turbo and a voltage of 150mV applied for 7 minutes.

2.2.8.2 Immunoblotting

The membrane was blocked in 5% (w/v) dried milk powder in TBS/0.2% Tween-20 for 1 hour. The primary antibody was diluted as appropriate (Table 2.1) in blocking solution and applied to the membrane overnight at 4°C. After washing, the membrane was incubated with secondary-HRP conjugated antibody for 1 hour prior to addition of the detection reagent, ECL (chemiluminescent substrate). The membrane blots were then visualised on the chemidoc. For re-probing with a different antibody, membranes were treated with stripping buffer (Bio-Rad), washed in TBS/0.2% Tween-20 and the immunoblotting procedure repeated.

2.2.9 Enzyme linked immunosorbent assay (ELISA)

The concentration of cytokines was measured by sandwich ELISA. All of the ELISA kits were purchased from BD biosciences (San Diego CA). ELISA plates were obtained from Nunc (Roskilde Denmark). Between each step, plates were washed three times with 1 x PBS Tween (0.01%) wash buffer. The capture antibody was diluted in assay diluent and 50µl used to coat each well of the 96 well plate and left over night at 4°C. The wells were washed and blocked in 100µl of diluent for 1 hour at room temperature. After washing, 50µl of appropriately diluted sample(s) was added to the plate, alongside a serial dilution of recombinant protein standards and incubated at room temperature from 2 hours. The plate was washed and 50µl of appropriate secondary biotinylated antibody was diluted, added to the wells and incubated for 1 hour. 100µl of strepavidin-HRP conjugate was added to the washed wells and after a further incubation for 1 hour the samples were developed using the TMB peroxidase substrate systems. TMB peroxidase substrate and peroxidase substrate solution were purchased from KPL Inc. (Maryland USA). The reaction was stopped with H_2SO_4 and the absorbance read at 450nm.

2.2.10 Chromatin Immunoprecipitation

Chromatin immunoprecipitation is a powerful genomics tool that allows the analysis of interactions between endogenous transcription factors and their promoters. The cells are subjected to formaldehyde crosslinking to freeze DNA and protein interactions in place, therefore taking a 'snapshot' of the genome-associated chromatin representing the conditions of the experiment. The nuclei are then isolated and sonicated to allow shearing of the chromatin, fragmenting the genome into small sequences of ~500 bp. The DNA

binding protein of interest is then immunopricipitated with specific antibodies and the associated DNA bound. After several washing procedures, the immune complexes are eluted and reverse crosslinked using heat. The DNA may then be purified and subject to real-time PCR using primers directed at various positions to assess the extent of protein recruitment. Antibodies used here are described in Table 2.3 and primers used for real-time PCE analysis are detailed in Table 2.7 Figure 2.3 is a schematic of the ChIP procedure.

2.2.10.1 Preparation of nuclear extracts

Primary cell cultures were seeded at a density of $10x10^6$ cells/ml whereas cell lines were seeded at $7x10^6$ cells/ml in 10cm tissue culture dishes and treated the following day, as appropriate. Cells were crosslinked with 1% (final concentration) formaldehyde for 10 minutes at room temperature. Fixed cells were then quenched with 125Mm (final concentration) Tris pH 7.5 and washed with ice-cold PBS. An additional three washes with ice-cold PBE removed formaldehyde. Cells were detached from the plate by scraping into PBS containing protease inhibitor cocktail (Roche), transferred to 1.5ml Eppendorf (Cambridge UK) Lobind tubes and recovered by centrifugation; 5 000 g for 5 minutes at 4°C. Once recovered and the supernatant discarded, pellets were resuspended in cell membrane lysis buffer 1 (LB1) and incubated on ice for 10 minutes before 2 min centrifugation at 5000 g. Cell detergents were removed and nuclei recovered by incubation with lysis buffer 2 (LB2) for 10 minutes at room temperature and subsequent centrifugation for 2 min at 5000 g. Pellets were resuspended in nuclear lysis buffer 3 (LB3).

2.2.10.2 Sonication

Isolated chromatin was sonicated using conditions optimised for each cell type (6 x 12 sec or 8 x 12 sec for primary macrophages or RAW 254.7 respectively at 20% amplitude). To ensure

that DNA was sheared to a suitable size, a 50µl aliquot was removed from each sample and analysed for electrophoresis as a standard practice, for quality control. The sonicate was reverse crosslinked at 65°C overnight and the DNA precipitated by phenol chloroform extraction and ethanol precipitation. The samples were resuspended in 30µl of H_2O, 10µl of which was loaded into a 1% agarose gel and run at 120v until sufficiently resolved to ascertain the DNA fragment size. The desired fragmentation was 200-500 base pairs; if concurrent, 10% triton was added to samples for storage at -80°C before immunoprecipitation.

2.2.10.3 Immunoprecipitation

Immunoprecipitation was carried out at 4°C, using magnetic capture by Dynabeads® Protein G (Invitrogen). Beads were washed 3 X in freshly made ice-cold, block solution (1 X PBS with 0.5% BSA, filtered). The appropriate antibody and isotype control were diluted as appropriate (Table 2.3) in the blocking solution and left rotating overnight at 4°C. Antibody removed and beads washed with blocking buffer, 100µl of lysate and 400µl of block were added to the beads and incubated overnight at 4°C.

Samples were spun at 18, 000 g for 1 minute, captured and 200µl of supernatant was kept for input DNA. The remaining magnetic bead-associated Immunoprecipitate was subsequently washed 5x 3 minutes in RIPA buffer plus protease inhibitor, followed by one wash in 1ml TE + 50mM NaCl. Beads were spun at 3000 g for 3 minutes and resuspended, gently, in 200µl of elution buffer (2% SDS in TE) then left at 65°C for 15 minutes, resuspending every 2 minutes. After spinning down and application to the magnetic bead separator, the elute was transferred to new tubes and reverse cross-linked over-night at 65°C.

2.2.10.4 DNA purification

Immunoprecipitated complexes and input fractions were purified using the QIAquick PCR purification kit (Qiagen) as per the manufacturer's instructions. DNA was eluted with 200µl of ddH_2O in Eppendorf Lobind tubes and stored at -20°C until used for real-time PCR analysis (Section 2.2.2.3).

2.2.10 Statistics

GraphPad Prism software (Version 5.03) was used for statistical analysis. Unpaired, two-tailed Student's t-test was applied for comparison of two groups. For analysis of multiple groups, ANOVA was used with Bonferroni correction for multiple comparisons. The following marks are used throughout: *,$p<0.05$; **,$p<0.01$;***,$p<0.005$

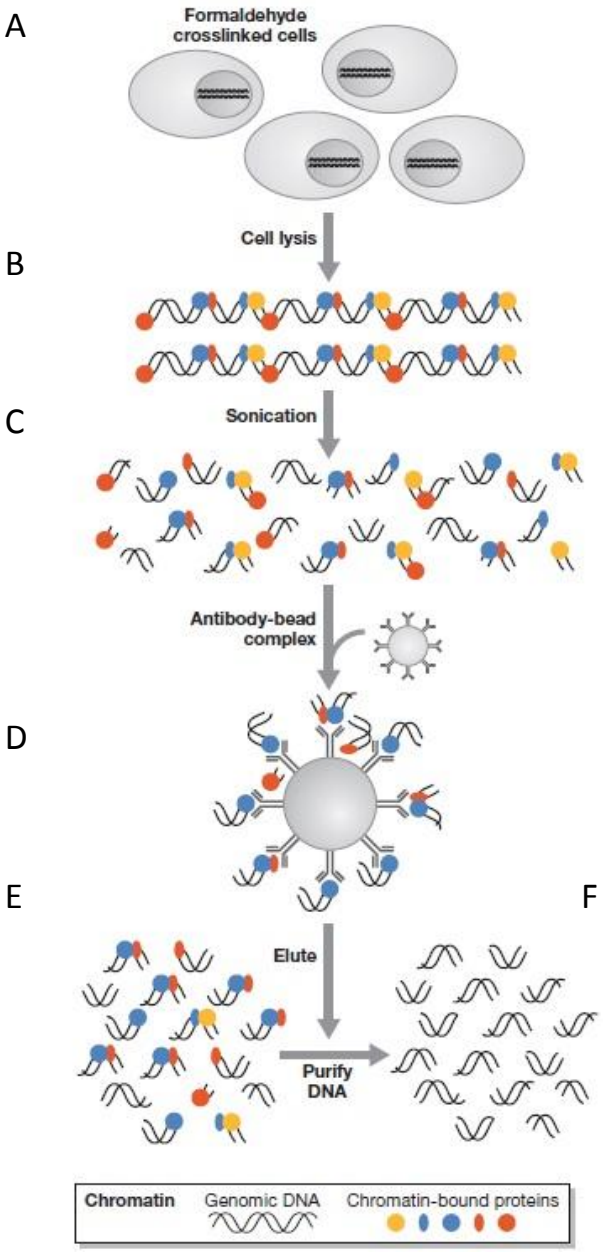


Figure 2.3 Schematic of Chromatin Immunoprecipitation procedure.

This figure has been adapted from Massie and Mills (2008). (A) Cells are treated with formaldehyde which cross-links chromatin/DNA/protein interaction. (B) The cells are lysed and the nuclei isolated. (C) The nuclear fraction is sonicated to shear chromatin and genomic DNA. (D) Immunoprecipitation of chromatin associated with the antibody of interest is carried out using magnetic capture of Dynabeads® with Protein G. The associated DNA is isolated (E) and purified (F) for real-time PCR analysis.

3.0 THE ROLE OF NF-κB IN LPS-INDUCED EXPRESSION OF FEEDBACK NODE GENES

3.1 Introduction

LPS is a potent microbial initiator of inflammation and in macrophages stimulates signalling by TLRs; ultimately leading to the activation of MAPKs and transcription factors like NFkB and AP1 (Bowie & O'Neill 2000). Nuclear factor κB consists of homo/heterodimers of the Rel family, which includes p50, p52, cRel, p65 (RelA) and RelB. Activation of NFκB complex proteins results in the rapid and transient production of inflammatory mediators due to the simultaneous synthesis of anti-inflammatory proteins that negatively feedback on pro-inflammatory agonists (Hayden & Ghosh 2014). A number of factors are involved in controlling this delicate balance between an effective immune reaction and the protection of host tissues. Canonical NFκB signaling is under regulatory control at a number of different molecular levels (Ruland 2011). In unstimulated cells, NFκB proteins are sequestered in the cytoplasm associated with inhibitors of κB proteins; IκBα, IκBβ, IκBγ and IκBε. Upon LPS stimulation IκBs become phosphorylated, ubiquitinated and targeted for degradation, thus resulting in activation and nuclear translocation of NFκB (Rao et al. 2010). Ubiquitination of the IκBs is directed by an E3-ligase complex comprised of multiple proteins including cullin-1, S-phase ligase-1 and Fbox (Soucy et al. 2010) and collectively termed cullin-RING (really interesting new gene) ligase-1. Activity of this complex is enhanced by the attachment of neuronal precursor cell expressed developmentally down-regulated -8 (Nedd8); that is, neddylation of cullin-1 (Watson et al. 2011; Read et al. 2000). Neddylation is catalyzed by Nedd8-activating enzyme (NAE). MLN4924 is a small molecule inhibitor of NAE and therefore causes cells to accumulate cullin-ring substrates including IκBs, leading to sustained sequestration of NFκB in the cytoplasm (Chang et al. 2012). Furthermore, MLN4924 has been shown to decrease phosphorylation and degradation of IκBs and

therefore nuclear accumulation of NFκB as well as directly reducing the expression of several target genes (Milhollen et al. 2010; Swords et al. 2010).

Additionally, deubiquitinases act upstream of IκB kinases (IKKs), so as to block activation of NFκB signalling (Ruland 2011). A20 is one such protein, directly induced by NFκB with dual ubiquitin-editing functions that promote the removal of K63- and addition of K48-polyubiquitin chains to RIP1; subsequently targeting it for proteasomal decay (J. C. Lee et al. 2000; Wertz, O'Rourke, Zhang, et al. 2004). A number of genome wide association studies have linked polymorphisms within the TNFAIP3 locus; that encodes the A20 gene, with susceptibility to various inflammatory and autoimmune diseases including SLE, Type 2 Diabetes, Sjogren syndrome, coronary artery disease, RA and IBD (Catrysse et al. 2014). Perhaps pertaining to the pathological potential of its aberrant activity, A20 may also target other components of the NF-kB signalling cascade including TRAF6 (Boone et al. 2004), IKKγ (Mauro et al. 2006), RIP2 (Hitotsumatsu et al. 2008) and MALT1 (Düwel et al. 2009). Additionally, A20 interacts with components of the DISC complex (Jin et al. 2009) and is a key regulator of apoptosis in a number of cell types (Bellail et al. 2012). Amongst numerous targets of NFκB, anti-inflammatory factors such as A20 feedback negatively upon the inflammatory signalling pathways as a means of restraint. In response to MAPK signalling the anti-inflammatory protein DUSP1 becomes active through phosphorylation, what's more DUSP1 transcript is up-regulated by NFκB. Through inhibition of MAPK signalling, DUSP1 leads to the timely dissociation of inflammatory signalling complexes including those that promote IKK activation and NFκB translocation to the nucleus (Winsauer & de Martin 2007). An important function of DUSP1 is to modulate the phosphorylation of Tristetraprolin (TTP), an additional NFκB/AP1 directly-responsive gene. The primary function of TTP is

widely considered to be the posttranscriptional down-regulation of gene expression. Therein lies a large proportion of its ability to quench the inflammatory response by targeting injurious cytokines for degradation. However a number of studies have highlighted the potential for TTP to directly inhibit NFκB signalling through interaction with p65, HDAC recruitment and suppression of NFκB dependent promoters (Zhang et al. 2013; Schichl et al. 2009; Liang et al. 2009).

The aim of this chapter was to investigate the mRNA expression profiles of DUSP1, TTP and A20 in response to LPS and the pharmacological inhibitor of NFκB MLN4924. With a particular focus on TTP, the objective was to identify the molecular mechanisms underlying LPS-mediated gene expression of feedback node genes.

3.2 Results

3.2.1 LPS-induced synthesis of *Tnfα* mRNA is inhibited by MLN4924

As a primary target of NFκB, we expect the potent inflammatory mediator TNFα to be rapidly and transiently up-regulated by LPS simulation in myeloid cells.

Primary human macrophages were isolated from peripheral blood monocytes whereas murine primary cells were derived from bone marrow; both cell types required M-CSF for polarisation. After 5 days of maturation, primary cells were seeded at a density of $1x10^6$cells/ml in 12 well plates whereas RAW264.7 macrophages were seeded at $5x10^5$cells/ml in 6 well plates. The next day, cells were left untreated, stimulated with LPS or LPS and MLN4924 simultaneously for 1, 2, 4 or 8 hours. At the appropriate time point, cells were harvested for mRNA and subject to QPCR with probes specifically designed for TNFα.

LPS strongly and rapidly induced expression of TNFα mRNA in all cell types (Figure 3.1). TNFα is a primary response gene (PRG) and under active transcription as early as 15 minutes post LPS-stimulation (Smallie et al. 2010). The expression was highest after 1 hour LPS stimulation and although slowly diminishing, remained elevated above basal levels until after the last time point at 8 hours. In the same experiments, cells were treated with LPS in combination with NFκB antagonist MLN4924. In response to MLN, the level of LPS-induced TNFα mRNA was strongly reduced, by approximately 90% in primary human and RAW macrophages and 50% in primary murine macrophages. Significant inhibition was observed in primary macrophages at 1 ($p<0.001$) and 2 ($p<0.001$) hours of incubation and from 1 to 8 hours ($p<0.001$) of stimulation in RAW264.7 cells. The observed significant inhibition of TNFα transcription by MLN4924 was an expected outcome and signified efficient inhibition of NFκB signaling in each cell type, using the novel pharmacological inhibitor MLN4924.

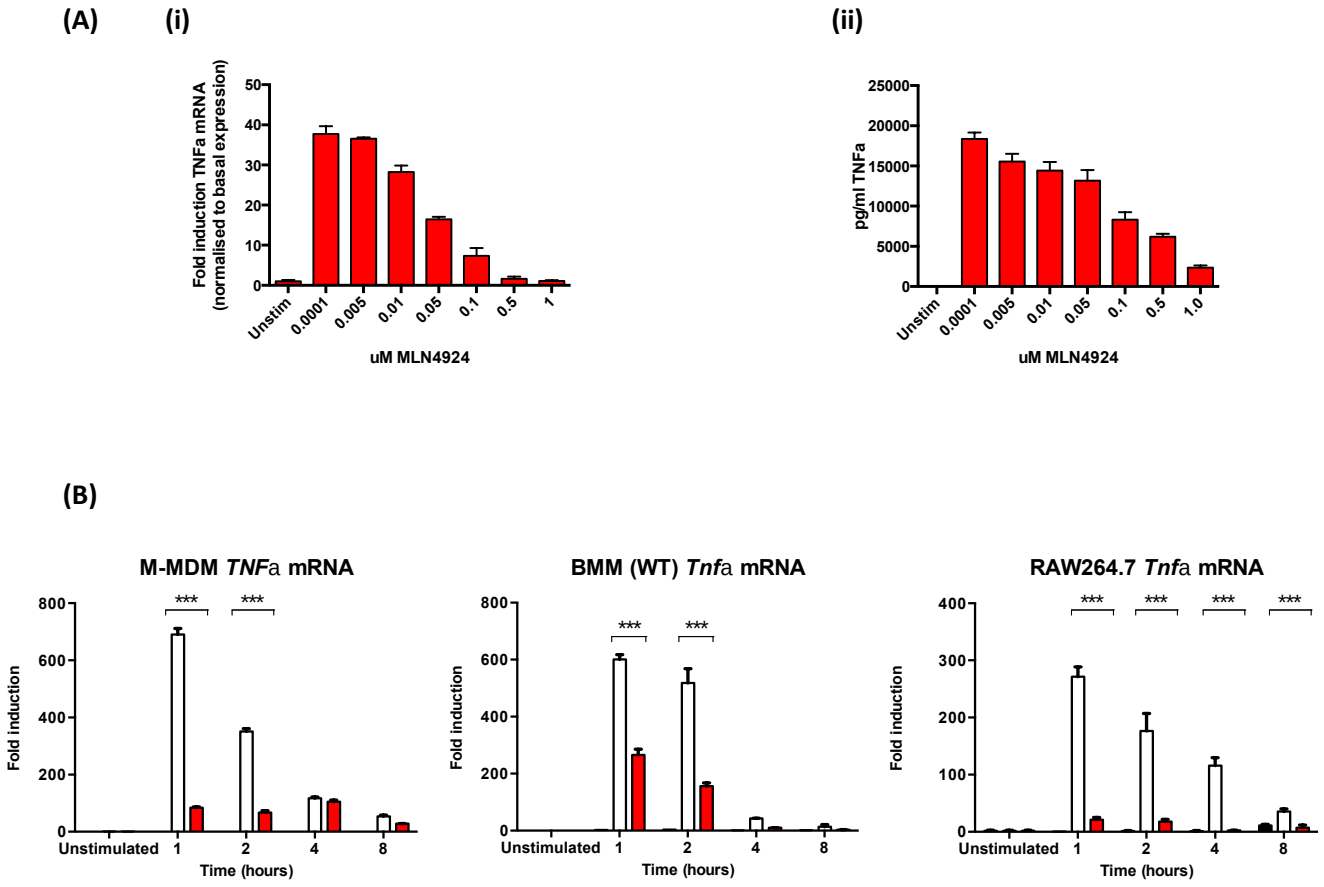


Figure 3.1 TNFα is inhibited by MLN4924 in primary macrophages and RAW cells

(A) Preliminary titrations of MLN4924 carried out in BMM seeded at a density of 1x106cells/ml in 12-well plates. For measurements of mRNA, whole cell lysates were harvested in RNA lysis buffer containing β-mercaptoethanol. Supernatant was collected and diluted accordingly for protein quantification by ELISA. MLN was effective at inhibiting LPS-induced TNFα at the level of mRNA expression (i) and protein secretion (ii) at each dose tested and an optimal concentration of 100nM was chosen for future experiments. **(B)** Primary macrophages and RAW cells were seeded at a density of $1x10^6$cells/ml and $5x10^5$cells/ml respectively and either left un-stimulated, treated with LPS (10ng/ml) or LPS and MLN4924 (100nM) for the times indicated. Data show the mean +/- SEM for three and four separate experiments in primary and RAW macrophages respectively. Each QPCR was carried out in triplicate. *** p< 0.001

Black bars = MLN (100nM); White bars = LPS (10ng/ml); Red bars = MLN + LPS

3.2.2 Chemical inhibition of NFκB enhances LPS-induced *Dusp1* mRNA expression

Using the material obtained from experiments in 3.1, the mRNA expression profile of *Dusp1* was observed over a period of 8 hours. Expressed at almost undetectable levels in un-stimulated macrophages, *Dusp1* precipitously responds to LPS, in a dose dependent manner (data not shown). In accordance with literature, *Dusp1* mRNA was up-regulated by the 1 hour time point in response to LPS-activation, in all cell types (Figure 3.2). Over a period of 8 hours the transcript expression profile of *Dusp1* gradually resolved back down to basal levels. These data confirm the responsiveness of *Dusp1* to inflammatory activation in an immediate early manner. The transcription of *Dusp1* is regulated by a number of factors and accordingly the promoter region contains a large number of transcription factor binding motifs. NFκB has a central role in the succinct activation of gene transcription in response to TLR activation. Indeed, at least two conserved NFκB consensus motifs are located within 2.2kb upstream of the human *DUSP1* promoter with exact sequence matches ~2.5kb uspstream of the murine *Dusp1* promoter (J. Wang et al. 2010; Huang & Tan 2012). Furthermore J Wang et al. (2010) showed that mutation of these sites within a *Dusp1*-promoter driven reporter strongly attenuated luciferase expression. Interestingly however, the addition of NFκB inhibitor MLN4924 (1nM) in fact significantly ($p<0.001$) enhanced and extended the amount of LPS-induced *Dusp1* mRNA in all cell types. Cooperativity between LPS and the NFκB inhibitor was three times the inducing effect of LPS alone on *Dusp1* gene expression at 2 and 4 hours in human primary macrophages. The same effect was not quite as striking in murine cells but was sustained over 4 and 8 hours in primary and RAW macrophages respectively. NFκB is a critical downstream effector of TLR and MAPK signalling, as is DUSP1 protein. It is generally conceived that functional κB elements within

the *Dusp1* promoter are primarily responsible for its up-regulation during inflammation. Chemical inhibition of MAPKs from ERK, p38 and JNK families was unable to effectively inhibit Dusp1 gene expression (J. Wang et al. 2010), whereas inhibition of NFκB or indeed mutation of promoter proximally located NFκB motifs abrogated *Dusp1* transcription in response to LPS. (King, Holden, et al. 2009a). The promoter of *Dusp1* contains numerous cis-acting motifs and therefore it is possible that in these experiments, the absence of nuclear components of NFκB facilitates binding of an orchestra of factors that may influence transcription more efficiently than in the presence of NFκB.

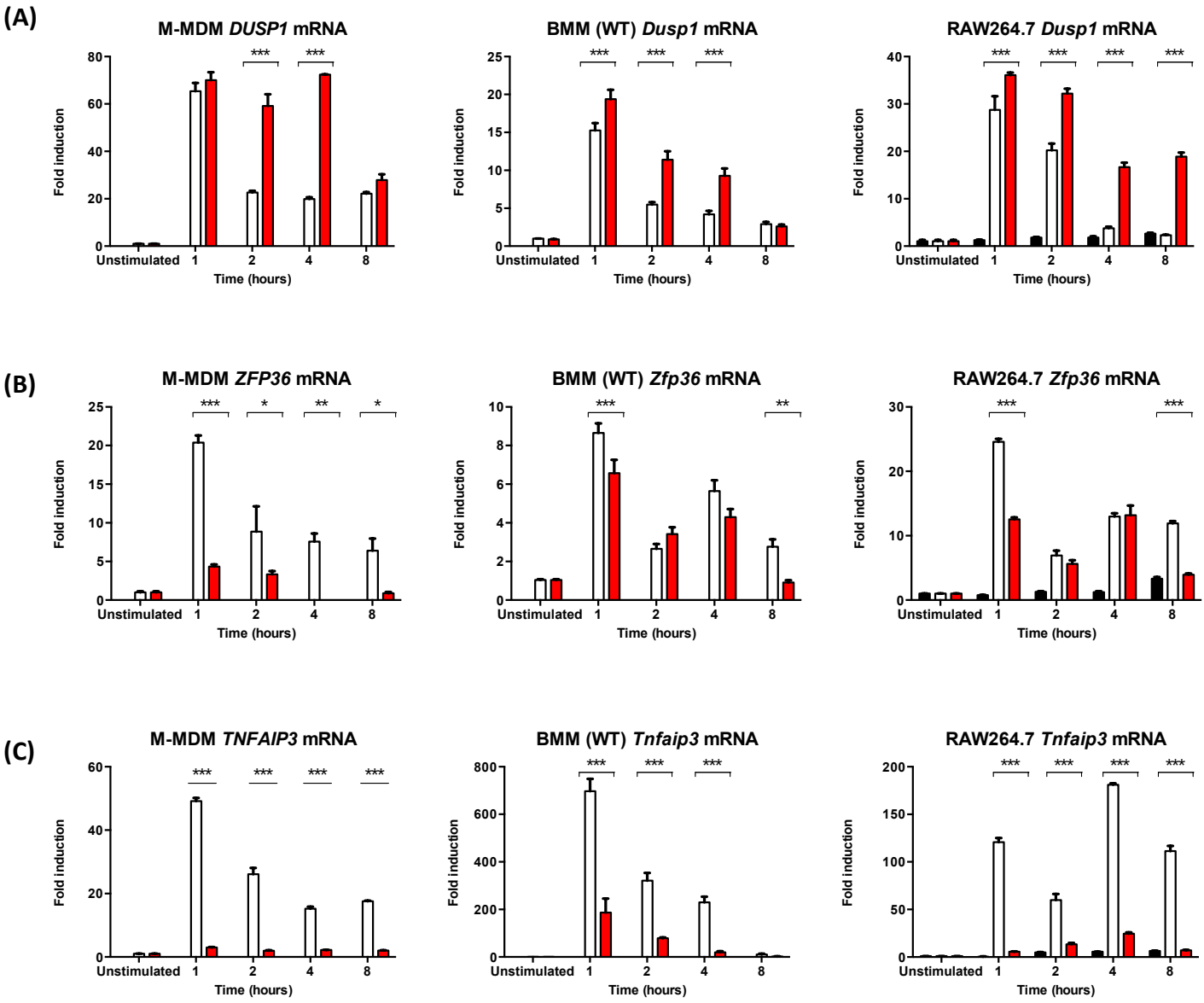


Figure 3.2 The effects of MLN on FNG transcript expression.

In parallel with Chapter 3.2.1 (Figure 3.1) the relative level of FNG mRNA expression was quantified by QPCR in macrophages un-stimulated, treated, with MLN (100nM) (black bars) activated with LPS (10ng/ml) (white bars) or LPS + MLN simultaneously (red bars) for the times indicated. Data represent the mean ± SEM for three and four separate experiments in primary and RAW macrophages respectively. *** p< 0.001 **p<0.01 *p<0.05.

(A) Expression of DUSP1 mRNA is significantly enhanced by MLN in primary macrophages and RAW cells.

(B) LPS-induced *ZFP36* (TTP) mRNA expression is partly dependent on nuclear NFκB

(C) LPS-induction of Tnfaip3 mRNA is strongly inhibited by MLN4924

3.2.3 LPS-induced *ZFP36* mRNA expression is partly dependent on nuclear NFκB

The expression of *Zfp36* mRNA was also quantified in macrophages stimulated with LPS, MLN or both in combination.

Zfp36 is an immediate early gene induced by LPS and TTP protein primarily acts post-transcriptionally to destabilise pro-inflammatory mRNAs. TTP has a multitude of targets, most notably TNFα. In addition, TTP also has direct negative effects on NFκB target gene transcription (Liang et al. 2009). Therefore as a classical negative regulator, TTP inhibits the pathways from which it was activated. The transcriptional regulation of TTP is not well understood. In order to characterise the *Zfp36* response to LPS, mRNA was quantified by QPCR. Typically, in the absence of inflammatory stimulus, macrophages expressed very low levels of *Zfp36* mRNA. A small number of studies have suggested that *Zfp36* transcription is dependent on NFκB (Lai et al. 1998; Jalonen et al. 2005; King, Kaur, et al. 2009; Chen et al. 2013). Carayol et al showed that IKK/NFκB signalling has a dominant role in the LPS-mediated early response in monocytic cells. Indeed, DUSP1 was included in their microarray as one of the immediate early genes dependent on NFκB activation and inhibited by a dominant negative mutant version of the IKKγ complex (Carayol & Wang 2006). However TTP was not identified as NFκB-dependent by the same microarray, contrary to previously discussed studies. Since the activation of NFκB requires an inflammatory agonist, it would corroborate that *Zfp36* is not actively transcribed in resting cells. Correspondingly, LPS rapidly induces *Zfp36* transcript within 60 minutes in a dose-dependent manner (data not shown). Further to this, LPS treated mRNA preparations from previously described experiments (Chapter3.2.1) were subject to QPCR (Figure 3.2(B)). *Zfp36* mRNA was increased significantly ($p<0.001$) by LPS as early as 30 minutes (not shown) and reached a maximal level at 1 hour in macrophages. As a primary response gene, *Zfp36* transcription is

initiated almost instantaneously following TLR activation. It is this mechanism that causes the peak of mRNA expression as early as 1-hour post LPS treatment. Thereafter, expression levels moderated before peaking again at 4 hours of incubation with LPS. At the four-hour time point *Zfp36* responds similarly to a secondary response gene and it could be that distinct mechanisms underlie this wave of transcript up-regulation. In fact, Sauer et al (2006) have shown that interferons strongly induce TTP via signaling through STAT1 and co-stimulation of p38 MAPK. Furthermore, the same study identified that IFN-driven up-regulation of the TTP protein reduced the expression of numerous LPS-induced pro-inflammatory genes including TNFα and IL6 (Ines Sauer et al. 2006). Following 8 hours of treatment, *Zfp36* mRNA remained elevated above the resting level. The rapid up regulation of immediate early genes like *Zfp36* often requires NFκB. Conversely, secondary response genes are often under the control of alternative transcription regulators. In order to determine the role of NFκB in the biosynthesis of TTP, mRNA from cells treated with LPS and MLN4924 was probed for *Zfp36* transcript by QPCR. In primary human macrophages MLN4924 almost abolished LPS-induced Zfp36, reducing transcript expression by over 70% at 1 and 2 hours and 90% at 4 and 8 hours post co-treatment. The effect of MLN4924 on *Zfp36* mRNA in murine macrophages however, was more complex. In primary and RAW murine macrophages, MLN4924 was inhibitory to LPS-dependent mRNA up-regulation by roughly 20% and 50% at 1 hour. After 2 and 4 hours of co-stimulation, MLN had no significant effect on the LPS-mediated increase in mRNA in either murine cell types. At the 8-hour time point MLN once again significantly lowered *Zfp36* mRNA in murine macrophages.

3.2.4 LPS-induction of TNFAIP3 requires NFκB

Another significant negative feedback regulator, A20 is transcribed directly downstream of TLR-signalling and subsequently targets the essential canonical NFκB activators RIP1 and TRAF6 for proteasomal degradation. Here we examined the LPS-induced mRNA expression profile of A20 in human and murine primary macrophages and RAW cells treated as previously described (Chapter 3.2.1). Within 1 hour of LPS activation, macrophages strongly expressed Tnfaip3 mRNA. The pattern of expression was transient and gradually decreased in primary macrophages. In contrast to primary macrophages, RAW264.7 cells expressed Tnfaip3 in a biphasic manner, with peaks at 1 and 4 h after an LPS stimulus. The addition of MLN4924 resulted in a dramatic inhibition of LPS-driven Tnfaip3 mRNA synthesis, particularly in primary human (~90%) and RAW macrophages (~80%). The LPS effect on gene expression was also significantly inhibited in murine primary macrophages, although to a lesser extent (~70%). These results suggest that NFκB is the principal transcription factor involved in A20 biosynthesis in response to LPS. Indeed, two highly conserved consensus binding sites for NFκB have been identified within the *TNFAIP3* promoter (Altonsy et al. 2014). However it has been documented that *Tnfaip3* may selectively escape transcriptional inhibition by the nuclear factor GR, which is known to obstruct NFκB-driven pro-inflammatory gene expression (Caldenhoven et al. 1995; Altonsy et al. 2014).

3.2.5 The effect of MLN on LPS-induction of *Tnfα*, *Zfp36* and *Tnfaip3* mRNA expression in *Dusp1-/-* macrophages

DUSP1 is key in modulating the inflammatory response and critically required for the timely inactivation of MAPK signalling. The effects of excessive MAPK p38 activation are highlighted in mice null for *Dusp1*; where the consequent build-up of phosphorylated TTP results in increased pro-inflammatory mRNA stability. In *Dusp1-/-* macrophages, TNFα is elevated at the posttranscriptional level when compared to WT macrophages but not at the level of gene expression. Overexpression of inflammatory cytokines driven by increased mRNA stability in mice null for *Dusp1* subsequently leads to severe hypotension and multiple organ failure due to an increased susceptibility to endotoxic shock (Zhao et al. 2006; Salojin et al. 2006).

In parallel with time-course experiments in WT primary murine macrophages, cells were also isolated from the bone marrow of age and sex matched *Dusp1-/-* knock out mice and treated with LPS, MLN or both as appropriate. Cells were harvested for mRNA, which was used as a template for QPCR of TNFα, A20 and TTP. The ΔC_t values for *Dusp1-/-* mRNA data were normalised against that of the WT unstimulated and therefore graphs in Figure 3.3 represent fold increase in mRNA compared to WT basal expression levels.

3.2.5.1 MLN-mediated inhibition of LPS-induced Tnfα synthesis is reduced in Dusp1-/- macrophages

Murine *Dusp1 -/-* macrophages expressed basal levels of TNFα far in excess of the negligible amount observed in WT cells. Consequently, on activation with LPS, TNFα transcript levels were dramatically augmented in knock out cells; at 1 hour reaching approximately 600 times the quantity observed in WTs. On addition of MLN, LPS-induced TNFα mRNA expression in

the knock out cells was reduced with significance at the 1-hour time point albeit to a far lesser extent than in WT macrophages. Thereafter MLN was not significantly inhibitory to TNFα production in response to LPS. One explanation for the reduced effectiveness of MLN in *Dusp1-/-* macrophages could be an increase in stability of the TNFα transcript due to enhanced p38 activity and TTP phosphorylation. In Fact, a number of cytokines and chemokines are overexpressed in mice null for *Dusp1*. Additionally, results from our group show that the rate of transcription of TNFα is not dissimilar between *Dusp1-/-* and WT macrophages.

3.2.5.2 The inhibitory effects of MLN on Tnfaip3 mRNA expression are lost in Dusp1-/- macrophages

LPS-activated *Dusp1-/-* macrophages expressed elevated *Tnfaip3* mRNA, up to 800 times the levels observed in WT cells, due to excessive stimulation of this heavily NFκB-responsive gene. Co-stimulation of these cells with LPS and MLN simultaneously reduced *Tnfaip3* transcript quantities at the 1-hour time point but, rather unexpectedly, amplified expression after 2 hours of incubation. Thereafter MLN had no effect on the level of LPS-induced *Tnfaip3* message. The *Tnfaip3* locus contains two upstream NFκB binding motifs and is a direct target of NFκB mediated transcription (Lai et al. 2013). Therefore we observe its enhanced expression as a result of inflammatory signalling; a response that is augmented in *Dusp1* knockout cells where p38 signalling is protracted. Unexpected however, is the reduced effectiveness of MLN inhibition in these cells, along with the significant augmentation of *Tnfaip3* gene expression after 2 hours of co-stimulation. The *Tnfaip3* transcript has two highly conserved adenine-uridine rich motifs, TTATTTATT and TTATTTATA motif; both of which are appropriate TTP binding sequences. Just as the stability of pro-inflammatory TNFα and CXCL1 message is elevated in *Dusp1-/-*, it could be that reduced TTP

activity is also responsible for an increase in *Tnfaip3* transcript levels. Additionally, *Tnfaip3* contains a highly p38 dependent C/EBPβ binding motif within its promoter (Litvak et al. 2009; Lai et al. 2013). It is possible that enhanced p38 MAPK activation due to a lack of *Dusp1* facilitates the binding of transcription factors, other than NFκB that regulate *Tnfaip3* transcript up-regulation.

3.2.5.3 MLN treatment induced Zfp36 transcript expression in DUSP1-/- macrophages

Again, *Zfp36* was more strongly induced in the knockout but not to the same extent as *Tnfα* or *Tnfaip3*. TTP is a potent anti-inflammatory protein capable of destabilising multiple mRNAs. Furthermore, the effectiveness of TTP depends on the equilibrium between active and inactive configurations, rather than on transcriptional up-regulation. Activated cells that lack *Dusp1* accumulate TTP in its inactive state due to extended p38 activity. Thus the natural dissipation of the inflammatory response and the cellular equilibrium that favours the active form of TTP, is retarded in *Dusp1-/-* macrophages.

When stimulated with MLN alone, *Dusp1-/-* macrophages showed a small and unexpected increase in *Zfp36* transcript level. *Dusp1-/-* macrophages display a higher basal level of cytokine expression due to increased mRNA stability and although could account for this slight increase in *Zfp36* message mice null for DUSP1 are normal under basal conditions and only show a hyperresponsive reaction under immune challenge. Other than this phenomenon, the patterns of *Zfp36* expression are broadly similar between WT and KO cells, with the exception that MLN enhances expression at 4 hours in KO.

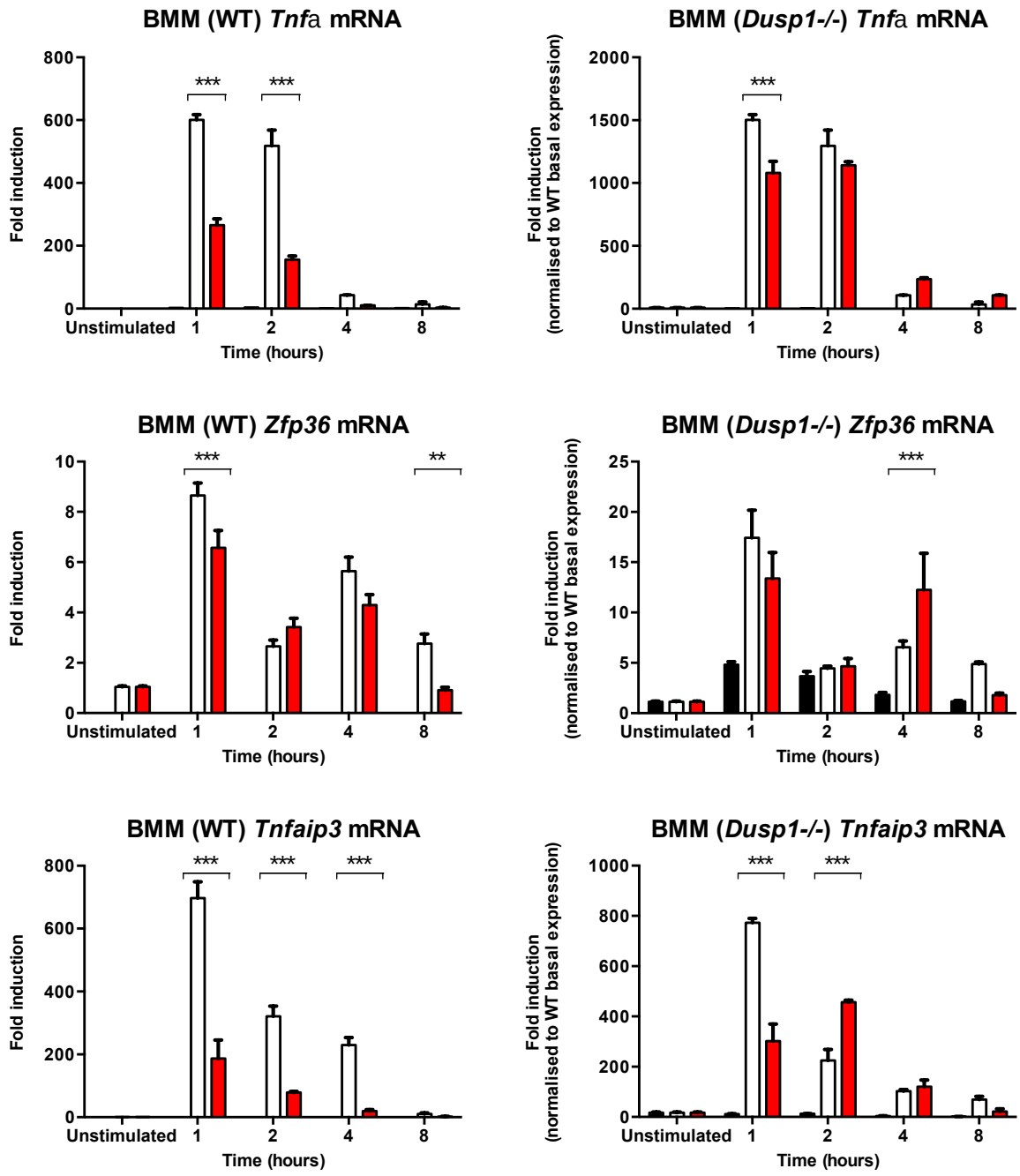


Figure 3.3 Quantification of *Tnfα*, Tnfai*p3* and *Zfp36* transcript expression in WT and Dusp1-/- macrophages treated with LPS and MLN

In parallel with WT experiments, macrophages were harvested and matured from age and sex matched *Dusp1-/-* mice and treated with LPS, MLN or both as appropriate. Cells were harvested for mRNA, of which *Tnfα*, *Tnfaip3* and *Zfp36* were quantified by QPCR. The ΔC_t values for *Dusp1-/-* mRNA data were normalised against that of the WT unstimulated and therefore graphs represent fold increase in mRNA compared to WT basal expression levels. . *** p< 0.001 **p<0.01 *p<0.05

Black bars = MLN (100nM); White bars = LPS (10ng/ml); Red bars = MLN + LPS

3.2.6 Design of reporter constructs containing upstream conserved regions of the human and murine *Zfp36* locus

In order to identify and characterise novel regulatory sites at the *Zfp36* locus and their responsiveness to various inducers of transcription, reporter constructs were assembled containing evolutionarily conserved regions (ECRs) of the murine and human *Zfp36/ZFP36* locus. By cloning putative regulatory and enhancer regions upstream of a reporter sequence, it is possible to assess the ability of these regions to respond transcriptionally in vitro to a number of stimuli. This is a conventional genetic approach to establishing the biological relevance of DNA segments. However in order to elucidate sequence function it is important to use complimentary information from a number of different methods designed to identify regions of interest at specific gene loci. Evolutionary conservation of non-coding regions is often a marker of elements involved in the control of gene expression. Phylogenetic foot-printing is an approach that uses sequence conservation to identify putative regulatory elements. Figure 3.4 is a VISTA plot of the *Zfp36* locus detailing conservation between mouse, human, rat and chimp for sequences coding exons, UTR's and non-coding nucleotides. At the top of the figure is a cartoon of the *Zfp36* gene structure, which is highly conserved between mouse and human. Although powerful, evolutionary mechanisms of identifying non-coding functional elements are limited. For example, transcription factor binding sites are mostly short sequences and often highly degenerate, making them hard to identify and align. Some biological gene categories are subject to rapid evolutionary turnover, even amongst closely related species. The immune-regulatory genes are identified within this example and therefore the absence of sequence conservation between species regarding this functional group should not be interpreted as evidence for lack of function. Nor does an evolutionary approach to sequence detection allude to the

molecular mechanisms under selection, the relevant cell types or the physiological processes involved. Further complimentary to the two aforementioned techniques are biochemical methods of identification which provide information about both the molecular function of underling DNA elements and the cell types in which they act and therefore offer a launching point from which to study human disease (Kellis et al. 2014).

Genome-wide analyses of chromatin structure can provide helpful clues to the location of regulatory elements. DNAse I hypersensitivity assays are a method of detection which takes advantage of the compromised chromatin structure surrounding transcription regulatory sites. Hypersensitive sites are regions of open chromatin conformation, which frees up the DNA to enhance the binding of transcription facilitators. Moreover, transcription factor binding displaces histone octamers, further increasing the sensitivity of these loci. Hypersensitive sites can either indicate transient remodelling of chromatin as in the case of transcriptional induction, or stable epigenetic inheritance of programmed changes to a locus during differentiation (Pipkin 2006). DNase I hypersensitive sites can be identified across the genome by high-throughput sequencing of the ends generated by limited digestion of genomic DNA (Song et al. 2011; Boyle et al. 2011). Some DNAse I hypersensitivity and evolutionarily conserved site maps are now publicly accessible via the University of California Santa Cruz (UCSC) genome browser.

The small pink peak of highly conserved sequence directly upstream of the TTP transcription start site indicates the promoter region, which is rich in multiple transcription factor binding motifs including κB, AP1, SP1, TAK and ERG1. Studies of *Zfp36* transcriptional regulation have previously tended to focus on this small but critical region of conservation (Chen et al. 2013; Ogawa et al. 2003; I Sauer et al. 2006; Gaba et al. 2012). Although Lai et al. (1998)

have investigated serum inducibility of the *Zfp36* intronic region, whilst Florkowska et al. (2012)have inspected the sequence further upstream and identified Elk-1 transcription factor binding. Still, there are a number of more distal loci that are also well preserved, which we have termed ECR1 (in the Med29 3'UTR; not shown) ECR2, ECR3 and ECR4. Of particular importance, given the role of NFκB in the transcription of TTP, ECR4 contains a small number of both perfectly and imperfectly conserved κB consensus sequence gggRNNYYcc (Carayol et al. 2006; King, Kaur, et al. 2009; Chen et al. 2013). These three regions were amplified from human and mouse genomic DNA (Figure 3.4) and cloned upstream of luciferase-containing pGL3P (SV40 promoter) and PGL4.26 (minimal promoter). ECR3 and ECR4 were amplified both singularly and juxtaposed to explore potential cooperativety between the sequences.

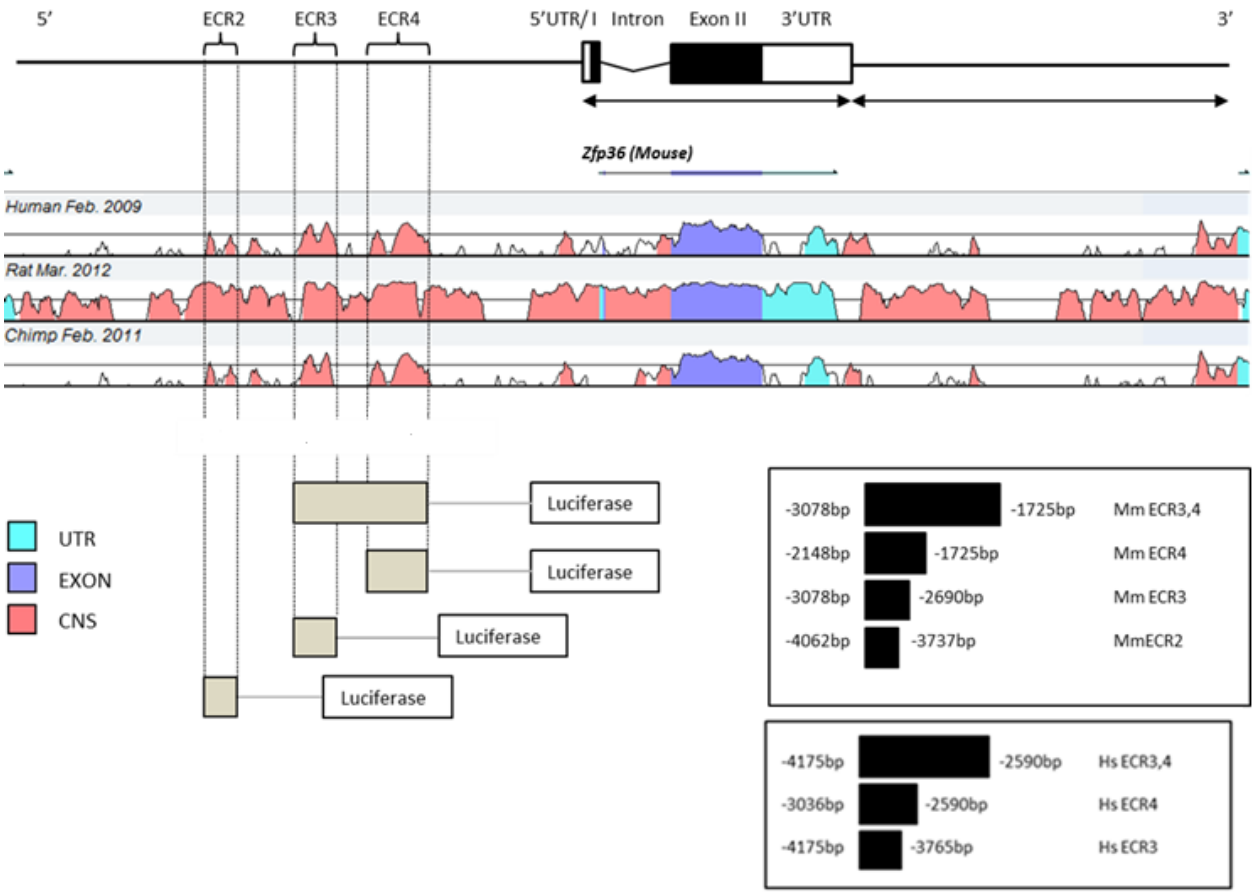


Figure 3.4 Evolutionarily conserved regions within the *Zfp36* locus

(A) VISTA plot of the mouse *Zfp36* gene showing the conserved regions between human, rat and chimp genomes. The X and Y axes represent nucleotide position and % identity respectively. Conservetion peaks coloured blue, purple and pink denote the untranslated region (UTR), Exons and extragenic conserved nucleotide sequence (CNS). Evolutionarily conserved regions (ECRs) of interest are indicated. ECRs were amplified using custom oligonucleotides listed in chapter 2; table 2.6 and cloned upstream of the luciferase reporter in a pGL3P vector containing a minimal promoter sequence. **(B)** Size and location of murine (Mm) and human (Hs) ECRs amplified and cloned into the luciferase reporter construct.

3.2.7 Three 5' putative TTP regulatory elements respond transcriptionally to LPS and one conserved region contains a functional NFκB element

Previously, studies have focused on the role of a 2kb upstream promoter region of TTP in LPS-mediated transcriptional regulation. In order to further explore NFκB-mediated *ZFP36* expression, three putative regulatory sequences identified by evolutionary conservation were analysed for their ability to induce gene transcription and luciferase activity. Human and Murine TTP 5' ECR-driven luciferase plasmids were transfected into HeLa and RAW cells respectively and cells were stimulated with IL1 or LPS for 8 hours. In addition to the appropriate human (Hs) ECR constructs, HeLa cells were, in parallel, transfected with positive (containing (κB)3-) or negative; (empty) pGL3P control vectors. The HeLa positive control vector contains six repeats of an NFκB motif ((κB)3-) cloned upstream of the luciferase sequence in the same minimal promoter containing vector used for the experimental constructs, pGL3P. Similarly, the RAW cell positive control vector contains the NFκB-responsive 5' and 3' un-translated sequences of *Tnfα*. This ensures that NFκB signalling is functional in the transfected system. The negative control is the 'empty' pGL3P vector and allows any background luciferase induction mediated by the minimal promoter sequence to be accounted for.

As can be seen in figure 3.5, the positive controls for each RAW264.7 (A) and HeLa (B) cells strongly induce luciferase expression and therefore the respective signalling pathways of the transfected cells remain intact. Using a positive control, we can confirm the experimental effects of chemical inhibition, for example by MLN are true. On the other hand, the empty pGL3P vector containing cells do not respond significantly to LPS stimulation as expected.

Figure 3.5 shows that the murine and human ECRs respond to pro-inflammatory agonists LPS and IL1α respectively by stimulating luciferase transcription and translation. A number of consistencies were identified between species. The longest conserved sequence containing ECR3-4 was the most responsive and brought about the largest induction of luciferase in both cell types, followed by ECR4, ECR3 and ECR2. Generally, the human constructs appear to induce a stronger luciferase response however could be an artefact of numerous factors including stimulus type and cell type. The effects of NFκB inhibiting compound MLN on luciferase induction by each ECR were also consistent between species.

The luciferase activity driven by ECR4 was blocked in both human and murine cells by MLN. This effect was also observed in ECR3-4. ECR4 contains a putative NFκB binding element located between base pairs −1859 and −1850 (Chen Jiang 2013) of the murine Zfp36 locus. This putative element is likely responsible for the observed inhibition of transcriptional activity by MLN. Neither ECR2 nor ECR3 contain identified NFκB binding motifs and accordingly in RAW cells neither reporter was affected by MLN. However HsECR 3 was negatively affected by MLN whereas HsECR2 was positively affected.

These functional studies suggest that the expression of TTP could be induced by NFκB signalling through an NFκB-binding site within ECR4 in macrophages and epithelial cells. Additionally, it may also outline sequence and/or functional differences between human and murine conserved regions. It should be noted that transfection of naked plasmid DNA does not mimic native chromatin environment and therefore the relative activity of constructs can often be marginal when compared to the induction of endogenous mRNA and protein. However in this study, transcriptional responses in both cell types are quite robust and not too dissimilar to the endogenous induction profile of *Zfp36.*

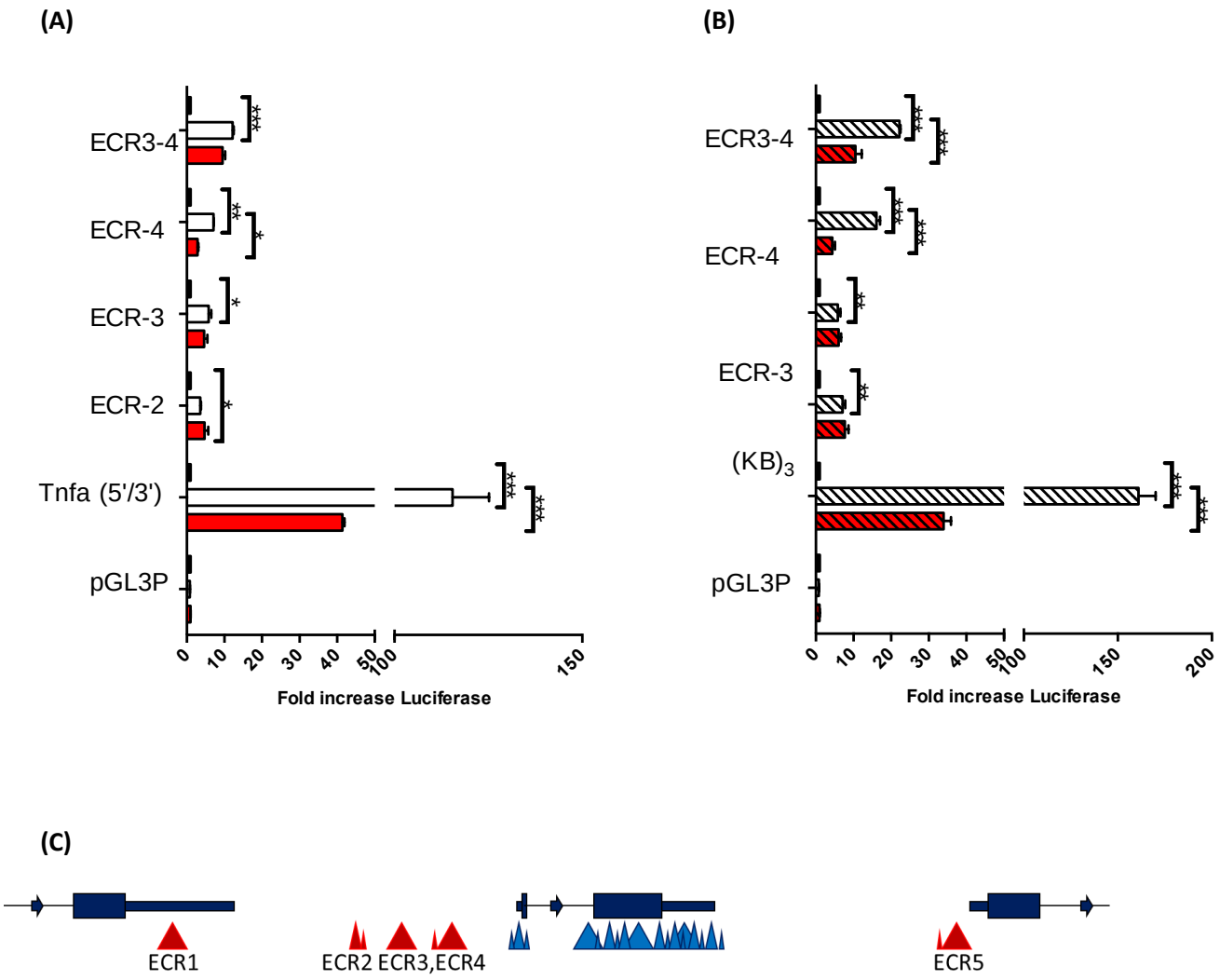


Figure 3.5 Human and murine *Zfp36* ECRs respond to transcriptional regulation by IL-1 and LPS respectively and ECR4 is sensitive to inhibition by MLN

(A) RAW264.7 and **(B)** HeLa cells were transiently transfected with luciferase reporter constructs. The following day, cells were left un-stimulated (black bars); RAW cells activated with 10ng/ml LPS (white bars) and HeLa cells with 1ng/ml IL1α (white-striped bars); 1nM MLN4924 (red bars) or in combination (red bars for RAW and red striped bars for HeLa) for an optimum of 8 hours. Following passive lysis, luciferase assays were performed. Data are expressed as mean ± SEM for three separate experiments. Cartoon **(C)** represents murine evolutionary conservation against a human-base genome and the ECRs 2-4, which were cloned for the reporter constructs above are represented here in red (blue is conservation of the Zfp36 coding sequence). . *** p< 0.001 **p<0.01 *p<0.05

3.2.8 Optimisation of ChIP in murine bone marrow derived macrophages

Due to the fact that MLN was able to reduce the LPS-response of *Zfp36* upstream ECR-containing constructs and the limitations of using reporter assays to investigate endogenous gene-regulation, the next step was to identify NFκB interactions with the TTP locus *in vivo*.

Chromatin immunoprecipitation is a powerful technique for identification of protein-DNA interaction in vivo. In combination with RNAi technology, ChIP is an invaluable tool for studying mechanisms of gene regulation (Li et al. 2006). Furthermore, the application of RNAPII ChIP can be used as a means to identify functional regions of chromatin involved in the regulation of transcription (Sandoval et al. 2004). This will prove useful in determining the mechanisms underlying the LPS-driven biphasic expression of *Zfp36* mRNA observed in this chapter (Figure 3.2) as well as previously (Tchen et al. 2004; Chen et al. 2013). A schematic of the ChIP procedure can be found in Chapter 2.2.9. Formaldehyde is initially used to fix the cells, freezing the architecture of the genome and ensuring that protein-DNA complexes are preserved. Transcription factors bound to their cognate sequences within the DNA can therefore be detected within the context of chromatin. Crosslinked chromatin is then subjected to sonication in order to shear or fragment the DNA, solubilising the chromatin in preparation for immunoprecipitation. Specific antibodies are used to complex target proteins, together with their fragments of DNA and thus enrich for DNA associated with the protein of interest. The crosslinks between the protein/DNA complexes are reversed, the DNA isolated and subject to PCR in order to detect specific target sequences. ChIP can therefore be used to identify regions of the genome where transcription factors or protein modifications are located (Massie & Mills 2008). We initially decided to probe for RNA polymerase II, to distinguish functional regulatory regions of the *Zfp36* locus in response to LPS-stimulation.

In order to design primers for the murine *Zfp36* gene region, the sequence pertaining to the target region was retrieved from UCSC Genome Browser. Primers were constructed to amplify targets of approximately 100-200bp based, generally 500bp apart. Oligonucleotide sequences were calculated to contain a similar GC content and have a predicted annealing temperature of 60°C. Each set of primers was initially tested for sequence specificity by RTPCR of input DNA isolated from the ChIP procedure, followed by melt curve analysis and agarose gel electrophoresis. Figure 3.7 details the location of each designed primer pair (black rectangles) in correlation to the murine *Zfp36* locus. Additionally, arrows mark the location of important genomic regions as well as the previously discussed ECRs. Upstream of *Zfp36* lies *Med29*; downstream is *Plekhg2*, both of which are non LPS-responsive as is evident from Figure 3.6, an experiment in which mRNA transcript was quantified for both genes in response to macrophage stimulation with LPS for the times indicated. ECRs 1 and 5 are associated with PU1; a marker of accessible chromatin in myeloid cells. Our hypothesis states that the entire gene region of *Zfp36* is transcriptionally active and for these reasons, primer pairs were designed across the locus and surrounding ECR1/5 which encroach on the 3'UTR and 5'UTR of *Med29* and *Plekhg2* respectively (Figures 3.5C and 3.7).

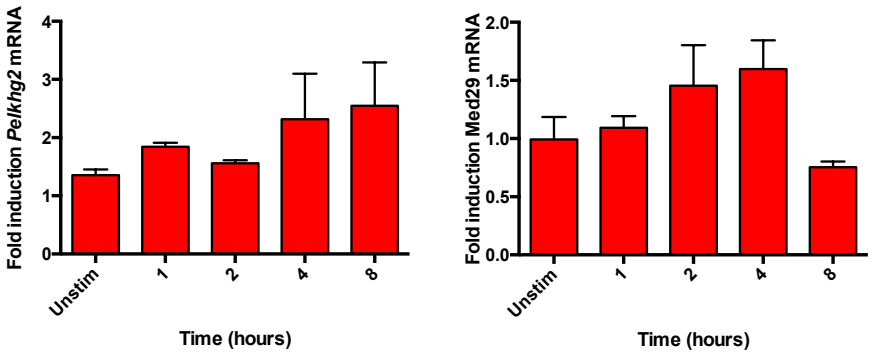


Figure 3.6 Upstream *Med29* and downstream *Plekhg2* transcripts are not LPS-induced in primary murine macrophages

Putative Zfp36-regulatory elements located within the Med29 3'UTR and Plekhg2 5'UTR have been proposed and ChIP with RNAPII revealed an enrichment for these sites in response to LPS. In order to identify whether these effects were due to up-regulation of either Zfp36 flanking gene, QPCR was carried out for Med29 and Plekhg2 on LPS-stimulation time courses. In parallel with previously described mRNA quantification experiments, the two Zfp36 flanking genes were measured for their response to induction by LPS 10ng/ml (red bars) over an 8 hour period.

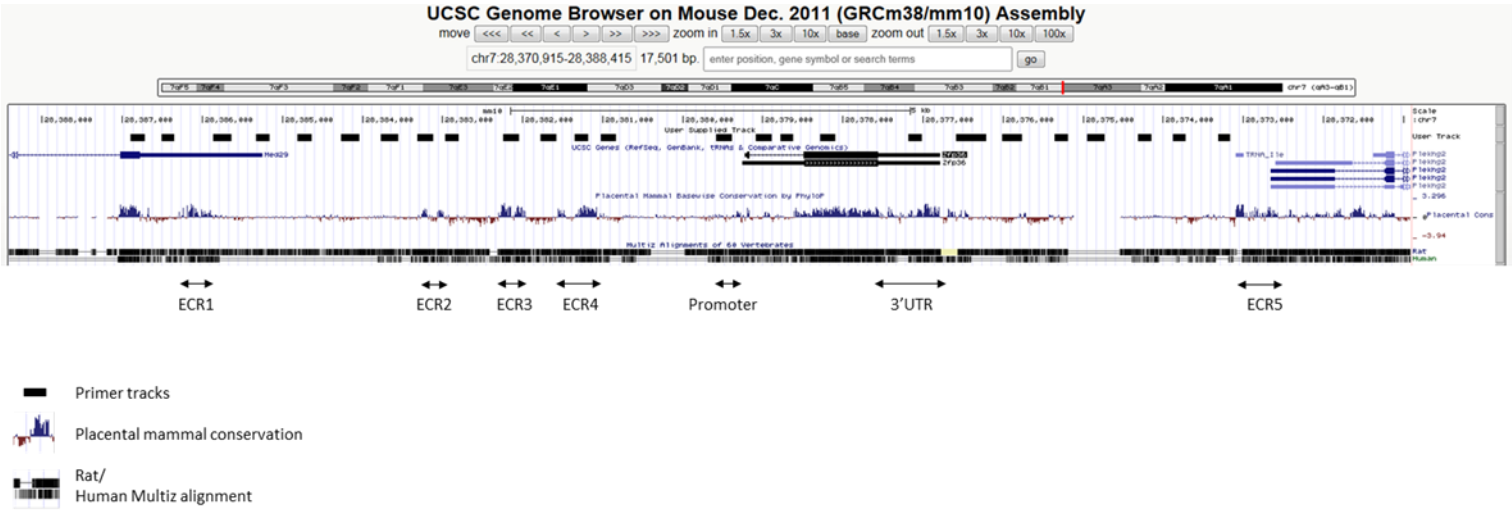


Figure 3.7 Design and sequence location of oligonucleotide primers targeting the mouse *Zfp36* locus

Primers (Chapter 2.1.4.2) were designed to target the mouse *Zfp36* locus (chr7:28371243 - 28386589) for use in ChIP analyses in RAW264.7 and primary bone marrow derived macrophages. Oligonucleotides were spaced approximately 500bp apart, each pair generating amplicons of roughly 300bp.

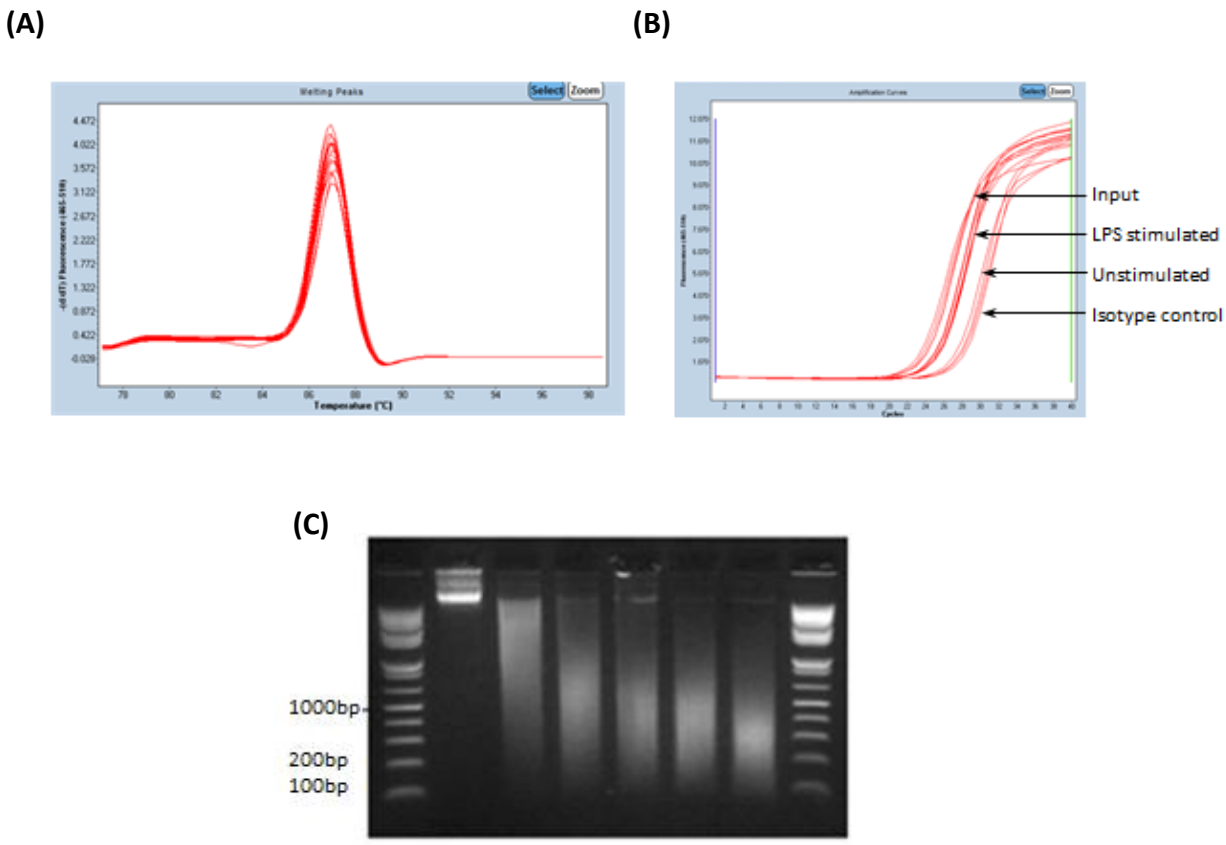


Figure 3.8 ChIP primer analysis and optimisation of chromatin sonication conditions for shearing myeloid cell chromatin

(A) Melt curve analysis of products generated during RT PCR. **(B)** RTPCR run profile showing input DNA and immunoprecipitated (IP) unstimulated, LPS treated and isotype control DNA. Input samples represent DNA not enriched by IP protein, contain more DNA and therefore are amplified at lower cycle numbers. Samples IP with IgG represent experimental noise and are used as a control for background and are unaffected by treatment conditions. Those samples IP for protein of interest (In this case RNAPII) change in cycle number for treatment conditions, reflecting the associated alterations in RNAPII enrichment at the locus. **(C)** Chromatin sonication optimisation. Cells were fixed with formaldehyde and chromatin isolated as described for ChIP (Chapter 2; Section). Chromatin was subject to an increasing number of sonication pulses in order to determine the appropriate number to use for ChIP. Following sonication, formaldehyde crosslinks were reversed by heat (65˚C) overnight. Total DNA was isolated before analysis using agarose gel electrophoresis.

As shown in Figure 3.8, the approach to primer design resulted in successful primer pairs for the amplification of specific target sequences.

The sonication of formaldehyde fixed chromatin is an important part of ChIP and involves shearing of the genome into fragments that are, in this case mostly 400-500bp in length. This ensures accurate resolution can be obtained to determine the position on the genome where the protein of interest is located. To achieve the appropriate level of genomic fragmentation, sonication was optimised for each cell type. Increasing amounts of 12 second pulses were applied to chromatin isolated from RAW264.7 and primary bone marrow derived macrophages. Total DNA was then isolated from these preparations and analysed by gel electrophoresis in order to visualise DNA fragment size (Figure 3.8). With an increasing number of pulses, shearing efficiency is enhanced and product size becomes smaller and more uniform. In this case, the optimum number of pulses for RAW cells and macrophages is 9 and 7 times respectively due to the mode of spread between 300-700bp.

Based on these conditions, a ChIP experiment was performed on 10 million RAW264.7 macrophages seeded overnight. The next day, cells were left un-stimulated, stimulated with LPS or LPS+MLN4924 for 1, 2 and 4 hours. Primers designed to the locus of Zfp36 were used to enrich DNA immunoprecipitated with antibodies specific for NFκB subunit RelA (p65), RNA polymerase II (RNAPII) or an immunoglobulin isotype control (IgG). The isotype control did not increase and remained constant, indicating the specificity of the RelA and RNAPII antibodies. The values for isotype control under each condition were used as a baseline to which the percent input quantities were normalised.

Run profiles of the real-time PCR for this experiment are shown in Figure 3.8 and show specific enrichment of the *Zfp36* locus DNA bound to RNAPII. The input DNA amplified at a

lower cycle number due to its relative abundance and represents DNA not enriched by the specific antibodies. Melt curve analysis was used to confirm that the PCR primers designed for this locus resulted in a specific amplification product. An aliquot of each chromatin sample used was checked for sonication efficiency.

3.2.9 Analysis of *Zfp36* chromatin involvement in RAW264.7 macrophage response to LPS; RNAPII ChIP

Once optimised, chromatin immunoprecipitation could be used to characterise the underlying mechanisms of LPS-mediated *Zfp36* expression through identification of protein-DNA interactions in vivo.

Figure 3.9 Shows ChIP of the TTP locus with an antibody specific for RNAPII in RAW cells that were stimulated for 1 hour with LPS or left untreated. Panel (A) depicts regions of the *Zfp36* locus (x axis) found to be enriched for RNAPII under resting conditions and when stimulated with LPS. Enrichment is calculated by normalisation of the per cent input values to the isotype control antibody, which essentially yields the amount of RNAPII accumulation at each primer position in relation to the background DNA-antibody association. TTP is a primary response gene (Schott et al. 2014)and even under basal conditions is associated with a relaxed chromatin configuration, which encourages the protein-DNA interactions that facilitate rapid gene transcription. Promoter proximal pausing is one such mechanism whereby RNAPII associates with the promoter sequence of PRGs and is held in a state of transcription initiation whilst elongation is inhibited, awaiting an appropriate activation signal (Hargreaves et al. 2009). Figure 3.9 shows a clear accumulation of RNAPII within the promoter region (x-axis p14) and much further upstream of *Zfp36*. Corresponding to p10-12,

the ECRs investigated previously (Chapter 3.2.7) are enriched for RNAPII in the absence of stimulus, which implicates them as putative transcription enhancer sequences. Enhancers facilitate gene transcription under varied and specific conditions of activation through increasing DNA-associations with co-activators, which might be proximal or distal to the gene region. Chromatin looping is an efficient mechanism whereby distal regulatory regions are brought into proximity with the target gene sequence.

For each ChIP, two control regions of the TNFα locus were probed by QPCR in parallel with Zfp36 target sequences. RNAPII accumulation at these two TNFα regulatory regions was considered before continuing with QPCR using experimental target primers.

Panel (B) of Figure 3.9 shows the RNAPII fold enrichment across the locus, which is calculated by normalising the LPS-stimulated values for each primer pair to the equivalent un-stimulated figure. The greatest increase in RNAPII association with the Zfp36 locus on stimulation with LPS happens within the coding region and downstream of the gene, illustrating the 5' – 3' movement of RNAPII as it transcribes the DNA. RNAPII proximal to the 3'UTR has already transcribed a molecule of mRNA whereas RNAPII in the gene region will be in the process of transcription.

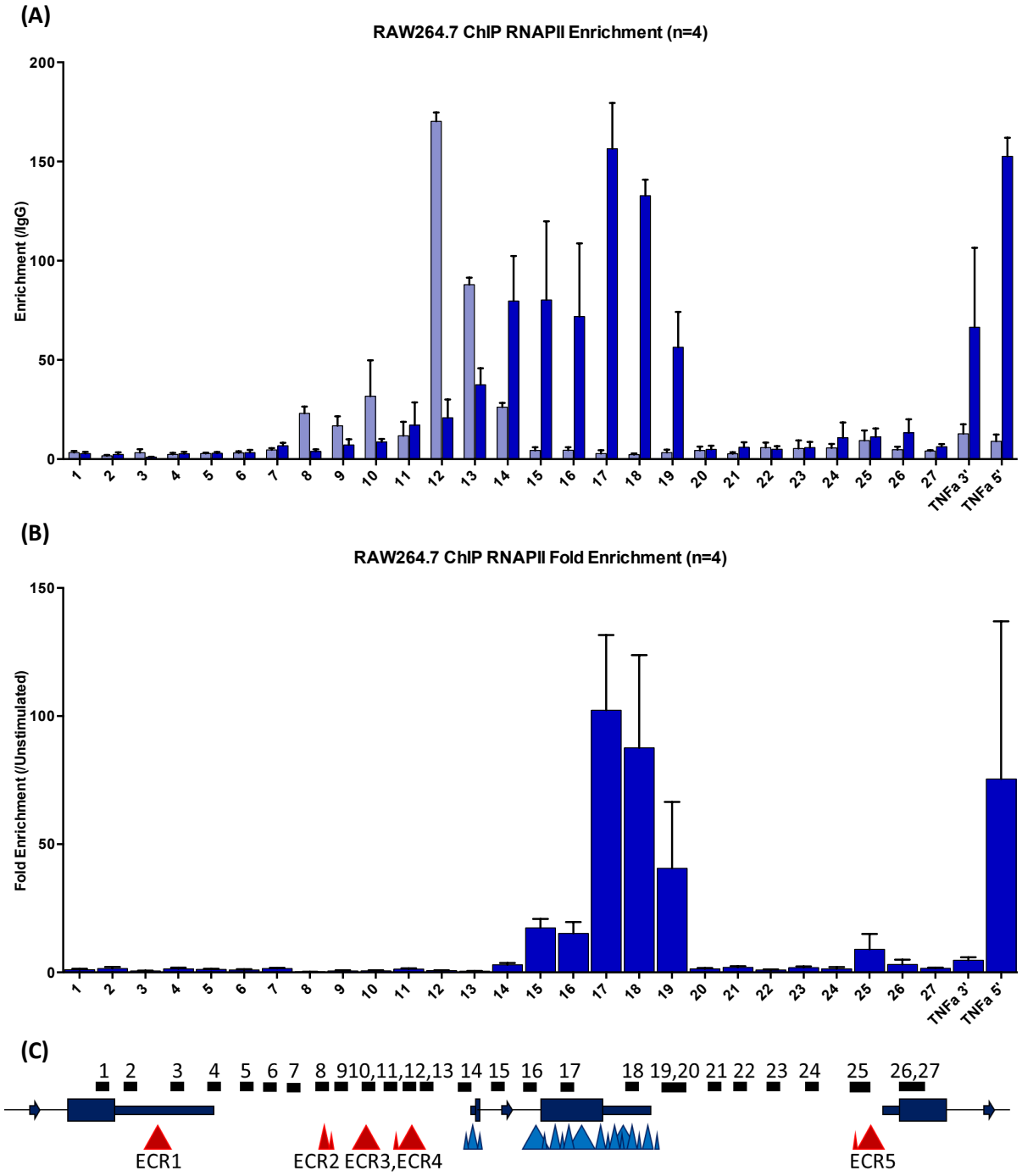


Figure 3.9 Enrichment of RNAPII at the Zfp36 locus in RAW264.7 murine macrophage cells left unstimulated or treated with LPS

RAW cells were seeded at a density of $7x10^5$ /ml overnight before being left unstimulated or treated with LPS (10ng/ml) for 1 hour. Subsequently, cells were subjected to ChIP with antibodies specific for RNAPII and an isotype control (IgG) before QPCR using oligonucleotides designed against the murine TTP locus (Figure 3.7). **(A)** Shows regions of the TTP locus enriched for RNAPII in unstimulated and LPS-treated cells, normalised to the percent input values of the isotype control. **(B)** Highlights the fold increase in sequence enriched for RNAPII on administration of LPS. Plotted are the mean values ± SEM of four separate experiments, for each of which QPCR was carried out in triplicate. **(C)** Cartoon schematic representing the murine Zfp36 gene locus including primer tracks in black (numbered 1-27) and ECRs as red triangles. Light and dark blue bars represent un-treated and LPS-stimulated cells respectively.

3.2.10 Association of RNAPII with the *Zfp36* locus in primary murine macrophages at 1, 2 and 4 hours of LPS stimulation

Further to RAW264.7 cells, ChIP was also carried out in primary murine BMM harvested at 1, 2 and 4 hours post-LPS treatment as well as for unstimulated cells. This was to address the mechanism behind the biphasic expression profile of TTP mRNA in response to LPS. In response to LPS, Zfp36 transcription is rapidly up-regulated and reaches maximum after 1 hour post-stimulation. Secondary to this, after an initial depression in LPS-mediated mRNA at 2 hours there is a succeeding increase in TTP transcript levels at 4 hours incubation in myeloid cells. ChIP of the Zfp36 locus using an RNAPII specific antibody allowed measurement of the abundance of transcription-facilitating machinery for each time point. Enrichment for RNAPII is shown for each time point in Figure 3.10. Again, we observe a large amount of RNAPII paused upstream of the TTP promoter in unstimulated cells. In cells treated with LPS, this association shifted in a 5'-3' direction and indicates active transcription of Zfp36. At one hour, the 5'-3' transition of RNAPII was smooth and enrichment was maximal at the most 3' end of TTP, which indicates that a large number of transcripts have already been produced in this time frame. At two hours the majority of RNAPII had dissociated from the coding region of *Zfp36* and re-associated in the upstream region, although coding sequence accumulation was still above basal levels. Similar to the one hour time point, at four hours once again RNAPII efficiently traffics along the DNA in a 5'-3' manner and accumulates towards the end of the 3'UTR. Thus the RNAPII ChIP in BMM concurs with the LPS-induced transcription profile of endogenous mRNA expression as depicted in Figure 3.3.

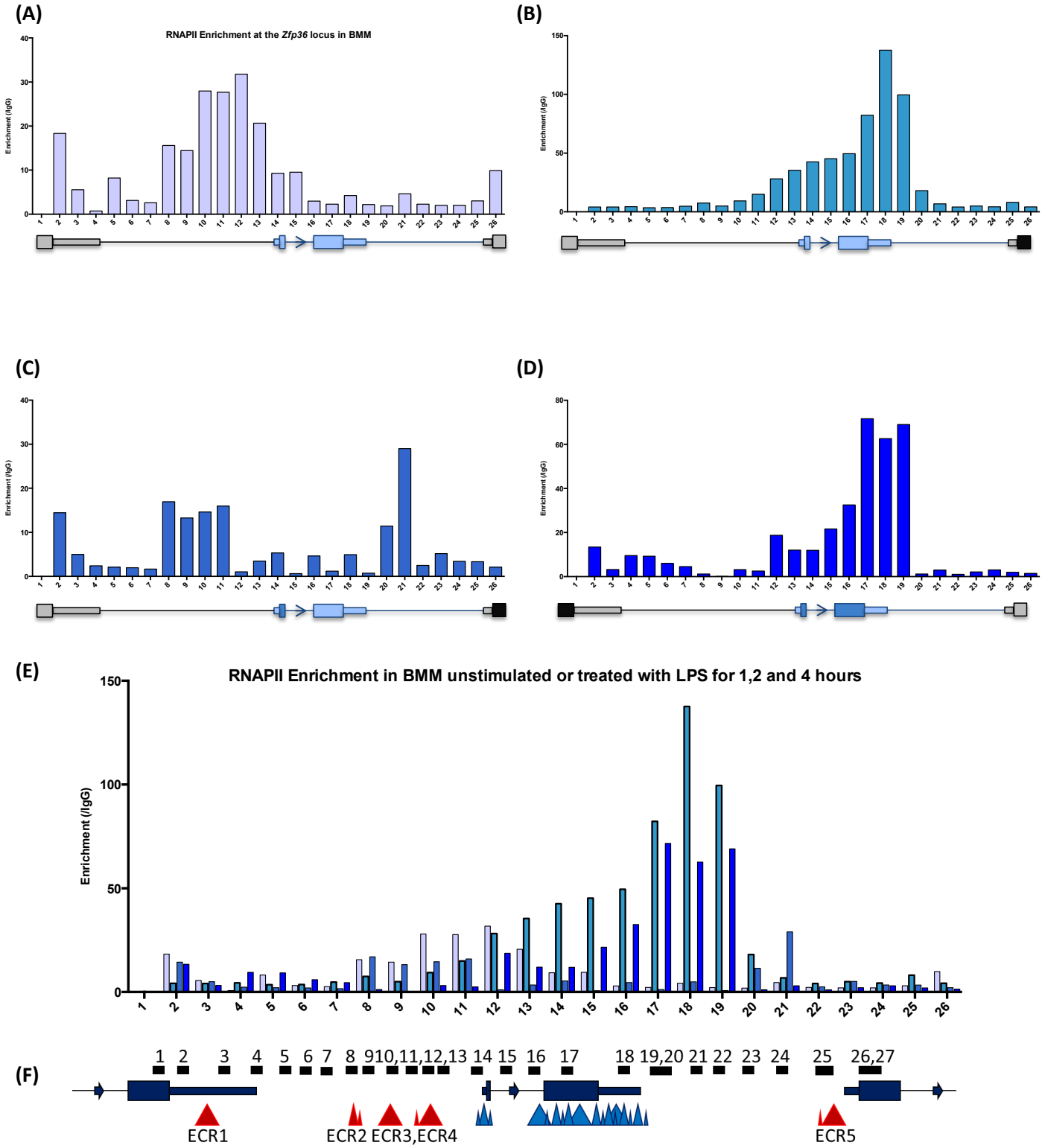


Figure 3.10 Enrichment of RNAPII at the *Zfp36* locus in murine bone marrow derived macrophages

Primary murine BMM were seeded at a density of $1x10^6$ /ml overnight before being left unstimulated or treated with LPS (10ng/ml) for the times indicated (**(A)** = unstimulated, **(B)** = 1h LPS, **(C)** = 2h LPS, **(D)** = 4h LPS). Subsequently, cells were subjected to ChIP with antibodies specific for RNAPII and an isotype control (IgG) before QPCR using oligonucleotides designed against the murine TTP locus (Figure 3.7). Plotted are the mean values for triplicate QPCRs of one experiment that is representative of three similar results. Cartoons below each figure represent the location of each primer pair denoted on the x-axis, with respect to the gene regions of *Zfp36* and *Med29* (upstream) and *Plekhg2* (downstream). Figure **(E)** Shows data from all measured time points (light blue = unstimulated; turquoise = 1h LPS; dark blue = 2h LPS; Indigo = 4h LPS) in one graphical representation for comparison and below **(F)**, is a schematic of the Zfp36 locus including primer locations, gene boundaries and evolutionarily conserved regions of interest.

3.2.11 Analysis of NFκB recruitment to the *Zfp36* gene locus in RAW274.7 murine macrophages using RelA ChIP

Supplementary aliquots of chromatin isolated from RAW cells (Chapter 3.2.9) were precipitated with antibodies specific for RelA; the major subunit of NFκB.

Panel (A) of figure 3.11 represents the regions of DNA, denoted by primer pairs (x axis) that were enriched for RelA antibody associations in vivo; under resting conditions (pale yellow) and when stimulated with LPS (orange). As described previously, in resting conditions, NFκB is mostly inactive and sequestered in the cytoplasm by IκB proteins. For that reason, RelA does not immunoprecipitate with regions of the *Zfp36* locus in unstimulated cells (Figure 3.12A). However, following stimulation with LPS, NFκB is permitted to translocate to the nucleus and bind to DNA. Since the basal level of RelA recruitment to the *Zfp36* locus is negligible, the values for fold enrichment are most representative of the mechanistic action during cell stimulation. *Zfp36*-proximal sequences enriched for by RelA ChIP are focused around ECR4 (primer pair 12), the promoter and within the intron, where known NFκB are recognised to reside. Additionally however, these data highlight a number of putative RelA-binding motifs upstream of *Zfp36* in ECRs 1 (primer pair 2), 2 (primer pairs 7-8) and 3 (primer pairs 10-11). Furthermore, we observe RelA associations downstream of *Zfp36* (primer pairs 25-26), within the *PlekHg2* 5'UTR where ECR5 is located. As aforementioned (Chapter 3.2.9) Chromatin looping is one mechanism which increased transcription potential by bringing enhancer regions into proximity with the promoter. Distal protein-DNA associations identified by Chromatin Immunoprecipitation can occasionally be a direct result of looping, whereby a protein bound to its cognate site might associate with a looped sequence through tethering.

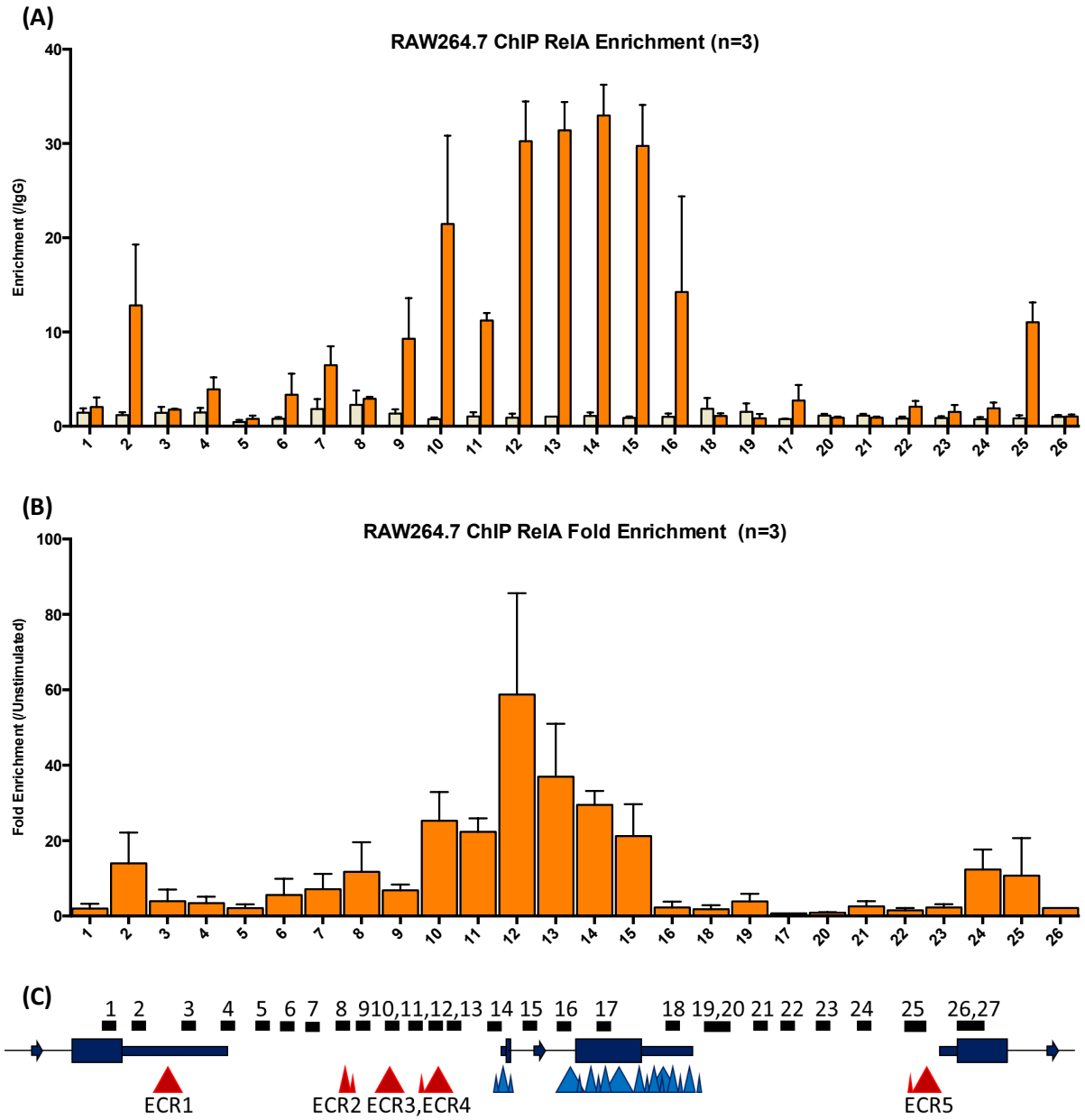


Figure 3.11 Enrichment of RelA at the *Zfp36* locus in RAW264.7 murine macrophage cells left unstimulated or treated with LPS

The same experiment as Figure 3.10. RAW cells were seeded at a density of $7x10^5$ /ml overnight before being left unstimulated or treated with LPS (10ng/ml) for 1 hour. Subsequently, cells were subjected to ChIP with antibodies specific for RelA and an isotype control (IgG) before QPCR using oligonucleotides designed against the murine TTP locus (Figure 3.8). Whie bars = unstilulated Orange bars = 1 hour LPS treated. **(A)** Shows regions of the TTP locus enriched for RNAPII in unstimulated and LPS-treated cells, normalised to the percent input values of the isotype control. **(B)** Highlights the fold increase in sequence enriched for RelA on administration of LPS. Plotted are the mean values ± SEM of three separate experiments, for each of which QPCR was carried out in triplicate. **(C)** Cartoon schematic representing the murine *Zfp36* gene locus including primer tracks in black (numbered 1-27) and ECRs as red triangles.

Tethered or looped DNA may then be enriched for by an antibody to the protein of interest indirectly. This could be the case for a number of the *Zfp36* promoter distal RelA associations. Equally, these associations might be genuine directly bound sequences containing imperfect or context-dependent κB sites.

3.2.11.2 NFκB is associated with the Zfp36 locus at the 1-hour time point but not thereafter

NFκB is activated early and negatively regulated in a rapid manner. It is clear from endogenous mRNA studies in which MLN only partially blocked LPS-mediated TTP transcription that there are other activators of *Zfp36* gene expression. We hypothesized that the second wave of TTP expression was not mediated by NFκB.

In the same experiment as previously described (Chapter 3.2.10), aliquots of chromatin prepared from BMMs untreated or stimulated with LPS for 1, 2 and 4 hours were probed for antibody specific for RelA (p65), a major subunit of NFκB. NFκB is not present in the cell nucleus of unstimulated cells, therefore in Figure 3.12 the data are shown as fold enrichment for RelA compared to the unstimulated chromatin. The greatest accumulation of RelA appears within the gene region. A number of κB motifs have previously been identified within the TTP promoter (Lai et al. 1995; Chen et al. 2013)and intron (Lai et al. 1998). Additionally, ECR4 is a functional element of the TTP locus and contains a putative NFκB binding site. We observed an accumulation of RelA at all of these positions and additionally in the locus 3' of *Med29*, distally upstream of TTP; a similar location to the observed accumulation of RNAPII. At the 2 hour time point the majority of RelA has dissociated from the locus and increasingly so at the 4 hour time point. RNAPII data from the same

experiment shows that RNAPII remains associated with the *Zfp36* locus at 2 and 4 hours following LPS stimulation (Figure 3.11); a similar observation as that for endogenous mRNA (Figure 3.3). This would suggest that other transcription factors and coactivators of transcription are involved in the up-regulation of TTP synthesis at these later time points. Given that NFκB signaling is inhibited by TTP (Liang, lei 2009), it is perhaps unsurprising that transcription initiation of early NFκB-driven feedback node genes later require induction by alternative, readily available factors.

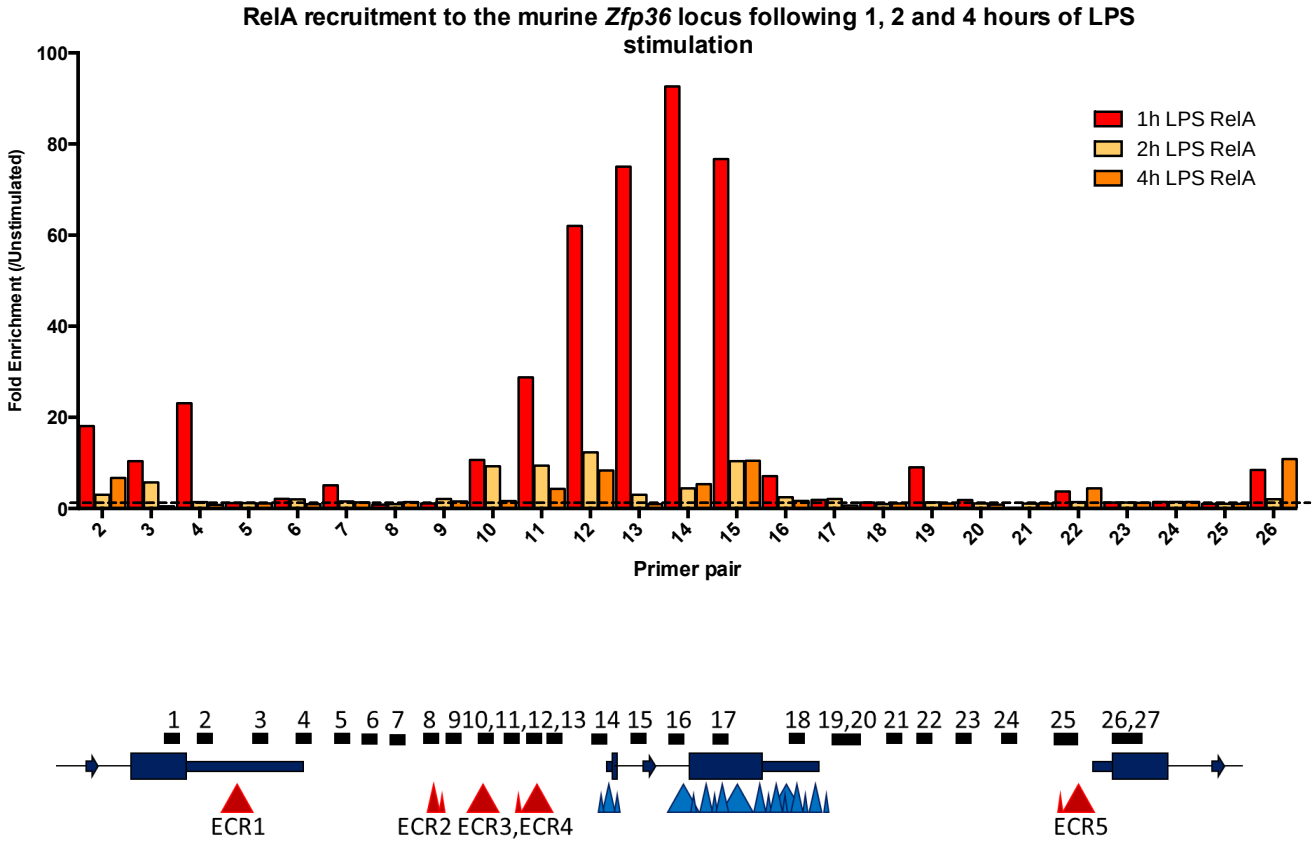


Figure 3.12 Enrichment of RelA at the Zfp36 locus in murine bone marrow derived macrophages

Primary murine BMM were seeded at a density of $1x10^6$ /ml overnight before being left unstimulated or treated with LPS (10ng/ml) for the times indicated (**(A)** = 1h LPS **(B)** = 2h LPS, **(C)** = 4h LPS. Subsequently, cells were subjected to ChIP with antibodies specific for RelA and an isotype control (IgG) before QPCR using oligonucleotides designed against the murine TTP locus (Figure 3.8). The fold induction is calculated as a normalisation of LPS-stimulated to unstimulated values for RelA antibody. Plotted are the mean values for triplicate QPCRs of one experiment that is representative of two similar results.

3.2.12 ChIP of the human ZFP36 locus and RelA knockdown by siRNA

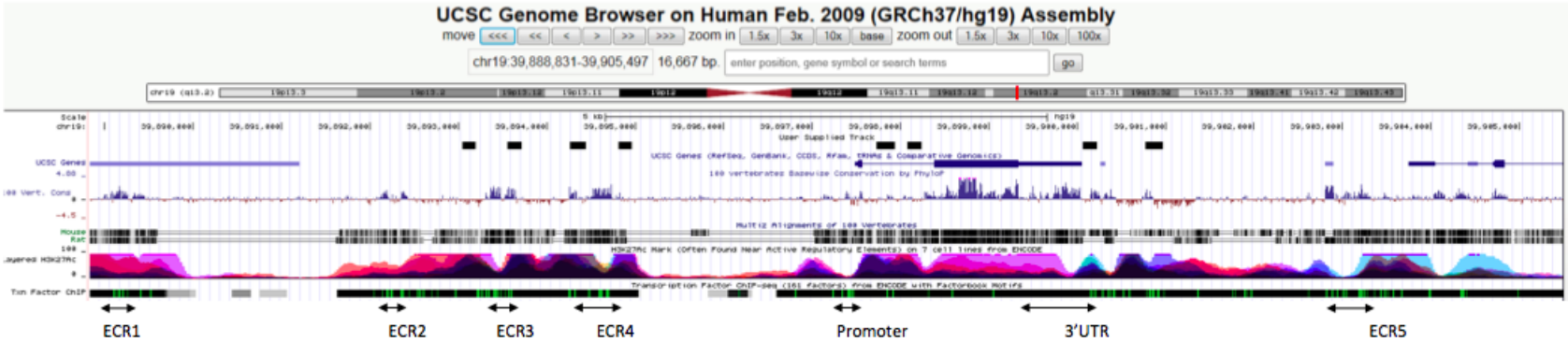
3.2.12.1

Further to the work on pharmacological inhibition of NFκB, primary human macrophages were subject to transfection with siRNA directed against human RelA in order to determine the effects on TTP transcription rate and RNAPII association.

Primary human macrophages were isolated as described in chapter 2.2.1.3 and on the third day transfected with RelA siRNA or scrambled oligonucleotide as a control. Transfection mixture was removed after 2 hours and cells allowed to recover for 48 hours before being subject to LPS activation or left un-stimulated.

As in 3.2.8, the ChIP procedure was optimised for human primary macrophages and QPCR primers were designed against specific regions of interest along the *ZFP36* locus (Figure 3.14). The focus of the human ChIP centred around 8 specific DNA regions of interest. This decision was partially based on time constraints, given that human cells were available only occasionally and therefore experimental timescales were lengthy. In addition and relatedly, the recovery of macrophage material was reduced in human cells when compared to murine primary macrophages and cell lines. Therefore it was not possible to recapture enough human DNA to carry out the amount of QPCR required to cover the human *ZFP36* locus in its entirety. The western blot in Figure 3.15(A) shows an approximate 80% knockdown in cells transfected with RelA siRNA versus scrambled oligonucleotide. In macrophages treated with a scrambled control siRNA, I detected strong association of RelA with ECR4, which contains putative NF-kB binding sites. There was also apparent association of RelA with ECR3, but not with the human ZFP36 intron, consistent with a previous report

(Chen 2013). Treatment of macrophages with siRNA directed against RelA virtually ablated all of these ChIP signals.



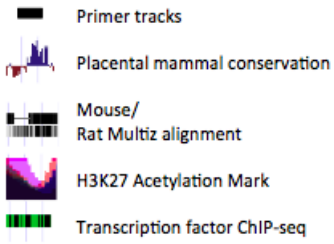


Figure 3.13 Design and sequence location of oligonucleotide primers targeting specific conserved regions of the human Zfp36 locus

(A) Primers were designed to target sequences of interest at the human Zfp36 locus, including ECR3 (chr19:39893046-39893195 and chr19:39893554-39893712), ECR4 (chr19:39894260-39894439 and chr19:39894807-39894964), the intron region (chr19:39897732-39897942 and chr19:39898081-39898238), 3' UTR (chr19:39900067-39900227) and a downstream region (chr19:39900769-39900967). Each primer pair amplified a single product of approximately 100-200bp. **(B)** Chromatin sonication was optimised for human monocyte derived macrophages. Six pulses of 12 seconds were found to be optimal for shearing chromatin into fragments approximately 200bp - 100bp in length.

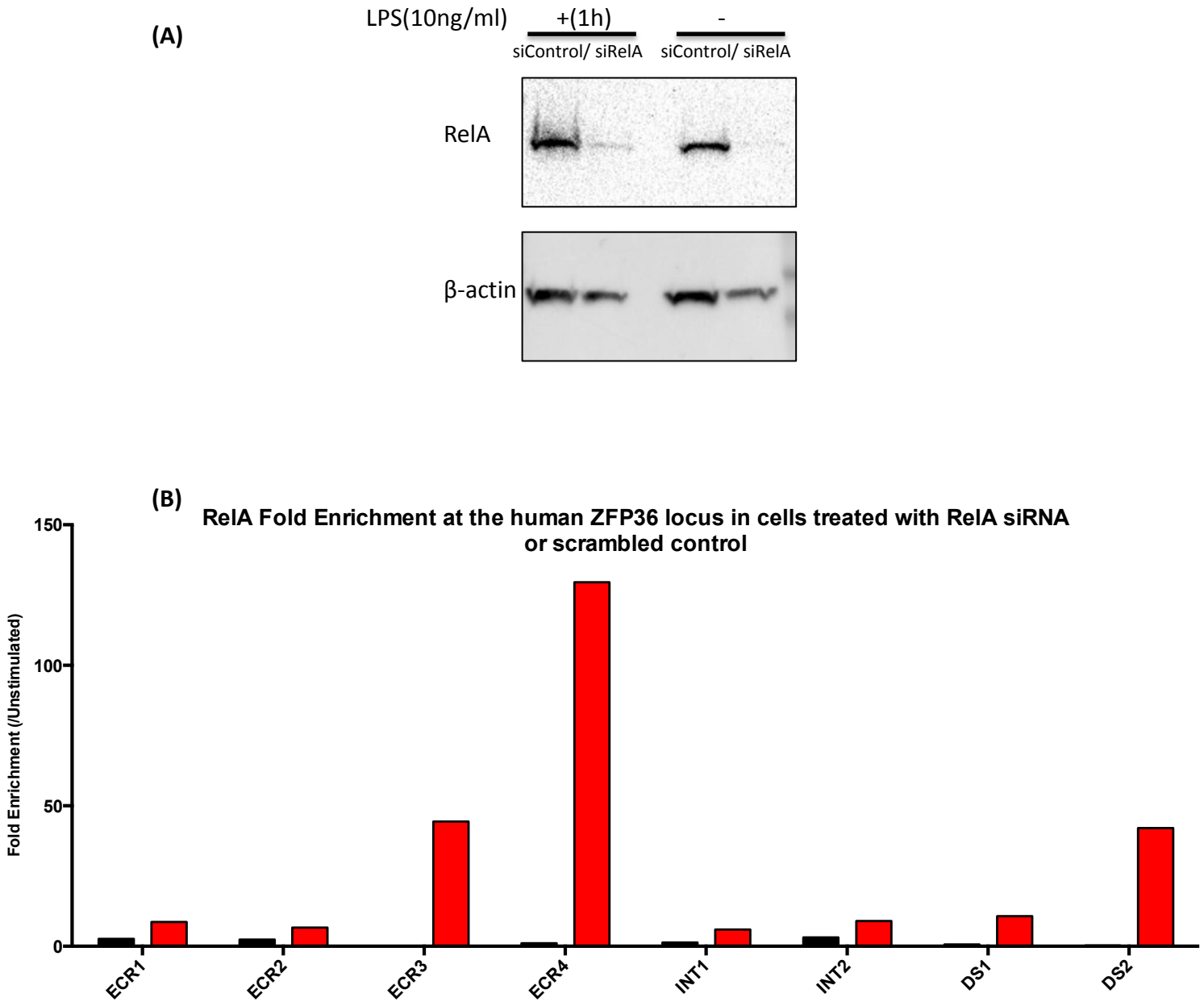


Figure 3.14 Knockdown of RelA & ChIP of the *ZFP36* locus in human primary macrophages

Primary human macrophages were seeded at a density of $10x10^6$. After 3 days, cells were transfected with either RelA siRNA or scrambled oligonucleotide. After transfection, cells were allowed to recover for two days before being stimulated with LPS and undergoing ChIP with antibody specific for RelA. In parallel, cells were harvested for RelA protein detection. **(A)** Western blot showing 80% RelA knockdown in primary human macrophages treated with RelA siRNA and scrambled oligonucleotide in the presence (1 hour) and absence of LPS. DNA was then subjected to QPCR using primers directed at regions of interest within the Zfp36 locus (Figure 3.12). **(B)** Confirmation of RelA knock down in ChIP experiments. RelA was not recovered from siRNA treated cells. Data shows mean values from QPCR in triplicate of a ChIP experiment from one donor. Black bars represent knockdown of RelA (siRNA) whereas red bars show scrambled control siRNA. ECR = evolutionarily conserved region; INT = intron; DS = downstream region.

3.3 Discussion

In this chapter, I have shown that although NFκB signalling plays a substantial role in the transcriptional regulation of FNGs in response to LPS, it is not indispensable. Whereas TNFα and A20 mRNA levels were significantly inhibited by MLN4924, The combination treatment had a cooperative effect on *DUSP1* transcript expression. This effect could be due to the association of additional immune-related transcription factors with cis-acting elements at the *DUSP1* locus that might become more readily available in the absence of NFκB. *DUSP-1* can be induced by a myriad of extracellular stimuli, including growth factors and serum (Keyse 2000; Noguchi et al. 1993; Li et al. 2001). Accordingly, the *DUSP1* promoter contains a large number of regulatory sequences and transcription factor binding motifs, including cMyc, cJun, cFos, Elk1 and ATF2 (Brunet et al. 1999; Davis 1995; Treisman 1996). Equally, it is possible that a negative regulator of *Dusp1* gene expression is induced by LPS in a strongly NFκB dependent manner. Noticeably, the stimulatory effect of MLN is stronger after 1 hour of incubation, which might allude to de novo protein synthesis of a DUSP1 inhibitor. It should be remarked, that MLN might have off-target effects that are not yet understood.

Despite previous studies highlighting the importance of NFκB in the transcriptional up-regulation of *Zfp36*, the inhibitory effect of MLN on LPS-induced *Zfp36* mRNA expression was underwhelming in WT macrophages. Furthermore, in *Dusp1*-/- cells the inhibitory effect of MLN was lost entirely. These data indicate that additional transcription factors are involved in the control of *Zfp36* up-regulation in response to pro-inflammatory stimuli and that prolonged MAPK activation may enhance the effect of trans-acting factors other than NFκB.

Previously, κB sites have been identified 5' of *Zfp36* in the region encapsulated by ECR4 and additionally within the *Zfp36* intron. Concomitantly, the murine ECR4 was the only element to show sensitivity to inhibition by MLN, whereas MmECR3-4, which contains the same sequence juxtaposed with MmECR3 was unresponsive to MLN. It would appear that the functional sequences within MmECR3 are able to overcome inhibition of MmECR4 by MLN. However, HsECR3-4 was significantly inhibited by MLN, therefore it is evident that there are differences in the regulation of *Zfp36* functional elements between the two species. These differences may be responsible for the transcriptional dependency on NFκB in humans that is not observed in murine macrophages treated with LPS and MLN. Indeed, *Zfp36* mRNA levels were inhibited to a higher degree in human macrophages when compared to murine cells.

The use of chromatin immunoprecipitation revealed a number of previously unidentified RelA - DNA associations. Aside from the recognised κB motifs at loci corresponding to primer pairs 12-13 (ECR4), 14 (promoter region) and 15-16 (Intron), enriched DNA was recovered using primer pairs 2-3 (ECR1), 7-8 (ECR2), 21-22 and 26 (ECR5). According to online databases, conserved regions 1 and 5 each contain imperfect κB consensus sequences and binding motifs for PU.1 – a myeloid marker of accessible chromatin domains. LPS induced enhancers are often bound to PU.1 and exhibit mono methylation of H3 lysine 4 (HSK4me1), which facilitates local histone depletion (Heintzman et al., 2007; Smale 2010). These two regions at either end of the *Zfp36* locus are therefore susceptible to enhancer activation and recruitment of inducible transcription factors including NFκB and, amongst others the IRF family of transcription activators following LPS stimulation. Subsequent binding of transcription co-activators and p300/ CBP protein complexes then allows

communication with promoter elements through looping and therefore enhances the frequency and efficiency of transcriptional elongation and transcript processing (Carey & Gentleman 2009; Medzhitov & Horng 2009; Smale 2010; Ghisletti et al. 2010; De Santa et al. 2010).

Often the presence or absence of a transcription factor cognate motif is neither sufficient nor necessary to configure binding or indeed activate, or repress transcription. Under varied conditions, sequences of DNA may be differentially responsive to a number of factors depending on protein associations induced by alternative stimuli, in different cell types, tissues and time frames depending on the milieu and absent or available effectors. Therefore most DNA sequences and putative binding motifs are context dependent and although well conserved, may not exhibit the expected consensus sequence. For example differential phosphorylation of p65 (RelA) is relevant to the capacity of NFκB to induce, or inhibit, transcription (Huang et al. 2011; Hochrainer et al. 2013). Post-translational modification of any of the 12 phosphorylation sites on RelA can enhance associations with cAMP-response element binding protein (Ser246/311) (Duran et al. 2003; Zhong et al. 1998)), HDAC1 (Thr435/505, Ser547) (O'Shea & Perkins 2010; Rocha et al. 2005) and alter associations with basal components of transcription machinery (Ser529/538) (Buss et al. 2004; Wang et al. 2000). These interactions influence NFκB binding or tethering to sequences of DNA that may or may not encode a recognised κB site therefore adding to the specificity of transcriptional control. Of course, DNA looping to achieve promoter-enhancer juxtaposition could also account for apparent RelA associations with distal loci.

It is clear that other factors have the ability to bind the *Zfp36* locus and induce transcription in response to LPS and other immune-related agonists. In fact a number of transcription

factor binding sites have been identified by computer analysis in mouse (DuBois et al. 1990; Lai et al. 1995), human (Heximer & Forsdyke 1993; Lai et al. 1995; Smoak & Cidlowski 2006), rat (Kaneda et al. 2000) and bovine (Lai et al. 1995) promoter and intron sequences (Lai 1998). Consensus sequences of AP2, SP1 and TPE1 have been identified directly 5' of the Zfp36 transcription start site as well as in the intron and have a confirmed role in serum inducibility of the TTP transcript. IL4 has been shown to stimulate TTP expression through signal transducer and activator of transcription 6 (STAT6) in mice (Suzuki et al. 2003) whereas TGFβ has been shown to up-regulate TTP expression through smad3 and smad4 (Ogawa et al. 2003) located in the promoter region (Smoak & Cidlowski 2006). A glucocorticoid-like response element has also been suggested to reside in the mouse TTP promoter (DuBois et al. 1990) as well as a hexameric glucocorticoid response element sequence in the human TTP 5' flanking region (Smoak & Cidlowski 2006). A functional GAS element that binds STAT1 has also been reported to mediate increases in TTP expression with interferon and p38 co-stimulatory stress signals (I Sauer et al. 2006). Figure 3.15, below, is a representation of current knowledge pertaining to the binding sites for numerous transcription factors which have been investigated as putative up-regulators of *Zfp36*.

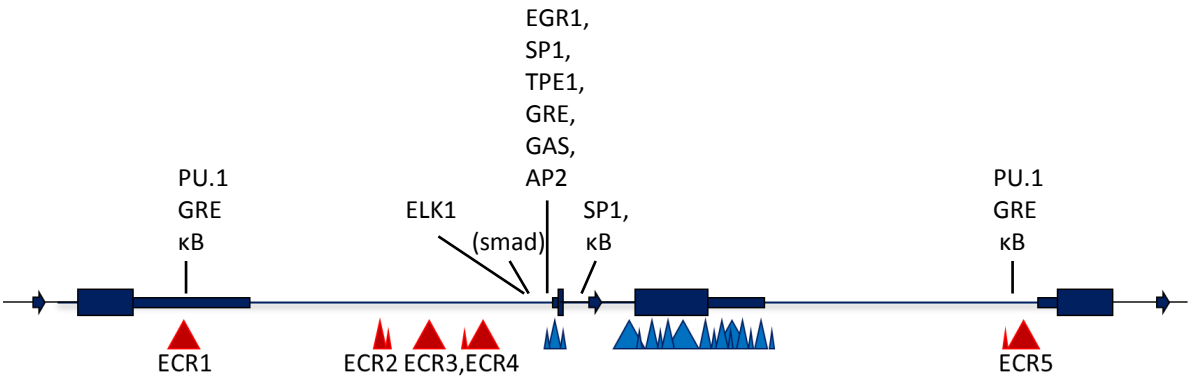


Figure 3.15 *Zfp36* locus transcription factor binding motifs

The *Zfp36* locus is small and compacted. However numerous agents have been suggested to induce *Zfp36* transcription. The above schematic illustrates our current knowledge of functional transcription factor binding motifs within the *Zfp36* locus. Also depicted are the evolutionary conserved regions discussed and used throughout this study, in relation to transcription factor binding sequences. ECR1 and ECR5 map directly onto two regions of significant DNAse hypersensitivity and are associated with PU.1, a transcriptional activator associated with macrophage activation and typically marks sites of accessible chromatin i.e. rapid response genes in macrophages.

4.0 THE ANTI-INFLAMMATORY EFFECTS OF DEXAMETHASONE SELECTIVELY SPARE FEEDBACK NODE GENE EXPRESSION

4.1 Introduction

Glucocoricoids represent the most effective anti-inflammatory treatment for chronic inflammatory diseases (Newton 2013). However the mechanisms by which glucocorticoids mediate their anti-inflammatory actions remain incompletely understood. A number of studies focused on identifying these mechanisms have sometimes uncovered contradictory findings and accommodated much debated schools of thought.

The feedback node genes *DUSP1*, *ZFP36* and *TNFAIP3* are induced by pro-inflammatory stimuli in macrophages and act negatively on their respective pathways of activation. Prior to this study, Lasa et al (Lasa et al. 2002) identified the cooperative up-regulation of *DUSP1* mRNA in HeLa cells co-stimulated with the pro-inflammatory cytokine IL1α and the anti-inflammatory synthetic glucocorticoid dexamethasone (DEX). Further to this, studies in *Dusp1-/-* mice revealed that the expression of *Dusp1* contributes to the anti-inflammatory effects of glucocorticoids (Abraham et al. 2006).

Tristetraprolin is a negative feedback regulator downstream of the MAPK p38 pathway and surprisingly, is also up-regulated by DEX treatment in A549 human alveolar epithelial cells (Smoak & Cidlowski 2006). This finding was contradictory to that of Jalonen et al. (2005), who showed that DEX inhibited tristetraprolin both at the level of mRNA and protein in a macrophage-like cell line. A handful of other groups have also highlighted inconsistencies regarding the effects of glucocorticoid on the regulation of *Zfp36*.

For example, when administered alone DEX was shown to induce TTP in A549 cells (King, Kaur, et al. 2009). However, in the same study and contradictory to Smoak & Cidlowski (2006), DEX inhibited TTP induction by the inflammatory cytokine IL1β (King, Kaur, et al. 2009). More recently, Brahma et al. (2012) revealed that glucocorticoid strongly induced TTP in adipocytes both alone and in combination with an inflammatory stimuli. The observed inconsistencies might be explained by the differential regulation of TTP according to cell type, stimulus and species.

With a strong transcriptional dependency on NFκB (Chapter 3) and a powerful ability to inhibit canonical NFκB signalling, A20 is a classic negative feedback regulator. However, only recently it has been reported that NFκB-mediated gene transcription at this locus may escape inhibition by GR (Altonsy et al. 2014).

The aim of this research chapter was to characterise FNG expression in response to the glucocorticoid dexamethasone in both resting and activated cells. In addition and drawing the focus to regulation of TTP expression, I investigated transcriptionally active sequences of the *Zfp36* locus and their response to stimulation with DEX and LPS.

4.2 Results

4.2.1 Dexamethasone inhibits pro-inflammatory gene mRNA production in macrophages

Initially, the kinetics and magnitude of dexamethasone's effect on the inflammatory mediators *TNFα*, *IL1α* and *IL6* was measured by QPCR in primary human monocyte and murine bone marrow derived and RAW264.7 macrophages. Figure 4.1 shows that *TNFα* and *IL1α* primary response genes (PRG) were rapidly and transiently induced upon stimulation with LPS. Forming part of the secondary response, *IL6* mRNA levels were up-regulated at the 2 hour time point and increased until 8 hours in primary macrophages, whereas expression was extended in RAW264.7 macrophage-like cell line. In resting macrophages, DEX alone had a small inhibitory effect on the expression of each pro-inflammatory mRNA. Dexamethasone was able to significantly inhibit LPS-induced expression of all pro-inflammatory mRNAs measured and at each time point as previously reported (Abraham et al. 2006). Glucocorticoids employ diverse inhibitory mechanisms and have transcriptional and posttranscriptional effects on gene expression. Thus GCs facilitate efficient down-regulation of PRGs and SRGs alike. Early inhibition of mRNA expression is often indicative of transcription inhibition, whereas late inhibition may signify post-transcriptional mechanisms of down-regulation (Hargreaves et al. 2009).

These data are consistent with similar studies and verify the anti-inflammatory outcome of dexamethasone treatment in these cell types.

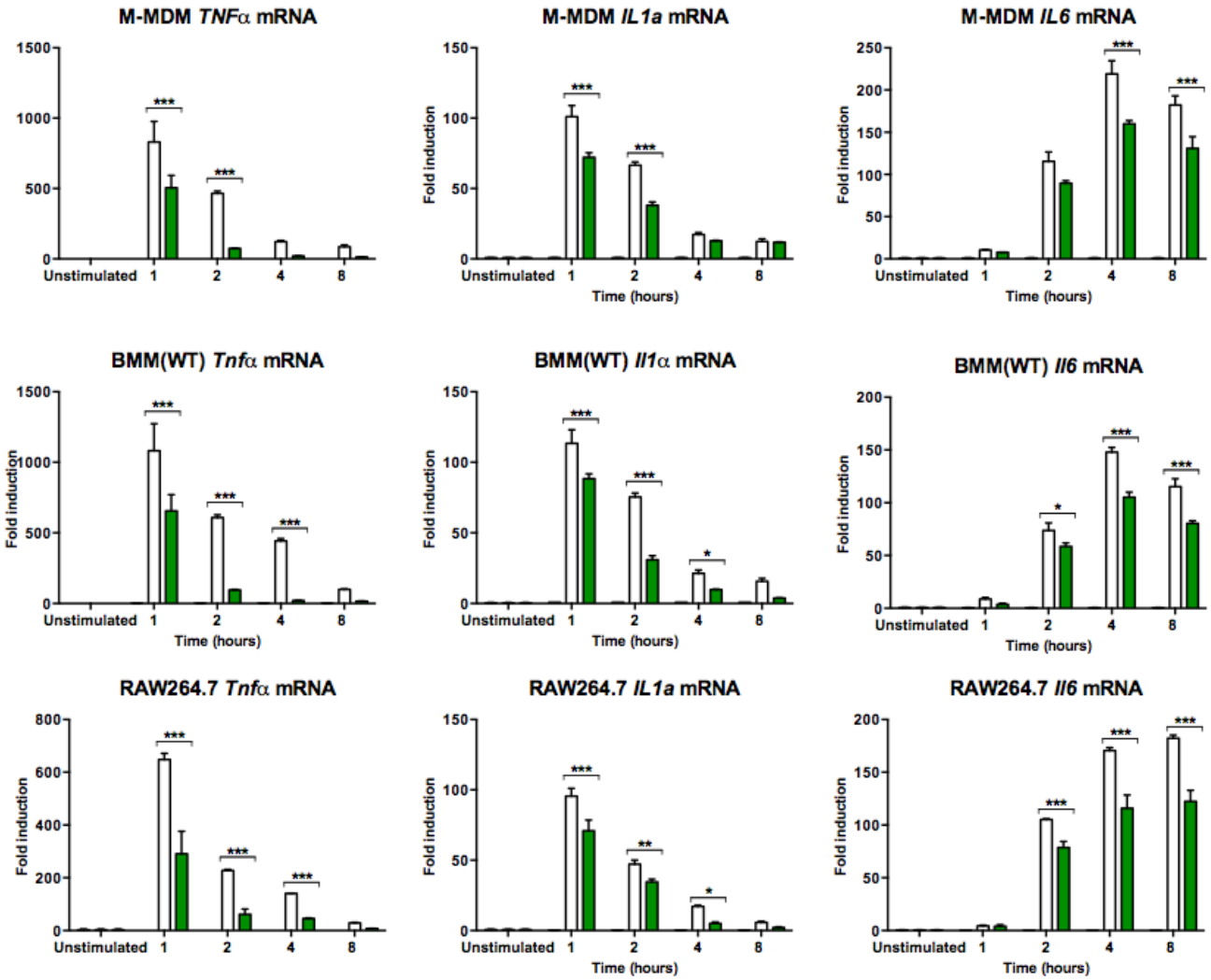


Figure 4.1 Dexamethasone inhibits mRNA expression of several pro-inflammatory genes in macrophages

Primary human and mouse macrophages and RAW264.6 macrophage-like cells were left untreated, stimulated with LPS (10ng/ml) (black bars), dexamethasone (100nM) (white bars) or LPS + DEX in combination (green bars) for 0, 1, 2, 4 and 8 hours. Total RNA was isolated and used as a template for cDNA synthesis and quantitative PCR of *TNFα*, *IL1α* and *IL6* gene expression, performed in triplicate. Mean values are shown with standard error and are representative of three separate experiments. Statistical significance was assessed using two-way ANOVA, *** ($p < 0.001$).

4.2.2 Dexamethasone and LPS cooperatively regulate *Dusp1* mRNA production

Consistent with studies previously conducted, the ability of dexamethasone to induce *DUSP1* in HeLa and A549 cell lines (Joanny et al. 2012; Lasa et al. 2002) was preserved in primary human and mouse macrophages and RAW264.7 macrophage-like cell line as well as HeLa and A549 epithelial cell lines.

Dexamethasone treatment alone was able to induce *DUSP1* at the level of mRNA in human epithelial cell lines approximately 5 fold, over an incubation period of 1 to 8 hours (Figure 4.2). This effect was stronger and more transient in the myeloid lineages; with an 8-fold induction at 1 hour in both human and mouse primary macrophages and 5 fold at 1 hour in the RAW264.7 macrophage cell line. Further to this, co-stimulation with biologically effective doses of LPS (10ng/ml) and DEX (100nM) significantly enhanced and extended *Dusp1* mRNA expression in macrophages. The synergistic induction was twice the magnitude of LPS alone and an effect more than additive of each agonist individually. A similar expression pattern was observed under co-treatment with IL-1α (1ng/ml) and DEX in the epithelial cell lines however the cooperative effect was not striking and may reflect the additive effect of IL-1α and DEX (Figure 4.2).

The up-regulation of *Dusp1/DUSP1* by glucocorticoids has previously been recognised (Abraham et al. 2006; Johansson-Haque et al. 2008; Tchen et al. 2010; Shipp et al. 2010; Joanny et al. 2012). Additionally, studies have shown that the anti-inflammatory potencies of glucocorticoids are partly dependent on the expression of *Dusp1* (Abraham et al. 2006; Lasa et al. 2002; Weinstein et al. 2011). Here, we observed the cooperative up-regulation of *Dusp1* by pro-inflammatory stimuli and the anti-inflammatory synthetic glucocorticoid DEX. The *Dusp1* locus contains a number of NFκB-like binding motifs as well as GR binding

sequences. Proximal GR association is generally considered to inhibit NFκB driven transcription through protein-protein interaction. However the expression of *Dusp1* in response to pro-inflammatory stimuli not only escaped GR-mediated inhibition but was significantly enhanced and extended by DEX treatment.

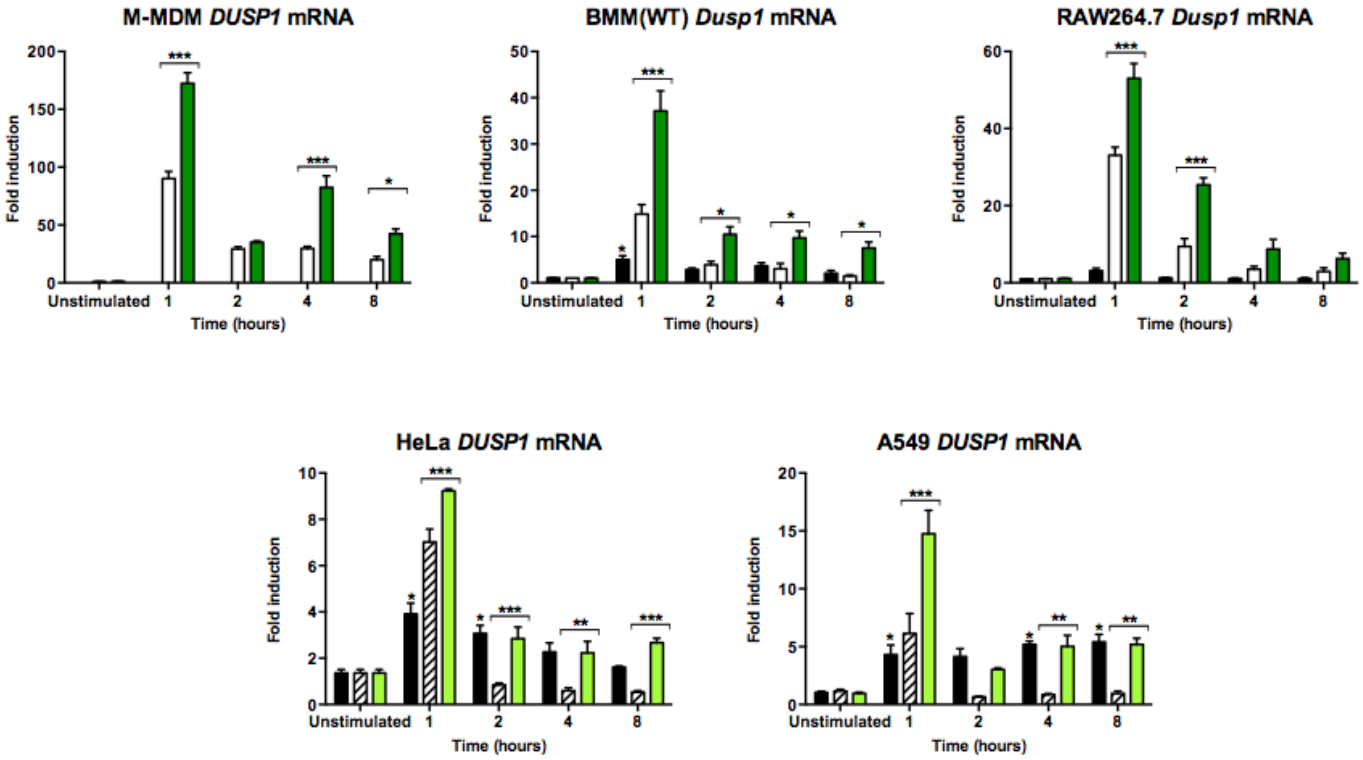


Figure 4.2 Dexamethasone and LPS cooperatively regulate *DUSP1* mRNA production.

Cells were left untreated, stimulated with LPS 10ng/ml (white bars) or IL1 1ng/ml (Striped bars), dexamethasone 100nM (black bars) or LPS/IL1 + DEX (dark/light green bars) in combination for 0, 1, 2, 4 and 8 hours. Total RNA was isolated and used as a template for cDNA synthesis and real time QPCR gene expression assay for *DUSP1*, performed in triplicate. Data shows mean values ± SEM. Significance was assessed with Two-way ANOVA. Top and bottom level statistical marks indicate significance between LPS/LPS+DEX and DEX/LPS respectively *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).

4.2.3 Dexamethasone and LPS cooperatively regulate *Zfp36* mRNA and protein expression

Regulation of gene transcription by glucocorticoids is a key but non-exclusive mechanism of their anti-inflammatory action. Indeed, glucocorticoids modulate gene expression by multiple mechanisms and a large proportion of glucocorticoid target genes are post-transcriptionally regulated. Transcriptional mechanisms are intricately coupled to posttranscriptional modification of gene expression at the level of mRNA transport, decay and translation and therefore a critical influence on the timing and magnitude of cellular responses (Brook et al. 2000; Stellato 2004). Posttranscriptional control of gene expression in inflammation is central to maintaining rapid protein output from primary response genes. Glucocorticoids have extensively been shown to act on tyrosine kinase pathways, which are key immunomodulatory 'switches'. The ERK pathway is strongly inhibited by glucocorticoid up-regulation of DUSP1 in murine mast cells and human osteoblasts (Kassel et al. 2001; Engelbrecht et al. 2003). In addition, glucocorticoid induced leucine zipper (GILZ) blocks phosphorylation of Raf1 and subsequent activation of MKK1/2 and ERK in T-cells (Ayroldi et al. 2002) but not in macrophages (Fernandes et al. 1999). In macrophages GCs are documented to inhibit JNK and, perhaps most relevant to posttranscriptional regulation in macrophages, MAPK p38 (Swantek et al. 1997; Kontoyiannis et al. 1999). Activated macrophages treated with inhibitors of p38 exhibit a sharp decrease in the mRNA of several genes including *IL1*, *VEGF*, *TNFα*, *IL6*, *IL8* and *COX2*; all of which are post-transcriptionally inhibited by glucocorticoids (Schwiebert et al. 1996; Amano et al. 1993; Tobler et al. 1992; Swantek et al. 1997; Lasa et al. 2001; Gille et al. 2001).

As aforementioned, the up-regulation of *DUSP1* by glucocorticoids and subsequent inhibition of MAPK signalling in macrophages may account for the mRNA destabilising effects of glucocorticoids. However this mechanism remains largely unclear.

Downstream of MAPK p38, TTP is inactivated by MK2-mediated phosphorylation of two specific and highly conserved serine residues. However *Zfp36* gene expression also lies downstream of inflammatory signalling and p38 MAPK -activation of numerous transcription factors including SP1, AP1, and NFκB (Chapter 3) to name just a few. Smoak and Cidlowski reported glucocorticoid-mediated up-regulation of tristetraprolin mRNA in the A549 human pulmonary alveolar cell line and in rat tissues, proposing a novel mechanism for glucocorticoid action. Our hypothesis was that, as with *Dusp1*, dexamethasone would 'superinduce' TTP mRNA when in combination with a pro-inflammatory stimulus; that is, LPS in myeloid cells and IL1α in epithelial cell lines.

Basally, TTP was expressed at very low levels, both in terms of mRNA and protein. In response to DEX treatment, *Zfp36* mRNA was transiently increased in the epithelial lineages (Figure 4.3). This effect was strongest in A549 cells, where we observed a 5 fold DEX-dependent increase in TTP RNA at the 1 hour time point, which had doubled to 10 fold by 4 hours of incubation preceding a decline after 8 hours. Gene induction in the HeLa cells followed a similar pattern and peaked with a 5-6 fold increase in *Zfp36* RNA expression at 4 hours post DEX treatment. However, DEX alone had very little effect on TTP mRNA levels in the myeloid cells.

Pro-inflammatory stimuli (LPS or IL-1α) alone induced *Zfp36* gene expression strongly and significantly in all cell types. At 1 hour in human cells, LPS-induced *Zfp36* was between 10

and 15 fold over basal levels. In murine cells, TTP induction was marginally stronger and between 20 to 30 times resting levels after 1 hour.

In combination with LPS, DEX was able to synergistically increase *Zfp36* mRNA synthesis to magnitudes that were significant at 1 and 4 hours in macrophages. Maximal induction of the *Zfp36* message with co-stimulation was up to 60 fold over basal levels in bone marrow derived primary murine macrophages. The murine derived myeloid cells showed the greatest synergistic effect where DEX treatment at least doubled the LPS induction of TTP mRNA at 1 hour and at 4 hours in RAW cells. After 4 hours, the effects of LPS or DEX treatment were little to non-existent in human macrophages. However simultaneous treatment increased *ZFP36* mRNA levels 5 fold more than LPS or DEX alone. The RNA expression profile of TTP at four hours of inflammatory stimulation is particularly interesting. The effects of DEX on LPS-induced *Zfp36* expression in the epithelial cells reflected a similar pattern to the myeloid cells at 1 and 2 hours in A549 cells and at 1 hour in HeLa cells. However it is less clear after the 1-hour mark whether some of the LPS plus DEX effects on *ZFP36* up-regulation are additive, rather than a result of the two agonists acting synergistically.

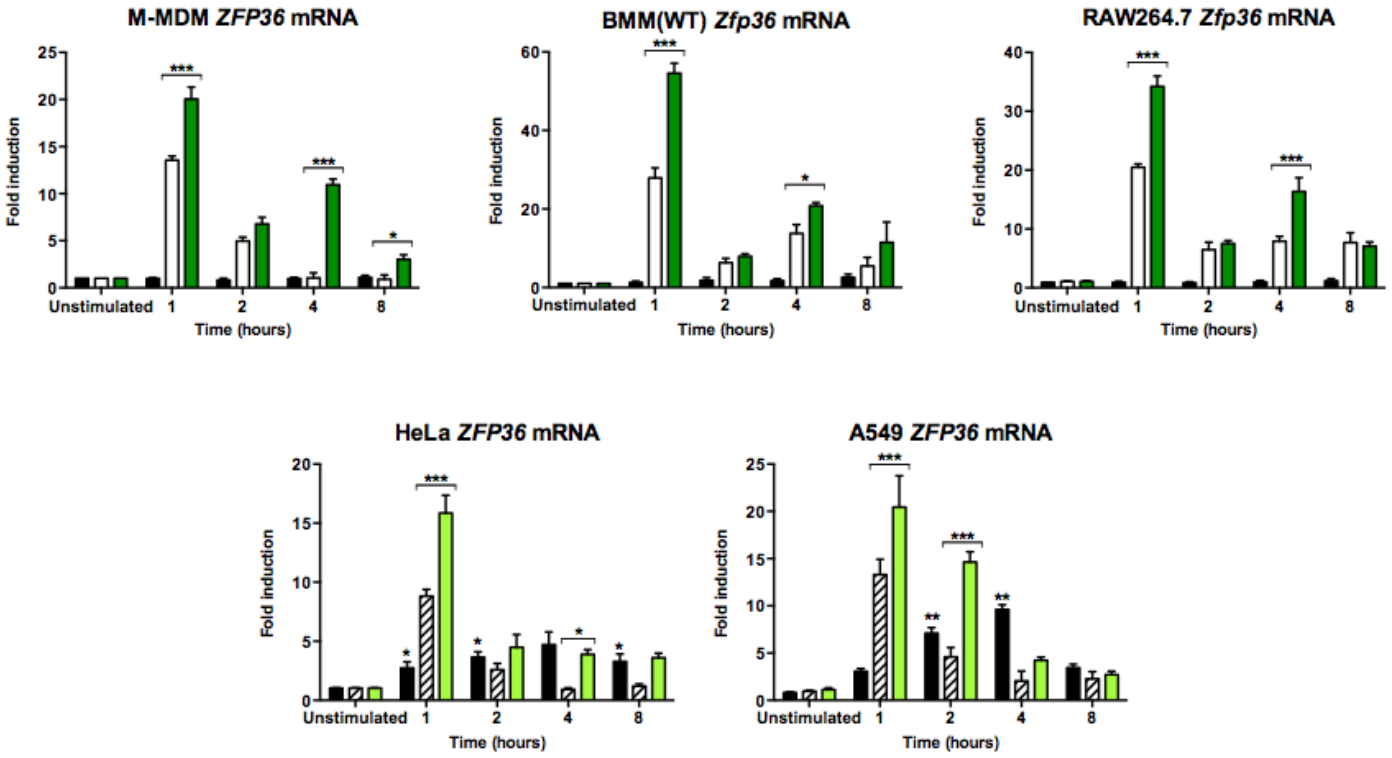


Figure 4.3 Dexamethasone and LPS cooperatively regulate *Zfp36* mRNA expression

Cells were left untreated, macropghages and epithelial cells were stimulated with LPS (10ng/ml) (white bars) or IL1 (white striped bars) respectively, dexamethasone (100nM) (black bars) or LPS/IL1 + DEX in combination (dark/light green bars) for 0, 1, 2, 4 and 8 hours. **(A)** Total RNA was isolated and used as a template for cDNA synthesis and RT PCR gene expression assay for *Zfp36*, performed in triplicate. Data shows mean values ± SEM of four separate experiments. Significance was assessed with Two-way ANOVA *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).

4.2.4 Dexamethasone and LPS cooperatively regulate *Tnfaip3* mRNA production

Another target of pro-inflammatory agonists, A20 (*TNFAIP3*) has recently been described to escape GR-mediated inhibition in airway epithelial cells co-stimulated with dexamethasone and TNFα (Altonsy et al. 2014; Rao et al. 2011). In this study, we showed the same effect in the A549 pulmonary alveolar cell line and primary human and murine macrophages co-treated with an inflammatory stimulus plus DEX.

LPS induction of *Tnfaip3* was greatest in murine cells; transcripts were detected at levels 170 and 100 times that of resting level in BMM and RAW respectively. LPS induction of *TNFAIP3* was more modest in human cells with a 50 fold induction observed at 1 hour in human macrophages and an approximately 80 fold in human epithelial cells. Treatment with dexamethasone alone was able to induce *Tnfaip3* 2 fold in macrophages and up to 6 fold in human epithelial cell lines. One hour of co-treatment significantly and cooperatively augmented *Tnfaip3* gene induction in macrophages and A549 cells. DEX also cooperatively enhanced the LPS effect at 2 hours in RAW murine macrophage-like cells. However, thereafter DEX had no significant augmenting effect on LPS induced *Tnfaip3* expression. In fact at two hours post co-stimulation, DEX was significantly inhibitory to *Tnfaip3* transcription in HeLa cells and at 2, 4 and 8 hours in BMMs. A20 gene expression was rapidly induced by pro-inflammatory stimuli and was shown to be strongly dependent on NF-kB (Chapter 3). This response constitutes a negative feedback loop that helps to determine the kinetics of NF-kB activation. It is possible that dexamethasone inhibits A20 expression at late time points indirectly, by altering the characteristics of the NF-kB - A20 negative feedback loop.

However, we did not observe DEX inhibition of *TNFAIP3* in every cell type. Additionally, *TNFAIP3* is a PRG, therefore late-stage inhibition after de novo protein synthesis should not restrict the early function of A20. These data indicate that, in addition to *DUSP1* and *Zfp36*, *Tnfaip3* is a feedback node gene that mostly escapes GR-mediated inhibition of inflammatory signalling-responsive genes.

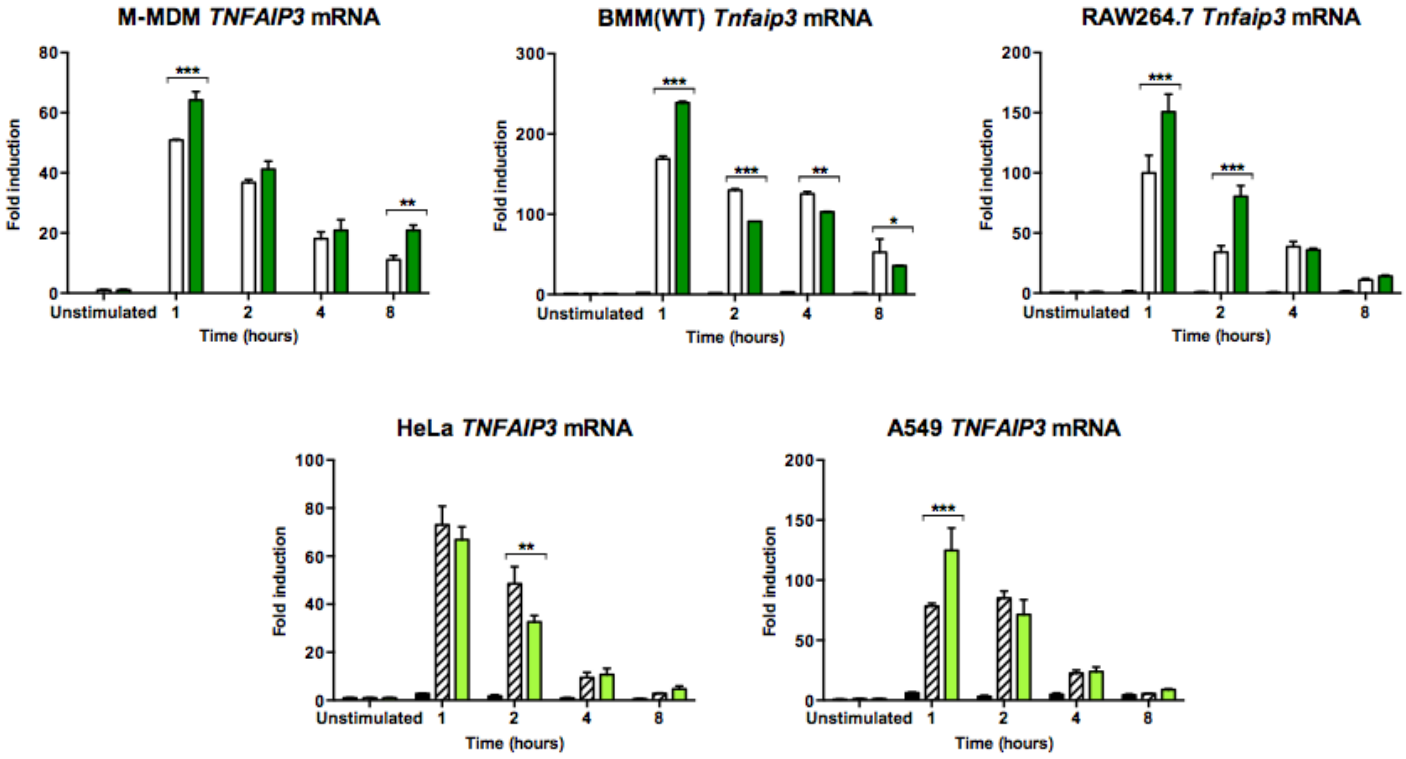


Figure 4.4 Dexamethasone and LPS cooperatively regulate *Tnfaip3* mRNA production

Cells were left untreated, macrophages and epithelial cells stimulated with LPS (10ng/ml) (white bars) or IL1 (1ng/ml) (white striped bars) respectively, dexamethasone (100nM) or LPS/IL1 + DEX in combination (dark/ light green bars) for 0, 1, 2, 4 and 8 hours. Total RNA was isolated and used as a template for cDNA synthesis and RT PCR gene expression assay for *Tnfaip3*, performed in triplicate. Data shows mean values ± SEM. Significance was assessed with Two-way ANOVA *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).

4.2.5 Cooperative up-regulation of *Zfp36* mRNA by LPS and DEX requires DUSP1

In order to highlight the anti-inflammatory effects of glucocorticoids, previously we used QPCR to quantify *Tnfα* mRNA under conditions of LPS, DEX or LPS+DEX. The down-regulation of numerous pro-inflammatory factors by glucocorticoids and their therapeutic potential is in part, dependent on the expression of DUSP1 (Abraham et al. 2006; Tchen et al. 2010; Joanny et al. 2012). Studies using *Dusp1-/-* mice or DUSP1 knock down have illustrated its importance in glucocorticoid-mediated transcriptional inhibition. Additionally, a number of pro-inflammatory genes up-regulated by p38-dependent mRNA stabilisation are inhibited by glucocorticoids via posttranscriptional mechanisms (Abraham et al. 2006). These mRNAs, including *Tnfα*, *Cox2*, *Il1α/β* and *Cxcl1* are encoded by labile transcripts and require continuous activation of the p38 pathway for expression (Abraham & Clark 2006).

Both MAPK p38 and downstream effector MK2 post-translationally modify and inactivate TTP, preventing destabilisation of pro-inflammatory transcripts (Marchese et al. 2010; Clement et al. 2011; Stoecklin et al. 2008; Ronkina et al. 2010; Tchen et al. 2004; Brook et al. 2006; Tudor et al. 2009). In *Dusp1-/-* cells p38 MAPK activity is significantly enhanced, meaning that TTP remains phosphorylated and inactive. Furthermore, p38 MAPK is insensitive to inhibition by DEX in *Dusp1-/-* cells suggesting that *Dusp1* plays a role in glucocorticoid anti-inflammatory function (Clark et al. 2003; Abraham et al. 2006; Abraham & Clark 2006). As we currently understand, one function of DUSP1 is to limit p38 signalling and release TTP from its inactive state.

In order to identify whether the cooperative regulation of TTP by pro-inflammatory stimuli and DEX is a mechanism dependent on DUSP1, experiments were carried out as previously and compared WT and *Dusp1-/-* BMM *Zfp36* gene expression in response to DEX.

In parallel to previous time courses, age- and sex- matched wild type (*Dusp1+/+*) and *Dusp1-/-* murine BMMs were stimulated over a period of 0-8 hours with either DEX, LPS or LPS+DEX. Cells were harvested for RNA and subject to QPCR.

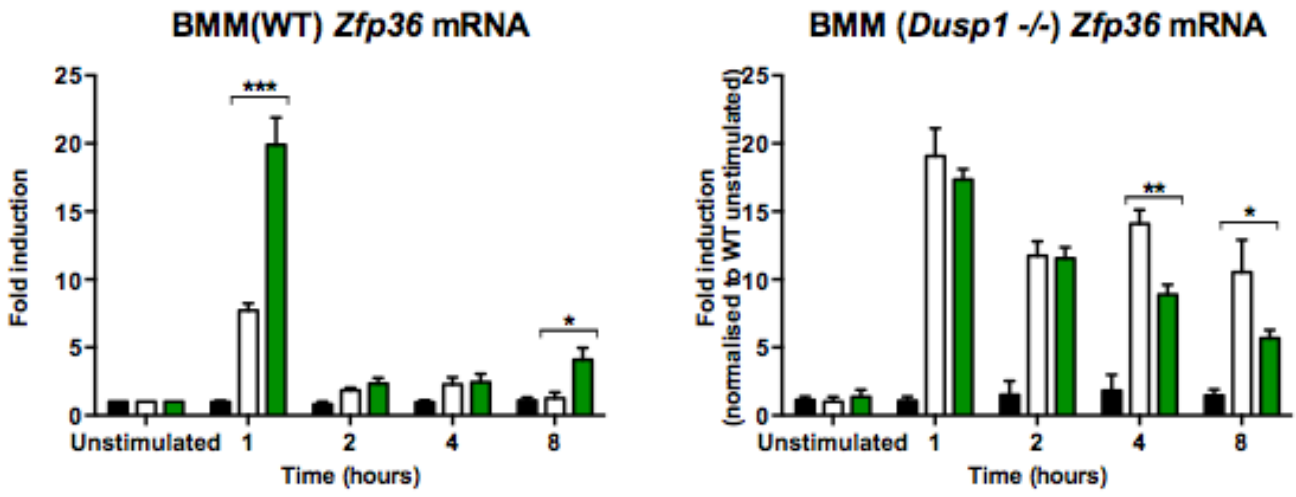


Figure 4.5 Synergistic induction of *Zfp36* mRNA by LPS and dexamethasone requires DSUP1

Primary macrophages were derived from the bone marrow of wild type (WT) and *Mkp1-/-* mice and cultured in GM-CSF for 5 days before seeding at a density of 10^6 cells/ ml in 12 well plates. Cells were left untreated, stimulated with LPS (10ng/ml), dexamethasone (100nM) or LPS + DEX in combination for 0, 1, 2, 4 and 8 hours. Total RNA was isolated and used as a template for cDNA synthesis and quantitative PCR of *Zfp36* gene expression, performed in triplicate. Data shows mean values of three independent experiments for each WT and Mkp1-/- primary macrophages ± SEM. Significance was assessed with Two-way ANOVA *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$).

The mechanisms dictating the normal expression of TTP are numerous, interrelated and therefore complex. Firstly, TTP binds to its own 3'UTR and subsequently regulates its own expression by mRNA destabilisation which couples its expression further to the activity of MAPK p38. Therefore, the initial gene induction of *Zfp36* requires NFκB-mediated transcriptional activation *and* p38 mediated mRNA stabilisation. Hence in wild type cells, as p38 activity declines TTP becomes active and destabilises its own mRNA accounting for the transient expression of *Zfp36* observed in WT BMMs (Figure 4.6). Additionally, in WT cells, DEX cooperated with LPS to activate *Zfp36* transcription but also induced *Dusp1* gene expression to inactivate p38 and enforce the off phase of the TTP. This effect is illustrated by a strong early peak of expression at 1 hour followed by a rapid decline thereafter.

In *Dusp1-/-* macrophages the initial transcriptional activation of *Zfp36* was enhanced by a minimum of two fold compared to WT, however DEX was unable to increase this expression further (Figure 4.6). In addition, the sustained elevation of LPS mediated *Zfp36* transcript expression in *Dusp1-/-* illustrates disruption to the off phase of TTP expression.

Unpublished data from our group showed that prolonged activation of p38 in these cells means that *Zfp36* mRNA remains stable, rather than becoming unstable and therefore accumulates. Indeed, results from the Clark group (currently unpublished data) have shown that increased stability of a set of pro-inflammatory cytokines and chemokines due to disruption of MAPK signalling in Dusp1-/- macrophages is entirely dependent on TTP phosphorylation.

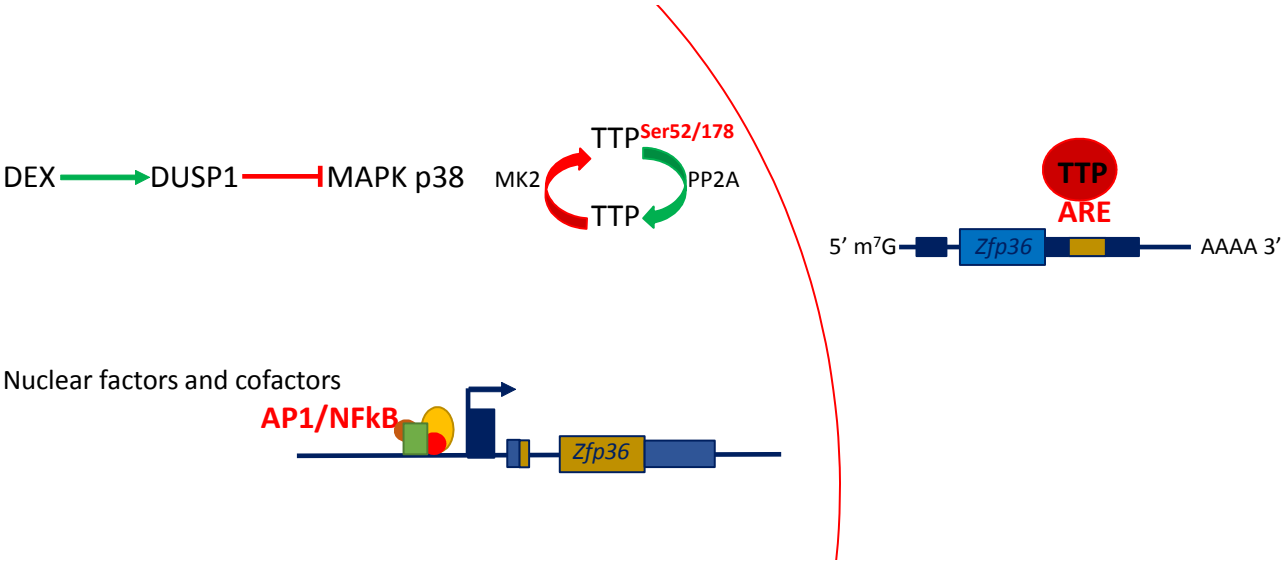


Figure 4.6 Complex mechanisms dictating expression of TTP

The inactivation of TTP by specific phosphorylation (Ser52/178 in mouse) is a downstream mechanism of p38 and MK2 (A), reversible by PP2A and the removal of p38 signalling. When active, TTP binds to AREs in its own 3'UTR (B) and promotes mRNA decay, furthermore coupling its expression to the actions of p38 MAPK. Transcriptionally, *Zfp36* is under the control of numerous transcription factors including NFκB (C). Therefore activation of both NFκB and p38 MAPK is required to raise the strong initial phase of TTP synthesis. DUSP1 blocks the p38 pathway and therefore facilitates not only TTP activation but also instability. DEX augments the effects of DUSP1 and has transcriptional effects on *Zfp36*, yet in doing so increases TTP protein and mRNA instability.

4.2.6 Synergistic regulation of Zfp36 reporter constructs by pro-inflammatory stimuli and dexamethasone LPS and dexamethasone

Using the reporter constructs detailed in Chapter 3, our aim was to identify the potential of DEX to regulate transcription via functional ECRs 2, 3 and 4.

HeLa cells were transfected with the appropriate experimental constructs and correspondingly with (κB)3- or pGL3P (empty)- luciferase vectors. Containing six tandem NFκB binding motifs, the (κB)3- containing luciferase reporter acts as a control ensuring inflammatory signalling remains in-tact in the transfected cells. Additionally, transfection of the rudimentary pGL3P vector is a control for potential background transcription of luciferase via the minimal promoter sequence. As previously described in Chapter 3, all of the constructs mounted a transcriptional response to stimulation with IL1α in HeLa cells (Figure 4.6). DEX alone increased luciferase activity in cells transfected with the HsECR3-4 expression vector but had no effect on transcription of the other constructs. The combination of IL1α and DEX augmented the expression of luciferase in HsECR3 and HsECR3-4 containing cells in a cooperative manner. On the other hand however, the transcriptional response of HsECR4 to IL1α was inhibited by treatment with DEX.

Similarly, RAW264.7 cells were transfected with the appropriate murine sequence (Mm) ECRs; 2; 3; 4; and 3-4. In this case the positive control expression vector contained a Tnfα 5' (TNF5') sequence. In response to LPS, all of the constructs were able to induce transcription and increased luciferase activity. Comparable to experiments in HeLa cells, DEX alone induced transcriptional activity of MmECR3-4 and additionally MmECR2 in RAW cells. Whereas co-stimulation enhanced the luciferase response additively in MmECR2, MmECR3-4 responded synergistically to DEX and LPS. In contrast to the HsECR3, the murine sequence

showed inhibited LPS-induced transcriptional activity when stimulated with DEX in combination, as did MmECR4.

In summary, we observed a number of consistencies between species and cell type. The ECR4 responded negatively to DEX, whereas ECR3-4 responded positively to DEX treatment and ECR3 showed relatively little response to DEX which was slightly positive in HeLa or negative RAW cells and therefore might be dependent on species or cell type. Sequence similarity between human and mouse within these regions is in the region of 70-79%. In both mammals, the ECR4 contains four overlapping sequences akin to κB-like binding motifs, whereas ECR3 contains one such motif and a putative GR sequence and ECR2 contains a GR motif. These data would suggest that cooperative regulation of transcription at the TTP locus requires the proximal binding of GR and NFκB in a way that does not facilitate transrepression. That is, NFkB sequences in isolation are subject to inhibition by GR, presumably through transrepression. However a number of recent studies have highlighted the complexity of GR and NFκB cross-talk (Joanny et al. 2012; Lannan et al. 2012; King et al. 2013) and when found in proximity, these two nuclear factors have been shown to work in concert to multiplicatively augment gene expression (Rao et al. 2011; Uhlenhaut et al. 2013). Coactivation of GR and NFκB has been the topic of recent studies exploring altered binding site repertoires in response to treatment with either agonist or both. Indeed crosstalk between glucocorticoids and pro-inflammatory stimuli may result in the loss or gain of GR-binding sites and their association with p65 (NFκB) in a mutually dependent manner (Rao et al. 2011). Furthermore we have identified these ECRs as putative GR, as well as NFκB binding sequences.

Here we have used transient transfection of luciferase reporter constructs as a tool to identify sequences responsive to GR, NFκB and the two in combination. However, it must be remarked that the actions of both GR and NFκB depended on epigenetic regulation, chromatin context, the recruitment of cofactors and associations with distal and proximal DNA sequence; none of which can be accounted for in this system (Uhlenhaut et al. 2013; Rao et al. 2011).

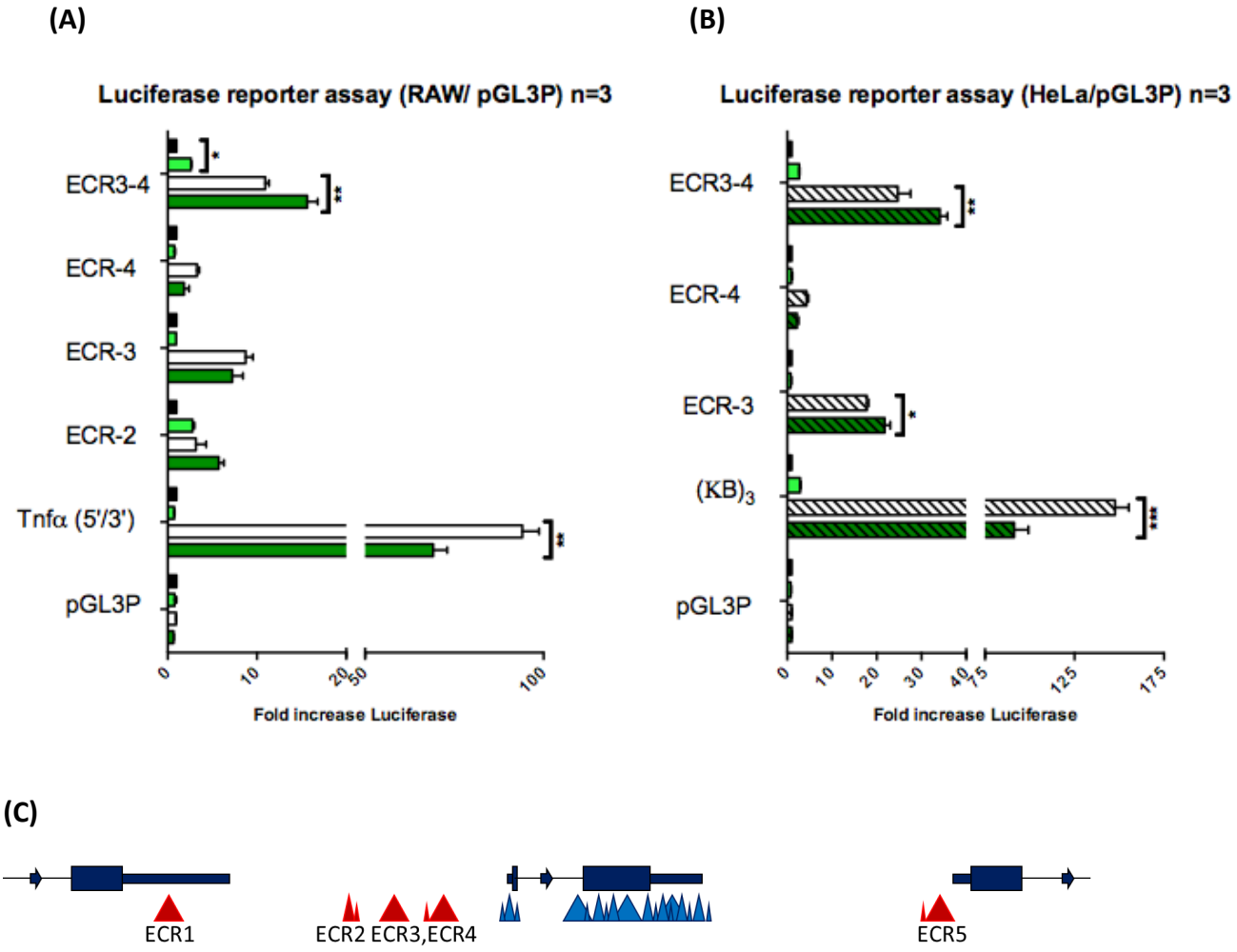


Figure 4.7 Dexamethasone and LPS co-induction of a luciferase reporter construct containing evolutionarily conserved regions of the Murine and Human *Zfp36* locus

(A) RAW264.7 and **(B)** HeLa cells were transiently transfected with luciferase reporter constructs. The following day cells were left unstimulated, activated with (A) LPS 10ng/ml (white bars) or (B) IL1α 1ng/ml (striped bars), dexamethasone 100nM (black bars) or mutually (dark green = LPS+dex striped green = IL1+dex) for an optimum of 6 hours. Following passive lysis, luciferase assays were performed. Data are expressed as mean ± SEM for four and three separate experiments in RAW264.6 and HeLa cells respectively.

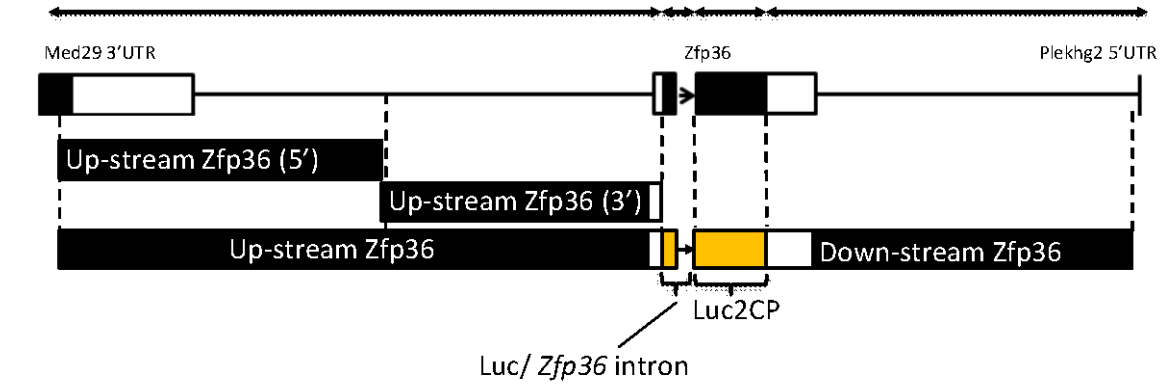
4.2.7 Dexamethasone has no significant effect on LPS-induction of a *Zfp36* mini-gene reporter.

In general, genes that are responsive to a wide variety of different agonists often have multiple, discrete and independent enhancers dispersed over large distances in the genome. Examples are *Pepck*, *Rank* and *Ifng*. However the *Zfp36* locus is relatively small, with only 6 and 5 kb separating it from its nearest 5' and 3' neighbours (*Med29* and *Plekhg2*, respectively). The whole mouse *Zfp36* locus occupies only 13.6 kb. The hypothesis states that important cis-acting elements involved in coordinating the expression of *Zfp36* in response to multiple agonists are located and are spread out within this small region. In addition, it cannot be ruled out that significant regulatory elements are located within the *Med29* or *Plekhg2* genes, or even further away.

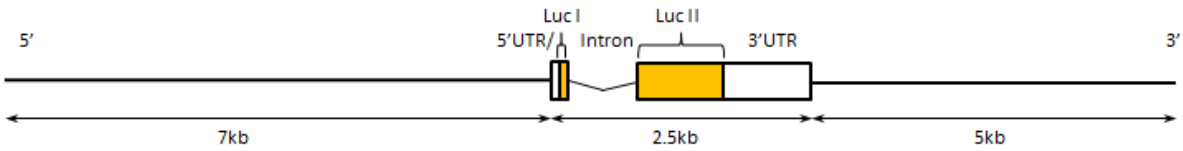
As a tool to investigate transcriptional responses of the *Zfp36* gene, we created a mimic-gene, based on the *Zfp36* locus but containing a luciferase cDNA in place of the two endogenous *Zfp36* exons. The construct contained the naturally occurring locus sequence from the 3' UTR of upstream Med29 (Chr7:28,378,549-28,393, 218) to the Zfp36 5'UTR, followed by a short 18bp LUC sequence, the endogenous *Zfp36* intron, the remainder of the LUC2CP sequence, *Zfp36* 3'UTR and downstream sequence leading up to the 5'UTR of Plekhg2 (Chr7:28,365,845-28,380,514). The interrupted Luciferase sequence was designed to splice exactly as the endogenous *Zfp36*. Following amplification of the appropriate sequences from murine genomic DNA using the primers described in Chapter 2.1.4, four cloning steps were required and are outlined in Figure 4.9. The final construct was transfected into RAW264.7 cells using the adapted protocol previously optimised for ECR-luciferase constructs and cells were stimulated with LPS, DEX or LPS+DEX for 6 hours.

Although luciferase was induced by LPS in RAW264.7 cells containing the mimic-gene, the results were not as expected. Most notably, the maximum quantified fluorescence following 6 hours of LPS stimulation was only induced 5 fold over the unstimulated control. This result was lower than that observed following LPS stimulation RAW264.7 containing ECR2, ECR3, ECR4 and ECR3-4, for which the relative luciferase were 5, 10, 5 and 12 fold the negative control respectively. Furthermore, endogenous *Zfp36* showed around 10-20 fold response to pro-inflammatory stimuli LPS and IL1. In order to determine whether an experimental error had occurred, the protocol was repeated. However, four independent experiments yielded the closely comparable results shown in Figure 4.9. The transcriptional response of the $(KB)_3$ construct following LPS stimulation was saturated, indicating that the unexpected result(s) were specific to the *Zfp-LUC* construct. Extensive diagnostic digestion of the *Zfp-LUC* reporter at various steps of construction revealed that the downstream region had, in the three way ligation step, become repeated and inverted. This significant rearrangement of regulatory sequences could potentially cause transcriptional interference, either by rendering the involved sequences inoperative or preventing the functionality of distally located motifs. Due to time constraints and given that this piece of work was late on in the project, we were unable to continue with this section of research, or indeed carry out any further diagnostic checks. For these reasons we could not confidently rely on the data produced from this part of the study.

(A)



(B)



(C)

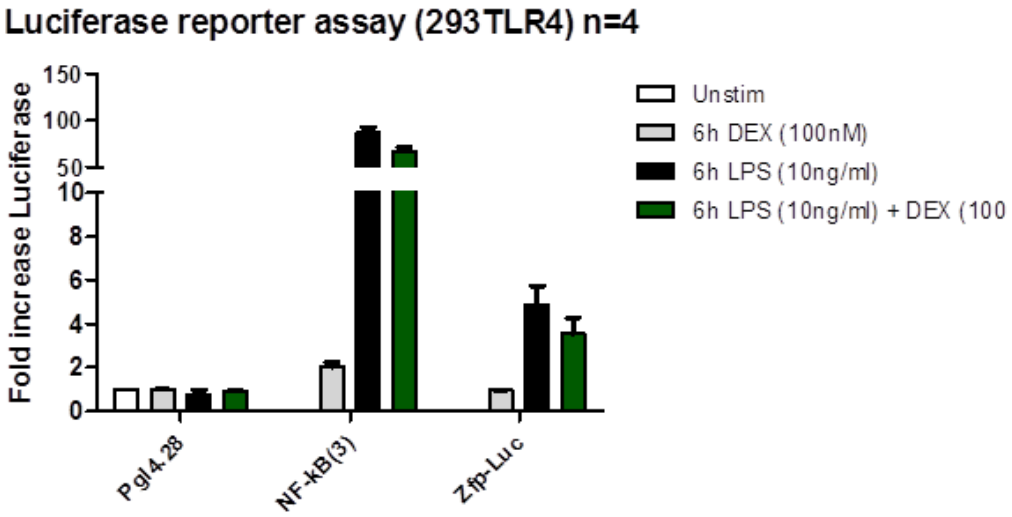


Figure 4.8 Effect of dexamethasone on LPS-induction of a *Zfp36* mini-gene luciferase reporter

(A) Schematic diagram of the *Zfp36* mini gene reporter construct (*Zfp-Luc*) comprising the entirety of the murine *Zfp36* locus (chr7:28371243 – 28386589) excluding the two exons, which were replaced by a Luc2CP luciferase sequence (pgl4.26), separated by the endogenous *Zfp36* intron at an appropriate splice motif. **(B)** *Zfp-Luc* mini-gene organisation. **(C)** Luciferase reporter assay. HEK293 human embryonic kidney epithelial cells transformed with pUNO-TLR4 and pDUO2-MD2-CD14 plasmids were transfected with *Zfp-Luc* and the following day stimulated with LPS (10ng/ml), DEX (100nM) or both for 6 hours. Following passive lysis, luciferase assays were performed. Data are expressed as mean ± SEM for four separate experiments.

4.2.8 Chromatin Immunoprecipitation (ChIP)

The effects of DEX on *Zfp36* transcription are most enhanced at the 1 hour time point, when maximum recruitment of RNAPII and RelA is observed (Chapter3.2.9-11). For this reason ChIP was carried out on cells that were left unstimulated or treated with DEX, LPS or LPS+DEX for 1 hour before fixation with formaldehyde. The optimisation of ChIP and the specifics of primer design are described in Chapter 3.2.8. The primers used for ChIP allow detection of transcription factor recruitment to within approximately 100-200bp of genomic DNA.

For each experiment an aliquot of chromatin was immunoprecipitated with an isotype control in order to account for background protein-DNA association. The values for protein enrichment were calculated by normalising the percent input values of experimental antibodies to that of the isotype control.

4.2.8.1 GR binds to discrete elements at the Zfp36 locus

Chromatin immunoprecipitation with glucocorticoid receptor was carried out in primary BMM treated with DEX (100nM), LPS (10ng/ml) or both for one hour (Figure 4.10). Included in the figure is a schematic corresponding to the relative location of the primers and *Zfp36* gene region.

In the absence of ligand, GR is found mostly cytoplasmic and bound to an inhibitory complex of chaperone proteins. Therefore in response to stimulation with LPS alone GR remains inactive in the cytoplasm and cannot interact with cognate DNA motifs. Nuclear GR has been observed at small concentrations in the absence of ligand in human monocyte derived primary macrophages; however the cytoplasmic-nuclear shift of GR is functional in response to DEX. Putative GR binding motifs are located distally upstream and downstream of *Zfp36*,

corresponding to primer pairs 2, 8 and 25 respectively. In this study, we showed that DEX facilitated GR binding to various sequences across the *Zfp36* locus in addition to the predicted sites. Of particular interest is the DEX-induced enrichment of GR observed at the upstream sites of evolutionary conservation previously investigated and analogous to sequences within primer amplicons 4, 6-7, and 10-12. Furthermore, GR also associated with the *Zfp36* intron (primer 15), which is an essential component of *Zfp36* transcription (Lai Blackshear 1998) and may have NFκB binding potential (Chen Chang 2013).

When stimulated with both agonists, GR binding was not only enhanced but shifted towards a more discrete pattern than observed in DEX-alone stimulated cells. Enrichment of GR was increased by LPS most significantly at MmECR2 (primers 6-7), a conserved region directly downstream of neighbouring gene Med29 (primer 4), MmECRs 3 and 4 and at the putative GR-motif associated primer pair 25. On the other hand LPS inhibited DEX-dependent enrichment of GR at numerous loci including at primers 8-9, 11, the intron region and the 3'UTR which corresponds to primers 16-17.

From these data, it is clear that GR motif classification and prediction alone is insufficient to determine regulatory binding. In the presence of DEX, GR was enriched for at the reputed upstream (primer pairs 2 & 8) and downstream (primer pair 25) sites. However this binding context was not optimally functional given that, according to the RNAPII readout, transcriptional elongation was enhanced by co-stimulation. The increased transcriptional elongation observed in co-treated macrophages was accompanied by a discrete re-distribution of GR to the putative enhancer regions previously described in this study. Furthermore and especially interesting are the sites of induced GR and NFκB co-occupancy and GR association with putative NFκB sites within the MmECR4 and intron.

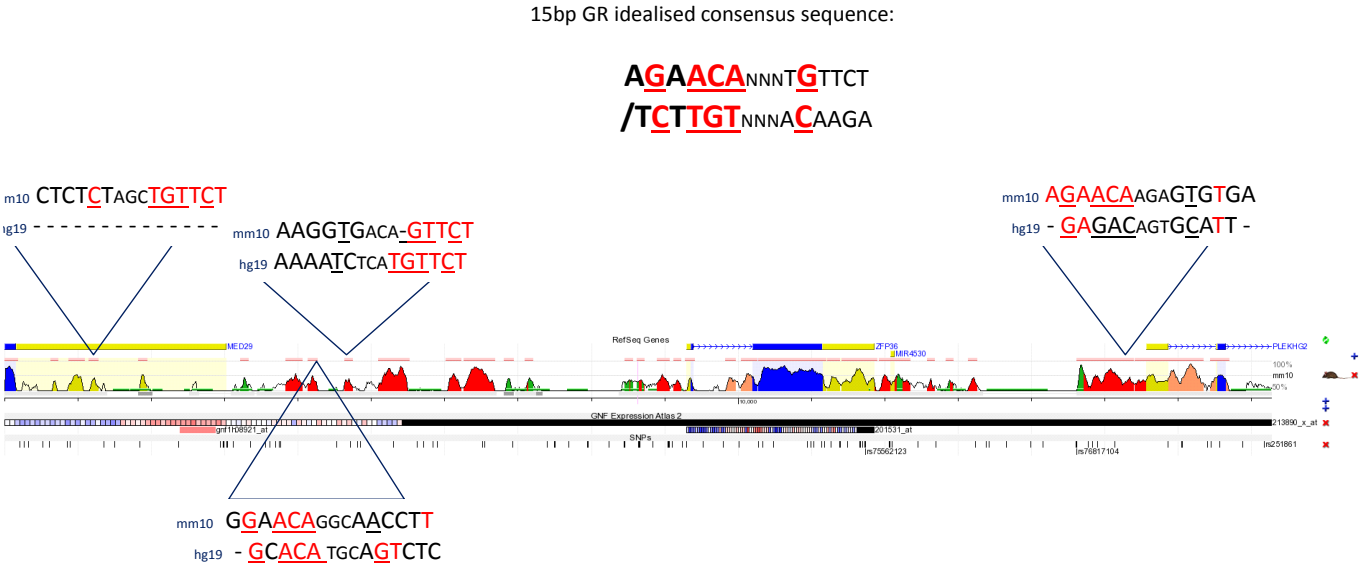
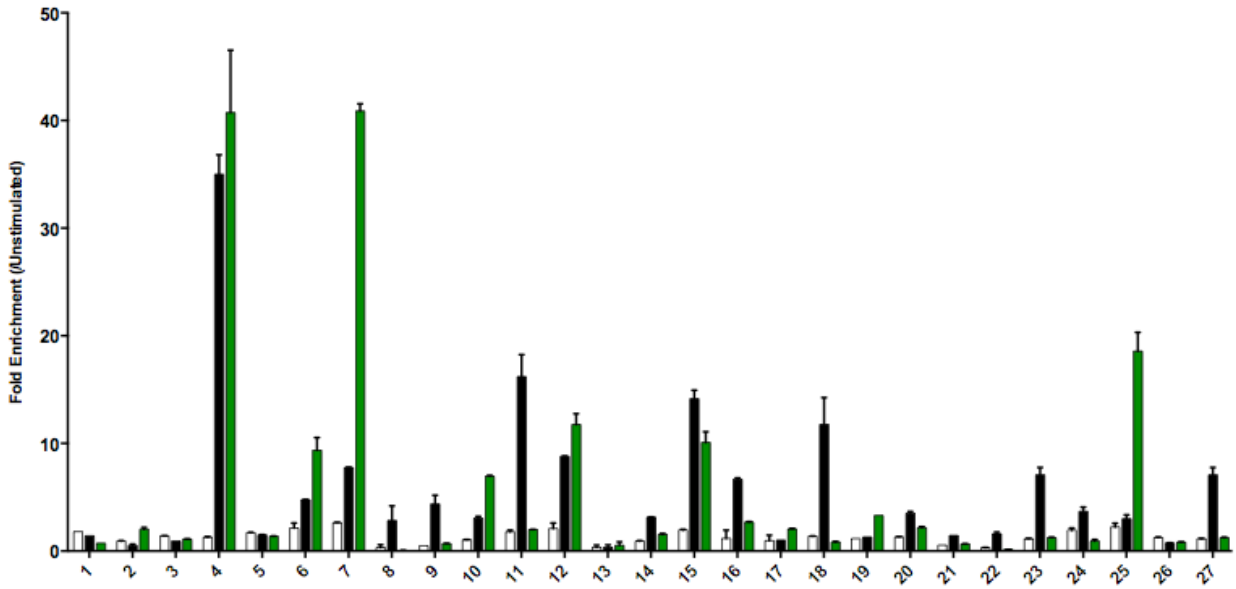


Figure 4.9 The GR consensus binding motif at the Zfp36 locus

Generally the GR binding motif exists as inverted repeats of the ½ site sequence AGAACA, with a separation of 3bp. However, in reality GR binding specificity is relaxed and only 5 bps (underlined) are consistently present at authentic binding sites with the idealised 15 bp consensus sequence AGAACANNNTGTTCT. On inspection of the Zfp36 locus there are 4 putative GR binding sites which imitate the consensus motif. The genome alignment above (taken from www.ecrbrowser.dcode.org) shows human *ZFP36* in parallel with the murine locus and highlights areas of evolutionary conservation between the two species. Text details the murine (mm10) and human (hg19) 'GR motif' aligned sequences (black text), 'essential' sites are underlined and consensus corresponding bps are in red.

(A)



(B)

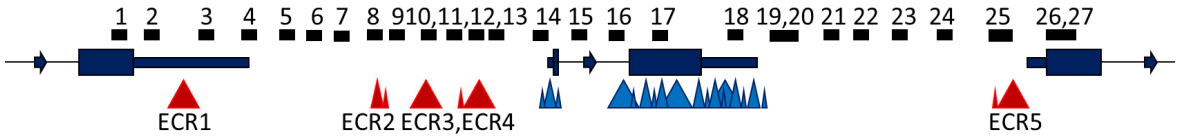


Figure 4.10 Cooperative transcription requires GR binding to discrete regions at the Zfp36 locus

(A) Cells were stimulated with LPS (white bars), DEX (black bars) or LPS + DEX (green bars) for 1 hour. After which ChIP was performed using antibodies to GR or an isotype control and primers spanning the entire locus of the murine Zfp36 gene. Data are represented as fold enrichment over the unstimulated control and represent the accumulation of GR antibody (y-axis) at each oligonucleotide (x-axis numbers) across the Zfp36 locus. Data points are expressed as the mean +/- SD of triplicate QPCR measurements and represent 3 similar separate experiments. **(B)** A schematic of the Zfp36 locus representing primer locations.

4.2.8.2 Dexamethasone alters the LPS-dependent RelA binding repertoire at the Zfp36 locus

Given the apparent mutual cooperativity of GR and NFκB, we considered the possibility that DEX exerts its synergistic effects on LPS-induced *Zfp36* transcription at the level of NFκB recruitment to target sites. We therefore used ChIP to investigate the recruitment of NFκB component RelA (p65) to the *Zfp36* locus in vivo upon stimulation with LPS, DEX or LPS+DEX. ChIP will provide insight into the mechanisms occurring in context of genome and chromatin and also generate information pertaining to multiple NFκB sites that are mutually responsive to GR.

To examine the effect of DEX on NFκB recruitment to the *Zfp36* locus, ChIP assays were performed as described in Chapter 3.2.11, using a RelA specific antibody and PCR primers designed across the *Zfp36* locus (Figure 4.11). Dexamethasone alone induced RelA binding adjacently upstream of and within ECR2; regions denoted by primer pairs 6-7 and 8-9 respectively. There are no identified NFκB predicted tracks within ECR2, there is however a consensus GR motif. What is remarkable is that this RelA association with the sequence determined by primers 6-9 was almost entirely dependent on DEX, given that LPS treatment does not replicate the amplitude of this accumulation. DEX also strongly induced RelA enrichment within the *Zfp36* promoter (primer 14) and intron (primer 15) both of which contain NFκB but not GR, motifs. Following administration of LPS for 1 hour, RelA was not observed to associate with ECR2, as with stimulation by DEX but, rather focused at discrete sites; upstream (primer 3); within ECR4 (primers 12-13), which contains a number of repetitive cognate NFκB motifs; and around consensus sequences within the promoter (primer 14) and intron (primer 15). Two specific downstream sequences, represented by

primers 21 and 24 were also enriched for by RelA ChIP. Neither of which two sequences are known to contain NFκB binding sites, although nearby primer 25 encloses a GR element.

After 1 hour under conditions of LPS + DEX, RelA associations with the *Zfp36* locus differed somewhat to those mediated by either stimulant alone. This is particularly interesting. Most strikingly, the effect of LPS+DEX stimulated RelA accumulation across ECR2, ECR3, ECR4, within the promoter and intron. The strong LPS+DEX mutual accrual of RelA at ECR2 is particularly prominent because either LPS or DEX alone were only able to induce protein binding by 2 and 5 fold respectively. Similarly, binding of RelA at primer pair 7 appears to be dependent on DEX. Altonsy et al. (2014) recently described the 'cooperative induction' of novel sites for either NFκB or GR, or both upstream of alternative feedback node gene *TNFAIP3*. The enrichment between primers 12 to 15 looks to be an additive effect of both agonists acting in concert. The relative underrepresentation of ECR4 is unexpected, since this region contains a repeat of NFκB consensus binding sequences. Conversely, although the nucleotide sequences at locations 3, 4, 21, 24 and 25 do not contain cognate sites there appears to be an association of NFκB with the locus. Primers 3 and 25 describe ECR1 and ECR5 respectively which is likely to be significant. Although there is a chance that chromatin looping and promoter-enhancer elements interactions result in false positive DNA-transcription factor associations. Overall, these data suggest that the cooperative up-regulation of *Zfp36* by LPS and DEX is driven by context-dependent NFκB association with novel DNA elements.

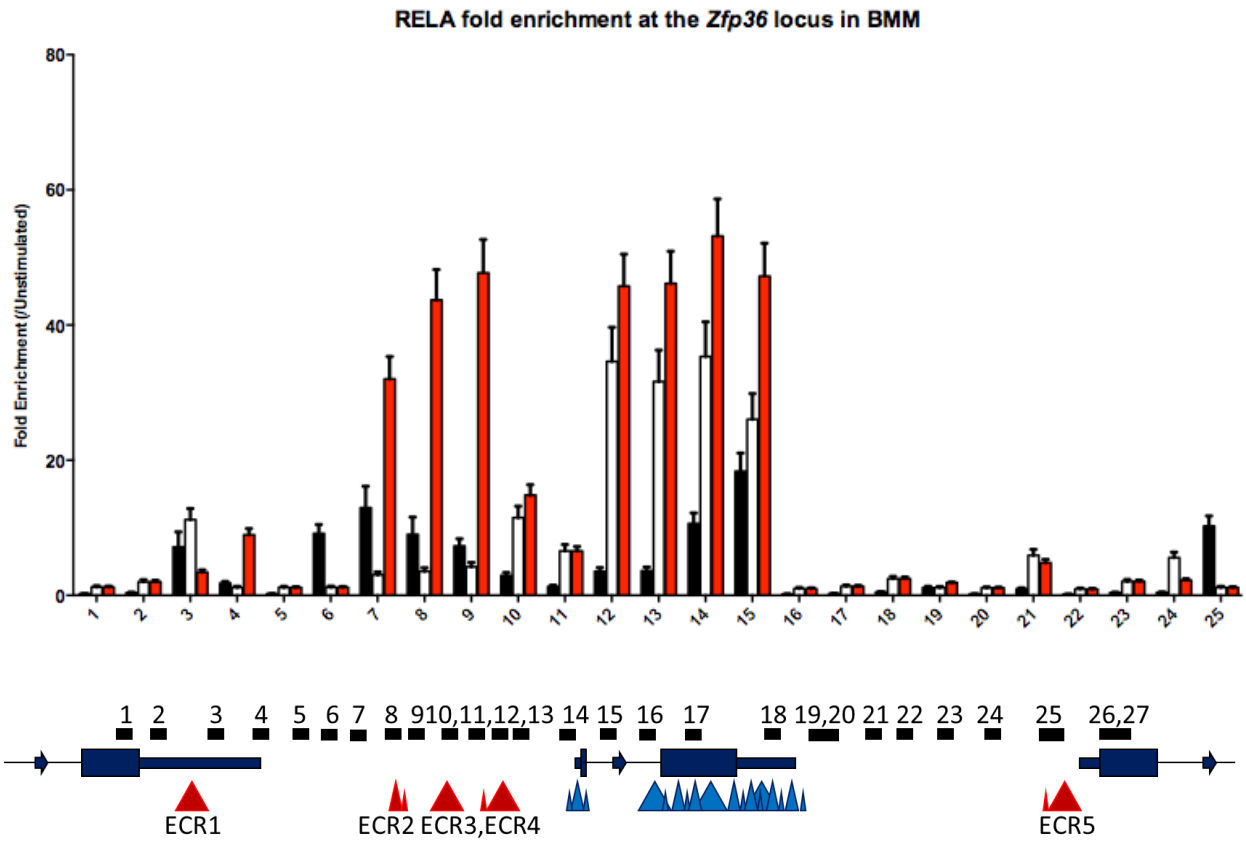


Figure 4.11 DEX increases the recruitment of RelA to κB and GR sites upstream of Zfp36.

Chromatin from the same experiment as Figure 4.10 was immunoprecipitated with antibody for RelA. The figure shows fold enrichment over unstimulated control in macrophages stimulated with dexamethasone (black bars), LPS (white bars), or both (red bars) for one hour. Data are represented as the fold enrichment of RelA in response to agonist over the unstimulated values. Data points are expressed as the mean +/- SEM of two QPCR separate experiments.

4.2.8.3 Dexamethasone enhances RNAPII binding at the Zfp36 locus

The RNAPII ChIP approach for analysing productivity of transcription consists of the detection of RNAPII – DNA interactions at specific sites, in this case at the *Zfp36* locus, designated by the primer pairs selected. RNAPII is essential for transcription and by quantifying its association with specific regions of DNA under discrete circumstances, we can analyse the effects on gene transcription. Specifically, an association of RNAPII with the coding region of a locus suggests active transcription and the elongation phase of nascent DNA sequences. Whereas interactions of RNAII with upstream sequences might be indicative of enhancer sequences, regulatory regions and sites of active chromatin. In order to determine if GR recruitment was able to enhance the transcription elongation stage of *Zfp36*, we used RNAPII ChIP and interpreted the result as a direct measure of transcriptional activity under conditions of LPS, DEX or both. DEX increased RelA binding to putative enhancer regions and the promoter of *Zfp36*, suggesting that the transcriptional response would be quickened. As a way of estimating the effects of DEX on *Zfp36* gene transcription, chromatin was immunoprecipiteded with antibody specific to RNAPII and probed with primers spanning the *Zfp36* locus. As highlighted previously (Chapter 3), we observed RNAPII pausing at the *Zfp36* promoter as well as at regions further upstream; a mechanism that allows for almost instantaneous gene transcription following stimulation (Hargreaves et al. 2009). A 5' to 3' progression of RNAPII represents transcript elongation and as RNAPII accumulates at the 3' end of the gene region it has already transcribed a molecule of RNA. An increase of RNAPII association within the gene region is associated with active transcription.

Shown in Figure 4.12 (A), RNAPII accumulated at the *Zfp36* locus in unstimulated macrophages, suggesting that they are 'paused' for the initiation of cofactors and the

elongation complex. On stimulation with DEX, the recruitment of RNAPII was enhanced within the *Zfp36* coding region and downstream of the 3'UTR, indicating that GR activation and proximal localisation (4.2.9.1) was able to enhance transcription elongation.

Figure 4.12 (B) shows enrichment of RNAPII at the *Zfp36* locus in macrophages stimulated with LPS or LPS + DEX. In response to LPS, RNAPII recruitment was both increased and shifted downstream as the active subunit of RNAPII (pSer2) progressed 5' to 3' and transcribed the RNA (Chapter 3.2.10) (Hargreaves et al. 2009). With the addition of DEX, RNAPII association with the coding region and the 3'UTR was greatly enhanced and indicated the cooperative increase in transcription kinetics that we observed in macrophages stimulated with both *Zfp36* agonists (Chapter 4.2.3). Enrichment of RNAPII was also observed upstream at primer regions 3 and 9 and within MmECR3-4, suggesting that these regions are associated with improved gene transcription. Distally located RNAPII enrichment could be a consequence of chromatin looping, whereby enhancer regions are brought into close proximity to the promoter favouring transcriptional elongation (Grzechnik et al. 2014; Stees et al. 2012). These data indicate that DEX, whether alone or in combination with LPS enhances RNAPII interaction with the *Zfp36* locus and subsequently increases the rate of transcription.

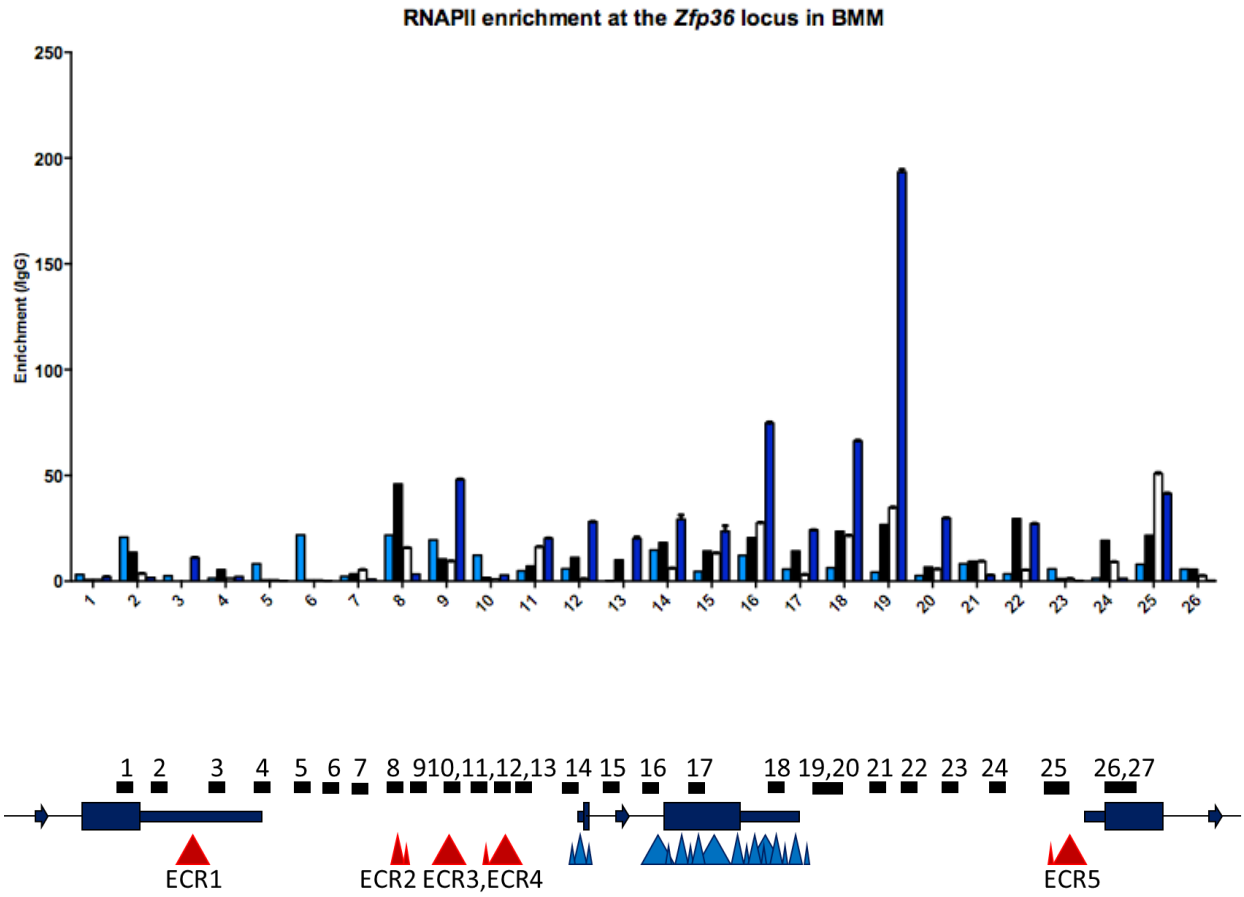


Figure 4.12 Effects of dexamethasone on RNAPII enrichment at the Zfp36 locus

Again, chromatin from the same experiment as Figure 4.10 was analysed by ChIP using antibodies specific to RNAPII or rabbit isotype control (IgG) and primers targeted at the mouse *Zfp36* locus. Primary murine macrophages were left unstimulated (light blue bars), stimulated for 1 hour with dexamethasone (Black bars), LPS (white bars) or a combination of both (dark blue bars) **(A)** RNAPII enrichment normalised to the isotype control for each data point. Data is shown as the mean ± SEM of two independent experiments using separate cultures of BMM. **(B)** Is a schematic of the Zfp36 locus which illustrates the location of each primer pair with respect to the ECRs and gene-coding regions.

4.3 Discussion

For decades, synthetic glucocorticoids have been the cornerstone of treatment for a multitude of autoimmune and immunosuppressive conditions. However, the anti-inflammatory mechanisms of glucocorticoid action are diverse and not fully understood.

In this chapter I have demonstrated that glucocorticoids may selectively spare and even augment the expression of three critical negative feed-back regulators of inflammation. This is contradictory to the well described repressive effects of GR on NFκB and pro-inflammatory gene transcription. When administered with LPS or IL1α, synthetic glucocorticoid dexamethasone synergistically induced the expression of DUSP1, TTP and A20 transcripts. Previous studies have described GR-mediated co-activation of NFκB driven promoters, including synergistic up-regulation of cellular inhibitor of apoptosis 2 (cIAP2) by DEX and TNFα (Webster et al. 2002), cooperative regulation of NFκB reporters (Hofmann & Schmitz 2002), super-induction of DUSP1, TNFAPI3, TNIP1 and NFKBIA (Abraham et al. 2006; Altonsy et al. 2014). However there have been no conclusive studies describing GR/NFκB cooperative induction of TTP.

Dysregulation of TTP is strongly associated with autoimmune disease and knockout studies have revealed its importance in post-transcriptional regulation of pro-inflammatory mediators, particularly *Tnfα*. The mechanism of TTP induction has not been studied in detail, although NFκB is implicated (King, Kaur, et al. 2009; Carayol et al. 2006; Chen et al. 2013) and Chapter 3.2.5). Previously, DEX has been shown to up-regulate *Zfp36* gene expression. However the mechanism of GR is unknown and must be context dependent given that NFκB and GR are generally considered to be mutually repressive. LPS and DEX co-treatment synergistically enhanced luciferase activity in expression vectors containing TTP-locus ECRs,

identifying three putative enhancer regions and their responsiveness to GR. When in isolation, reputed κB motifs upstream of *Zfp36* were inhibited by DEX. However, when proximally located to glucocorticoid responsive enhancer sequences NFκB binding element containing ECR4 super-induced luciferase activity. Endogenously, GREs are often found in close proximity to NFκB and AP1 binding sites that may be associated with glucocorticoid-activated or repressed genes. Thus the transcriptional outcome of GR association cannot be predicted based simply on sequence or the proximity of NFκB but is context dependent. That is, GR occupancy may confer gene-responsiveness but the receptor itself may not participate in the regulatory effects (Uhlenhaut et al. 2013).

Chromatin immunoprecipitation is an invaluable tool for identifying protein-DNA interactions in vivo and unlike reporter constructs represents the result of endogenous interactions between DNA, transcription factors, cofactors and the chromatin environment. In the presence of LPS, DEX directed GR and RelA co-localisation at 'gained' GR sites within ECR3-4, upstream of *Zfp36*. The mutual dependence of GR and p65 binding at shared sites in response to TNFα and the synthetic GC triamcinolone acetonide (TA) has been described previously on a genome wide scale (Rao et al. 2011). The same study identified a number of common binding sites that were 'maintained' i.e. binding of GR and p65 was unaffected by treatment with TA, TNFα or both. These maintained elements were associated with anti-inflammatory genes, including NFKBIA and TNFAIP3 (Rao et al. 2011). Through reorganisation of chromatin structure, inflammatory signalling may create additional domains of GR accessibility therefore priming for subsequent termination of the inflammatory response (Uhlenhaut et al. 2013).

ChIP also highlighted accumulation of GR, RelA and RNAPII, upstream of *Zfp36* in the distal ECR1 (primers 2-3). Distant genomic elements can selectively promote gene transcription through chromatin looping which creates a 'hub' for the recruitment of transcription factors, cofactors and ancillary proteins. Remotely located GR has been shown to promote transcription through chromatin looping and may be a mechanism for enabling co-occupancy with NFκB which facilitates cooperative regulation of gene expression in a stimulus and cell specific manner (Grzechnik et al. 2014; Rao et al. 2011; Hakim et al. 2009).

The transcriptional outcome of GR is not indicated by motif classification but rather depends on a number of context-dependent factors including epigenetic regulation, chromatin context and so far unrecognised regulatory determinants. LPS induction of TLR signalling causes a change in chromatin environment that favours transcription factor binding. Additionally to this, in response to DEX, GR-activated genes are enriched for H3K9 acetylation marks whereas GR-repressed genes are not (Uhlenhaut et al. 2013); therefore providing a cooperative mechanism for localised relaxation of chromatin configuration and expression of anti-inflammatory genes including *DUSP1*, *TNFAIP3* and *ZFP36* in a context-dependent manner.

5.0 EFFECTS OF PGE2 IN INFLAMMATORY RESPONSES OF MACROPHAGES

5.1 Introduction

Prostaglandin E2 (PGE_2) is an autocrine lipid mediator derived from the successive metabolism of arachidonic acid (AA) and PGH_2 by cyclooxygenase (COX) and PGE synthase enzymes respectively (Hata & Breyer 2004). The most ubiquitous prostanoid, PGE_2 has central roles in a number of physiological systems and biological processes including pain, fever, renal function, angiogenesis, vascular tone and inflammation (Konya et al. 2013). Not surprisingly therefore, aberrant expression of PGE_2 is associated with complications such as atherosclerosis (Tang et al. 2012), myocardial inflammation & cardiovascular disease (Hishikari et al. 2009; Xiao et al. 2004), cancer, hypertension and immune dysfunction (Konya et al. 2013).

Cyclooxygenase conversion of arachidonic acid to PGH_2 is the rate-limiting step of prostaglandin synthesis. Constitutively expressed, COX1 is responsible for basal prostanoid biosynthesis, whereas COX2 is inducible and increases arachidonic acid metabolism in response to cellular stresses and TLR agonists like LPS (Díaz-Muñoz et al. 2012; Higaki et al. 2012). Generally, prostaglandins are considered as pro-inflammatory. PGE_2 expression is elevated at sites of inflammation (Amer et al.) and under certain conditions may be pyrogenic and induce hyperalgesia. PGE2 also stimulates macrophage mobility (Tajima Murata 2009) yet, however at the same time reduces secretion of inflammatory cytokines (Tang et al. 2015). PGD_2 is bronchoconstrictive and correlates strongly with the pathogenesis of asthma and PGI_2 is vasodilatory and activates immune cell function.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a very successful group of therapeutic compounds used for their powerful analgesic, antipyretic and anti-inflammatory properties. Given that NSAIDs function by instigating blockage of COX activity and therefore directly

inhibit prostanoid synthesis (Amer et al.), it is logical to assume that prostanoids are inflammation promoting. However, whilst the analgesic properties of NSAIDs undoubtedly reduce arthritic joint pain and improve the mobility of rheumatoid patients they do not slow disease progression(Page et al. 2010). In fact, NSAIDs have been shown to induce TNFα production in synovial fibroblasts and LPS-stimulated monocytes. Furthermore, these effects were reversed by administration of exogenous PGE_2 and subsequent signalling via the E-type prostanoid receptor-2 (EP_2). The biological effects of prostanoids are clearly diverse and spatiotemporally specific. PGE_2 alone mediates its effects through four divergent receptors, each differing in affinity, sensitivity and coupled to separate signalling pathways (Kalinski 2012). Therefore, the diversity of receptors responsive to PGE_2 is likely to account for its multitude of actions, which may be mutually overlapping and often contradictory.

Specific to activation by PGE_2, EPs_{1-4} are G-protein coupled receptors which, with the exception of EP_1, may have either stimulatory ($G_{\alpha s}$) or inhibitory ($G_{\alpha i}$) effects on adenylate cyclase and intracellular cAMP (Kalinski 2012). Receptor EP_1 is ubiquitously expressed and signals predominantly through $G_{\alpha q}$ proteins, which elevate intracellular Ca^{2+} and activate phospholipase Cβ and Protein kinase C (PKC). Coupled to $G_{\alpha i}$ and, less predominantly $G_{\alpha s}/_{q}$, EP_3 is represented in cells of the pancreas, kidney and the vena cava. On activation, both EP_1 and EP_3 facilitate immune processes, increasing mast cell degranulation and T-cell proliferation as well as causing inflammatory hyperalgesia (Regard et al. 2008). Both EP_2 and EP_4 on the other hand, are associated with $G_{\alpha s}$ and elevate intracellular cAMP through enhanced adenylate cyclase metabolism (Alfranca et al. 2006). Transcripts encoding EP_4 have been identified in the uterus, skin, gastrointestinal and hematopoietic tissues whilst

EP_2 is expressed predominantly in airway, ovary, bone marrow and olfactory epithelium (Regard et al. 2008; Konya et al. 2013). The resultant increase in cAMP following $EP_{2/4}$ $G_{\alpha s}$ stimulation in turn initiates protein kinase A (PKA) phosphorylation and activation of cAMP responsive element binding (CREB) transcription factor (Tang et al. 2012; Konya et al. 2013). Contributing to the up-regulation of numerous immune related genes including *IL12*, *IL6*, *IL10* and *TNFα*, CREB promotes the survival signal in macrophages and, when phosphorylated can inhibit NFκB signaling and pro-inflammatory gene expression (Wen et al. 2010; Fujino et al. 2005). Despite sharing similar nominal functions, EP_2 and EP_4 differ not only in tissue-specificity but also in ligand affinity and susceptibility to receptor sensitisation. Like EP_3, EP_4 has a high affinity for PGE_2 and is activated by much lower concentrations of ligand than EP_2. Perhaps consequently, EP_4 is also subject to receptor sensitisation whereas EP_2 is not. Interestingly, EP_2 has been shown to stimulate GSK3β which, through phosphorylation of CREB and inhibition of CBP binding to RelA may inhibit NFκB mediated gene activation (Wen et al. 2010; Ollivier et al. 1996; Parry & Mackman 1997). Furthermore, EP_4 alternatively triggers PI3K dependent ERK1/2 signaling (Fujino et al. 2003) at the expense of PKA activation (Fujino et al. 2005). There is some evidence to implicate PI3K-dependent signaling by EP_4 and a lack of detectable increase in cAMP in the growth and motility of mouse colon adenocarcinoma cells (Sheng et al. 2001; Pozzi et al. 2004; Fujino et al. 2005). Therefore, it is thought to be stimulation of cAMP and subsequent signaling through the PKA/CREB pathway that dominates the anti-inflammatory and suppressive actions of PGE_2 and $EP_{2/4}$ in myeloid cells (Fujino et al. 2005; Regan et al. 1994; Honda et al. 1993; Fujino et al. 2003).

Based on microarray data from our lab, the transcript encoding EP_2, that is *Ptger2*, is expressed only at low levels in mouse macrophages, whereas *Ptger4* is strongly expressed and upregulated by LPS. The EP_1 and EP_3 transcripts *Ptger1* and *Ptger3* respectively, are not expressed in mouse macrophages. Therefore, at least in murine macrophages, it is likely that anti-inflammatory effects of PGE_2 are mediated by EP_4.

Compounds that elevate cAMP have been used in combination with glucocorticoids to treat patients with asthma and COPD, with significant beneficial effects (Giembycz et al. 2009). MAPKs are critical to the airway remodelling and chronic-inflammation attributed to the pathogenesis of asthma (Pelaia et al. 2005; Pelaia et al. 2011; Duan & Wong 2006). A negative regulator of MAPK signalling, *Dusp1* is induced by both glucocorticoids (Quante et al. 2008; Issa et al. 2007; Kang et al. 2008) and long-acting β_2-agonists (Giembycz et al. 2009). Furthermore, dex-induced *Dusp1* expression is enhanced and extended by co-stimulation with the long-acting β_2-adrenoceptor agonist Formoterol via the PKA pathway (Manetsch et al. 2012). The activity of cAMP is degraded by phosphodiesterase type 4 (PDE4), a hydrolyzing enzyme prevalent in immune cells and cells of the central nervous system. Due to the anti-inflammatory potential of compounds that enhance cAMP signaling, inhibitors of PDE4 are being increasingly investigated for use as novel anti-inflammatory drugs. Rolipram is a prototypical PDE4 inhibitor and has been investigated for the treatment of a diverse group of diseases including inflammatory conditions such as COPD, asthma and rheumatoid arthritis. In a study by Korhonen et al. (2013), Rolipram was shown to significantly down regulate TNFα expression in activated macrophages and furthermore attenuated carrageenan-induced paw inflammation in WT mice but not in *Dusp1-/-*. Therefore indicating that the therapeutic effects of this cAMP-enhancing compound require DUSP1

(Korhonen et al. 2013). TTP is a downstream target, activated by DUSP1 and rather interestingly, there is tentative data to suggest that activators of adenylate cyclase might increase *Zfp36* mRNA levels (Jalonen et al. 2007). In fact, the anti-inflammatory, mRNA down-regulating effects of long-acting β_2-adrenoceptor agonists and cholinergic stimulation require TTP expression (Joe et al. 2011; Geyer et al. 2012; Jalonen et al. 2007; Brahma et al. 2012).

Studies on cAMP-induced expression of A20 are few. However, Litvak et al. (2009) identified a C/EBPβ motif within the TNFAIP3 promoter and showed that C/EBPβ acts as an amplifier of NFκB signaling in activated murine primary macrophages. C/EBPβ acts as a co-activator of transcription and has been shown to be dependent on interaction with other transcription factors, including NFκB, SP1 and Fos/Jun (Lekstrom-Himes & Xanthopoulos 1998; Lee et al. 1997; Serio et al. 2005). Furthermore, Tnfaip3 is co-regulated by both NFκB and C/EBPβ in LPS-activated macrophages (Lai et al. 2013).

In this chapter we investigated the effects of PGE_2 on feedback node gene expression and its potential to initiate transcription via three putative *Zfp36* enhancer sequences.

5.2 Results

5.2.1 PGE2 inhibits expression of pro-inflammatory mediators at the level of mRNA and protein

In order to identify the effects of PGE_2 in our system of cells, firstly the effect of PGE_2 on expression of inflammatory mediators was examined. As can be seen in Figure 5.1, PGE_2 had no effect on resting levels of TNFα but was able to significantly enhance COX2 and IL6 transcript up to 12 and 8 times basal levels of expression respectively. These effects however are masked by the strong fold induction observed in response to LPS and LPS + PGE_2 in combination. PGE_2 has previously been described as IL-6 inducing in a number of cell types (P. Wang et al. 2010; Pu Wang et al. 2010) including macrophages (Shacter et al. 1992; Ogle et al. 1994; McCoy et al. 2002) and DCs pre-treated with IL-10 neutralising antibody (Harizi et al. 2004). In addition, PGE_2 has been suggested to induce COX2 expression as a positive feedback mechanism (Mancini & Di Battista 2011).

When administered in combination, PGE_2 had a significant inhibitory effect on the LPS-mediated induction of both TNFα and COX2 mRNA in myeloid cells. These results reflect - previous findings by Pang & Hoult (1997). Interestingly, PGE2 acted cooperatively with the LPS-induced production of IL6 transcript, whereas TNFα and COX2 transcript levels were strongly inhibited by the same treatment.

The amount of secreted inflammatory protein was quantified by sandwich ELISA. This was to determine whether the observed regulation at the level of mRNA was translated to the protein level and whether the effects of PGE2 were functional. Protein secretion was measured from primary macrophage cell supernatants collected at 4 and 8 hours of stimulation as well as from unstimulated cells. COX2 is not a secreted protein and the

method used to detect relative levels of COX2 is to quantify PGE2 in cell supernatants by ELISA. However clearly this was not a suitable method of detection in this experiment since cells were stimulated with exogenous PGE2 by direct addition to the cell media. However, TNFα, CXCL1 and IL6 were all measured (Figure 5.2). Again, we observed significant PGE_2-mediated inhibition of the LPS-driven pro-inflammatory TNFα and CXCL1 release in primary murine macrophages. However, PGE_2 alone did not induce significant expression of IL6 protein, neither did PGE_2 significantly affect LPS-induced IL6 release.

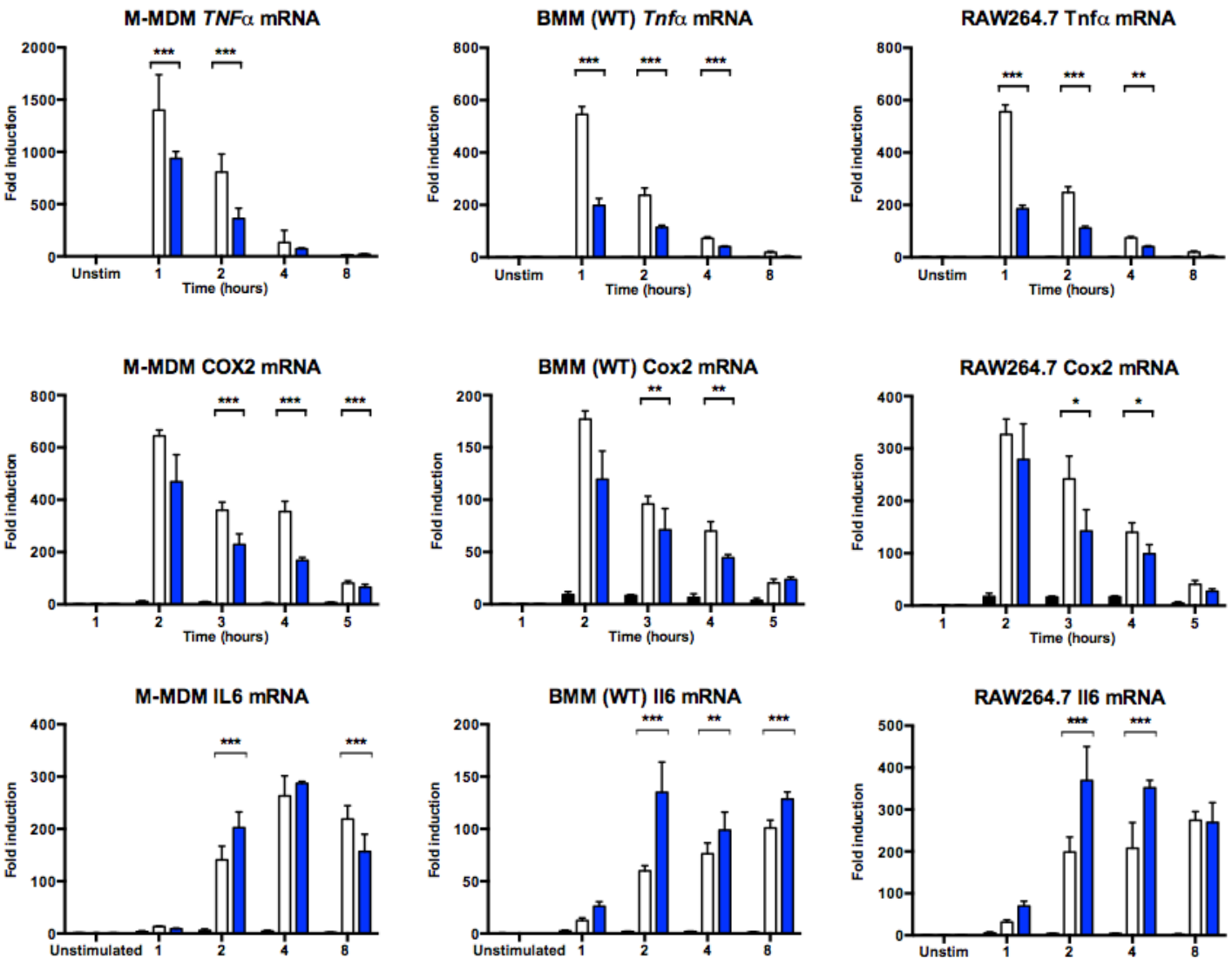


Figure 5.1 PGE_2 up-regulates *IL6* and inhibits LPS-induced *TNFα* and *IL1α* at the level of mRNA in macrophages

Primary macrophages and RAW264.7 cells were seeded at a density of $1x10^6$/ml and $6x10^5$/ml and established adherence overnight. The next day, macrophages were unstimulated (black bars), treated with LPS 10ng/ml (white bars) or LPS and PGE_2 10ng/ml (blue bars) for the times indicated. Whereas PGE_2 alone had no effect on TNFα transcript expression, it was inhibitory to LPS-induced transcription when cells were co-treated. Conversely, PGE_2 was able to up-regulate Cox2 and IL6 mRNA and had a cooperative effect on the LPS-induced IL6 message. Data represent mean ± SEM of three and four separate experiments in primary macrophages and RAW264.7 respectively. **p<0.01, ***p<0.001

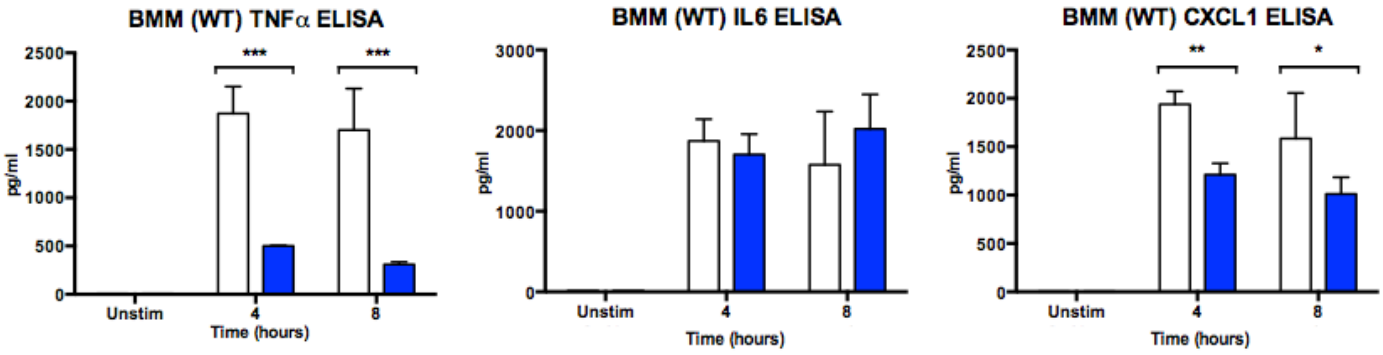


Figure 5.2 PGE_2 inhibits LPS-mediated secretion of pro-inflammatory proteins IL6, TNFα and CXCL1 in primary macrophages

Supernatant was collected from time course experiments in primary human and murine macrophages either unstimulated (black bars unseen), stimulated with LPS (white bars) or LPS+PGE2 (blue bars). Supernatants were diluted accordingly and applied to sandwich ELISA with antibodies for TNF, IL6 and CXCL1 (KC). Data show mean ± SEM for three separate experiments in both cell types.

5.2.2 PGE_2 cooperatively induces DUSP1, ZFP36 and TNFAIP3 transcript expression in primary macrophages and RAW cells

The anti-inflammatory effects of PGE_2 were also investigated by identifying the effect on anti-inflammatory feedback node gene expression. PGE_2 alone strongly induced *DUSP1* gene expression up to 10, 8 and 3 fold in human, murine and RAW macrophages respectively. This effect of PGE_2 was also observed at the level of protein in murine primary macrophages activated with LPS (Figure 5.2). The promoter of *Dusp1* contains two functional cAMP-responsive elements (Zhang et al. 2005) that have been reported to induce *Dusp1* expression through a cAMP-PKA-CREB dependent pathway (Shipp et al. 2010; Brion et al. 2011; Lee et al. 2012; Korhonen et al. 2013).

Additionally, in response to co-activation with LPS and PGE_2 the expression of *Dusp1* mRNA was both enhanced and extended as the two agonists acted synergistically. This effect was more significant ($p<0.001$) at the one hour time point in all cell types used and was also observed after two hours incubation in primary murine macrophages.

The effects of PGE_2 on *TNFAIP3* gene expression were also determined by QPCR (Figure 5.4). Although PGE_2 alone did not up-regulate *Tnfaip3* mRNA levels, when in combination with LPS, we observed a significant increase the amount of LPS-induced message after one and two hours in primary macrophages. The transformed mouse macrophage like cell line RAW264.7 did not demonstrate significant cooperative regulation of Tnfaip3 expression by LPS and PGE_2. Protein data shows potential cooperative up-regulation of A20 by PGE_2 and LPS at 4 hours post stimulation in primary murine macrophages (Figure 5.3). Agonists of cAMP, for example β2 adrenoceptor ligands, are known to have post-transcriptional effects on gene expression, particularly of TNFα and GM-CSF (Seldon et al. 1995; Clarke et al. 2004),

both of which are principal targets of TTP. Additionally, (Korhonen et al. 2013) showed that the effects of a phosphodiesterase inhibitor, Rolipram were downstream of DUSP1 signalling. Therefore we hypothesised that PGE_2 might up-regulate *Zfp36* transcription and furthermore, act cooperatively with LPS.

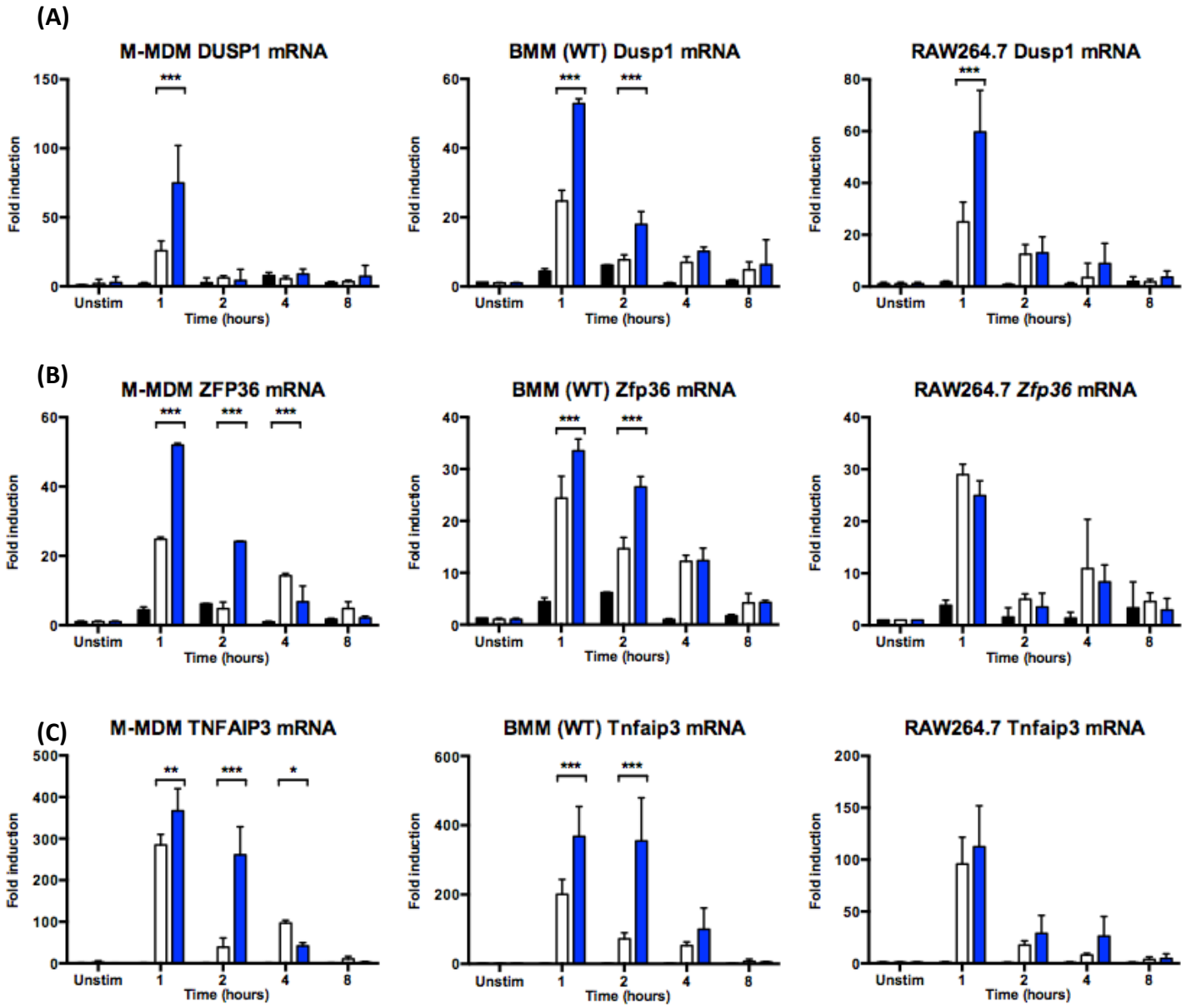


Figure 5.3 The effects of PGE_2 on feedback node gene transcript expression in macrophages

Primary macrophages and RAW264.7 cells were seeded at a density of $1x10^6$/ml and $6x10^5$/ml and established adherence overnight. The next day, macrophages were unstimulated (striped bars), treated with PGE_2 (black bars) LPS 10ng/ml (white bars) or LPS and PGE_2 10ng/ml (blue bars) for the times indicated. **(A)** PGE_2 increased *Dusp1* alone and acted cooperatively with LPS to super-induce gene expression. **(B)** *Zfp36* was up-regulated by PGE_2 alone and in cooperation with LPS at the level of mRNA and protein in primary macrophages **(C)** *Tnfaip3* transcript levels were unaffected by PGE_2 alone but were significantly increased by co-treatment with LPS. Data represent mean ± SEM of three and four separate experiments in primary macrophages and RAW264.7 respectively. ***p<0.001

5.2.3 The anti-inflammatory effects of PGE_2 are largely dependent on DUSP1

As aforementioned, the attenuation of inflammatory signalling by Rolipram requires DUSP1. In lieu of this, the effects of PGE_2 that we observed in WT macrophages were tested in *Dusp1-/-* cells. Time course experiments were carried out in parallel with WT experiments detailed earlier in this chapter.

The repressive effect of PGE_2 on TNFα observed in WT cells were abrogated by the 2 hour time point and thereafter in *Dusp1-/-* macrophages (Figure 5.4A). In fact, at later time points, simultaneous activation of *Dusp1-/-* cells with the two agonists appeared to have a cooperatively augmenting effect on *Tnfα* transcript production. These data are reflected similarly in ELISA experiments, where PGE_2 was less able to reduce TNFα protein secretion in *Dusp1-/-* cells.

In *Dusp1-/-* BMMs the LPS-induced expression of *Cox2* mRNA was enhanced and prolonged and inhibitory effects of PGE_2 were not evident.

Although CXCL1 protein secretion was greatly increased in *Dusp1* knock out macrophages, there was little observed difference between the relative inhibition of LPS-induction mediated by PGE_2 (Figure 5.4B).

In wild type BMMs the induction of *Il6* mRNA by LPS was characteristically delayed, reflecting the requirement for chromatin remodelling for maximal expression. From the 2 h time point onwards there was cooperative up-regulation of *Il6* mRNA by LPS and PGE_2, as previously shown (Fig. 5.1). Several aspects of this response were altered in *Dusp1-/-* BMMs. Maximal induction of *Il6* mRNA occurred more rapidly, and reached a higher level than in wild type control cells. Furthermore, the cooperative induction of Il6 mRNA by LPS and PGE_2

was no longer evident. However, when secreted IL6 protein was measured, I did not find significant effects of PGE_2 in either *Dusp1+/+* or *Dusp1-/-* BMMs, or significant differences of LPS response between the two genotypes.

The cooperative up-regulation of FNG mRNA expression by PGE_2 and LPS simulation observed in WT macrophages (Figure 5.5) was significantly altered and at times almost completely abrogated in *Dusp1-/-* cells. For example, after 1 hour of co-treatment, PGE_2 had no effect on the quantity of LPS-induced *Tnfaip3* mRNA in knockouts. After 2 hours of incubation however, cooperative induction of *Tnfaip3* was observed in both WT and *Dusp1* knockouts. Co-regulation at the 2-hour time point thus does not appear to depend on *Dusp1* expression. Then again, post 4 hours of co-stimulation PGE_2 has a significant inhibitory effect on the LPS-mediated regulation of *Tnfaip3*. With respect to these data it is reasonable to suggest that *Tnfaip3* is under differential regulatory control depending on time after LPS stimulation.

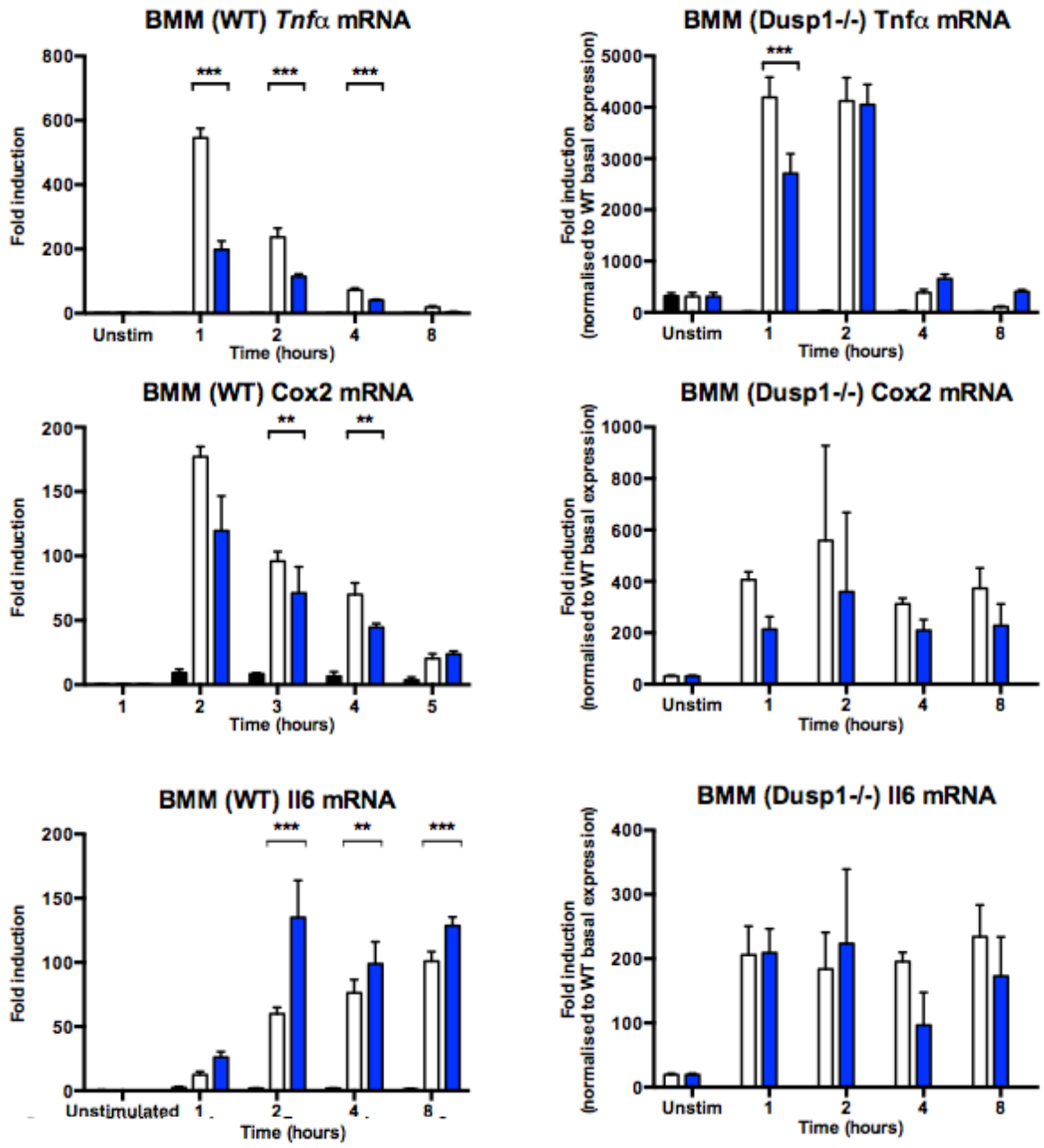


Figure 5.4(A) The PGE_2-mediated regulation of inflammatory mediators is abrogated in *Dusp1-/-* primary macrophages

In parallel with WT experiments, primary macrophages were isolated from age and sex-matched *Dusp1-/-* mice. Cells were seeded at a density of $1x10^6$/ml overnight. The next day, cultures were either unstimulated (striped bars), treated with PGE_2 (black bars) LPS 10ng/ml (white bars) or LPS and PGE_2 10ng/ml (blue bars) for the times indicated. mRNA was harvested and subjected to QPCR using probes for TNFα, COX2 and IL6.

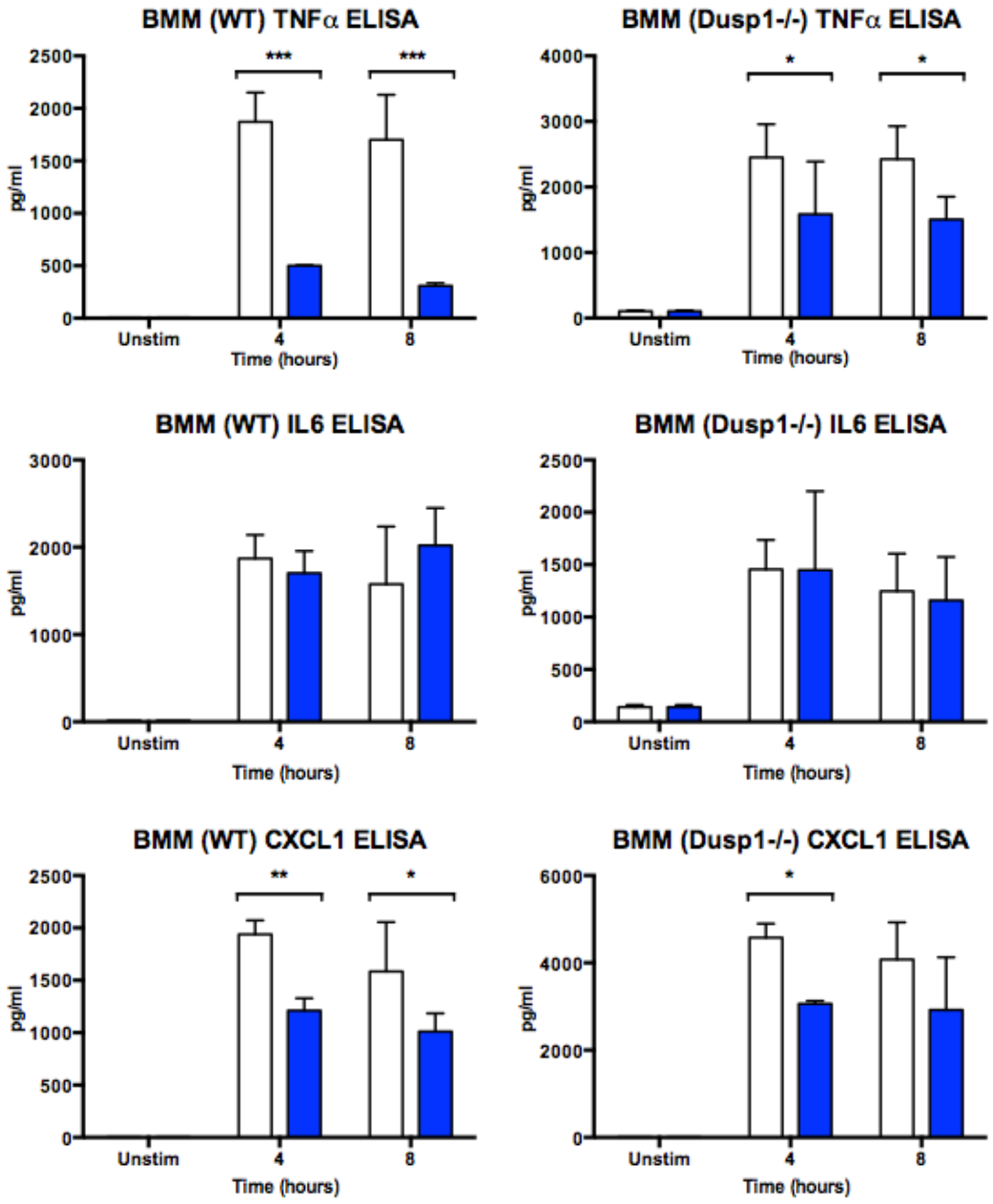


Figure 5.4(B) The PGE_2-mediated regulation of inflammatory mediators is abrogated in *Dusp1-/-* primary macrophages

In parallel with WT experiments, primary macrophages were isolated from age and sex-matched *Dusp1-/-* mice. Cells were seeded at a density of $1x10^6$/ml overnight. The next day, cultures were either unstimulated (striped bars), treated with PGE_2 (black bars) LPS 10ng/ml (white bars) or LPS and PGE_2 10ng/ml (blue bars) for the times indicated. Supernatant was collected from each experiment and applied to ELISAs for TNFα, CXCL1 (KC) and IL6. Data represent mean ± SEM of three separate experiments. Both QPCR and ELISA were carried out in triplicate within one experiment. *p<0.05, ***p<0.001

As described previously, LPS induction of *Zfp36* was both enhanced and extended in *Dusp1-/-* macrophages when compared to WT. This was also observed at the level of protein (Figure 5.6). In the absence of *Dusp1*, prolonged activation of p38 MAPK leads to accumulation of inactive TTP. *Zfp36* mRNA contains an ARE and is a target of its own protein product. We know from data in our lab (as yet unpublished) that *Dusp1-/-* induced TTP inactivity increases the stability of *Zfp36* transcript levels in these macrophages when compared to WT macrophages. When normalised to the WT data for unstimulated cells, PGE_2 had a significantly greater effect on *Zfp36* transcript induction in Dusp1-/- macrophages compared to WT. This effect was approximately two to three fold WT expression and observed at one and two hours following PGE_2 treatment. In WT macrophages, PGE_2 may stimulate *Dusp1* to inactivate p38 MAPK signalling and subsequently the dephosphorylation that activates TTP. In the knockout macrophages however, PGE_2 cannot drive TTP activation via *Dusp1* and therefore the *Zfp36* transcript becomes stabilised and more highly expressed in comparison to WT. In addition the β-adrenergic receptor agonist isoproterenol has been shown to induce *Zfp36* expression in adipocytes, via cAMP-dependent signalling (Brahma et al. 2012). This mechanism may or may not be differentially affected by the WT and *Dusp1-/-* genotypes.

The effects of PGE_2 on *Zfp36* mRNA expression in activated macrophages differ depending on genotype and time point. At the one-hour time point of PGE_2 agonism, *Zfp36* expression is comparable between the two genotypes. However, following this initial quantification of transcript expression, the effects of PGE_2 on the LPS-induction of *Zfp36* are significantly inhibitory in *Dusp1-/-* macrophages, which is in contrast to the observed co-operative induction observed in WT macrophages. Compounds that enhance the cAMP pathway are

increasingly being investigated for the treatment of various inflammatory and autoimmune conditions. A number of these novel therapeutic agents mediate their anti-inflammatory effects via mechanisms dependent on the up-regulation of DUSP1 (Korhonen et al. 2013). It is possible that the effects of PGE_2 on *Zfp36* transcript expression in an inflammatory setting i.e. under conditions of LPS, act via DUSP1 to enhance TTP activation and therefore pro-inflammatory cytokine destabilisation. At the same time, via additional mechanisms pro-inflammatory stimuli and PGE_2 must cooperate to drive *Zfp36* transcript expression, perhaps at the level of NFκB, AP1, AP2 or C/EBPβ. Indeed, TTP expression is critical to the posttranscriptional anti-inflammatory effects of cAMP-stimulating compounds (Joe et al. 2011; Geyer et al. 2012; Jalonen et al. 2007; Brahma et al. 2012).

5.2.3 Cooperative up-regulation of DUSP1 protein expression by PGE_2 and LPS in primary murine macrophages

In the previous section, we showed that PGE_2 was able to up-regulate LPS-induced FNG expression at the level of mRNA and also that this expression appeared to be partly dependent on DUSP1. Therefore in order to complete this set of experimental data and observe the biological implications, protein quantification was performed.

As expected, the expression of DUSP1 is absent in knock out mice. LPS was able to induce all three proteins in a transient manner and was similar to the expression profile for each FNG mRNA. PGE_2 was able to augment the LPS-induced expression of DUSP1 and TTP from as early as 1 hour in WT macrophages, however this effect was less evident and perhaps unconvincing for A20. However, in Dusp1-/- macrophages, the cooperative effect of PGE_2 on LPS-induced TTP was abrogated.

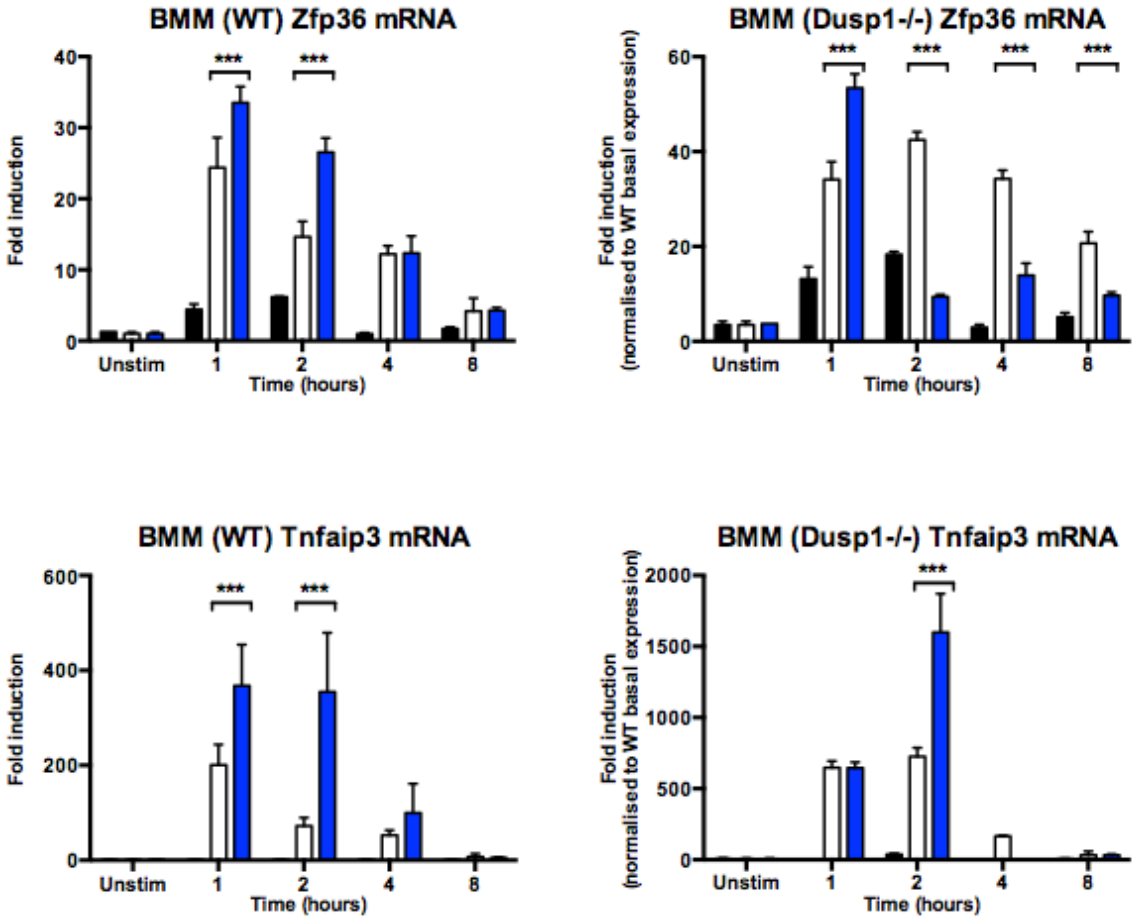


Figure 5.5 Cooperative regulation of anti-inflammatory feedback node genes by LPS and PGE_2 is abrogated in *Dusp1-/-* macrophages.

Tnfaip3 and *Zfp36* transcripts were quantified by QPCR of cDNA from *Dusp1-/-* macrophages unstimulated (striped bars) or incubated with PGE_2 (black bars) LPS 10ng/ml (white bars) or LPS and PGE_2 10ng/ml (blue bars) for the times indicated. **(A)** Cooperative induction of *Tnfaip3* by PGE_2 and LPS was abolished at the 1 hour time point but maintained at 2 hours in macrophages null for *Dusp*1. **(B)** PGE_2-mediated induction of *Zfp36* was augmented in *Dusp1-/-* macrophages and although synergistic regulation of *Zfp36* remained at 1 hour of co-stimulation, this effect was reversed from two hours and onward. ***p<0.001

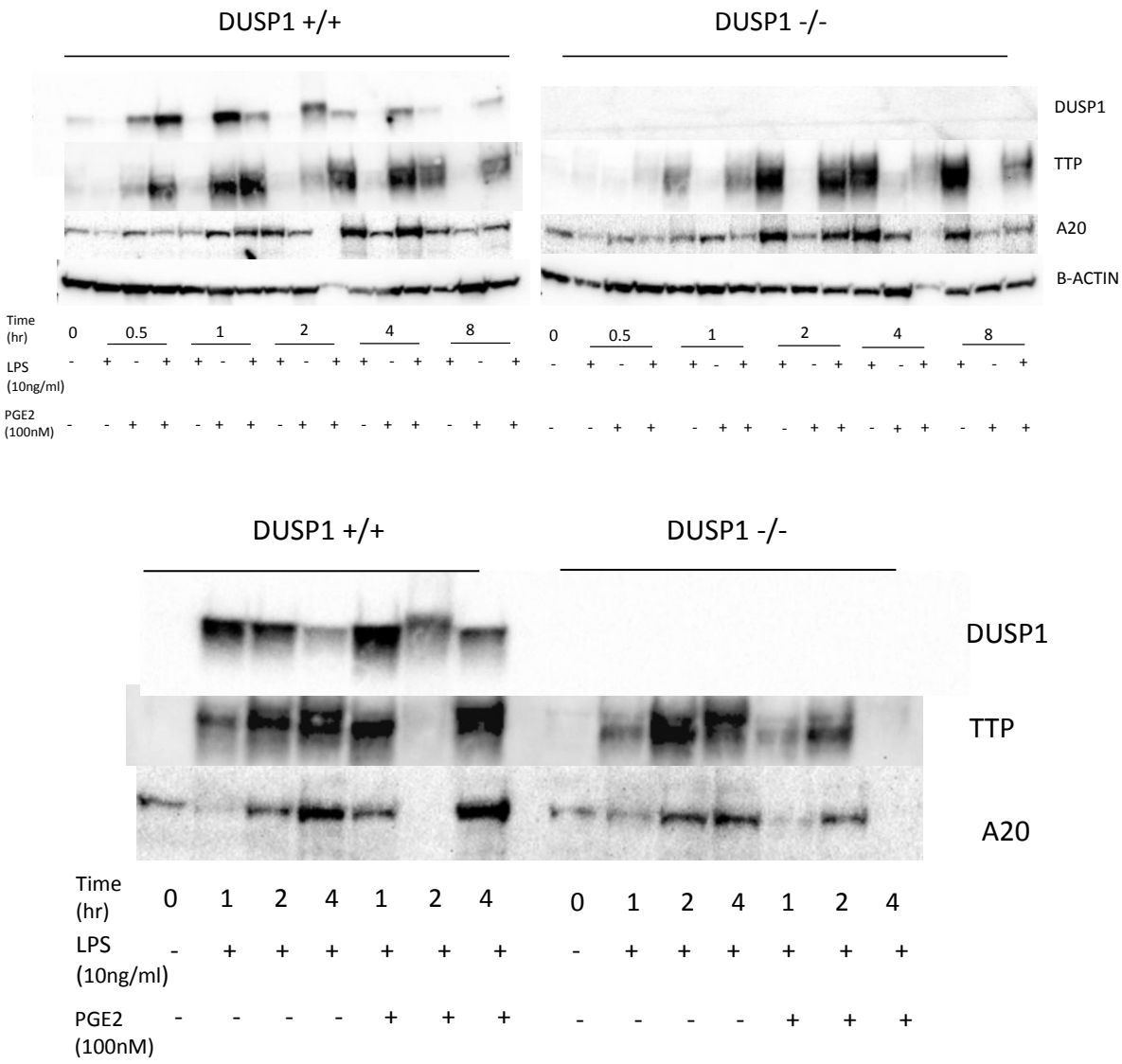


Figure 5.6 PGE_2 enhanced LPS-induced DUSP1 and TTP in WT but not in *Dusp1-/-* primary murine macrophages

Experiments were carried out on primary macrophages isolated from age and sex-matched WT and *Dusp1-/-* mice. Macrophages were seeded at a density of $1x10^6$/ml in 6 well plates. Following stimulation with LPS, PGE_2 or both agonists for the times indicated, cells were harvested form protein by cell lysis. Antibodies against DUSP1, TTP (SAK21) and A20 were used subsequently on the same blot. Protein concentration was controlled for by β-actin as a loading standard.

(Figure courtesy of T.Tang; Clark group)

5.2.4 PGE_2 transcriptional induction of a reporter containing upstream ECRs of TTP

Using the reporter constructs described previously (Chapter 3.2.6-7), our aim was to identify whether PGE_2 alone or in combination with a pro-inflammatory stimulus, was capable of inducing a luciferase response via functional elements within ECRs 2, 3 and 4.

The initial observation, when considering the luciferase activity of cells containing the control plasmids, was the effect of PGE_2 on LPS and IL1 induction of these constructs in both cell lines. PGE_2 significantly inhibited the relative luciferase by approximately 30% in RAW264.7 macrophages and 75% in HeLa cells. These data highlight the ability of PGE_2 to down regulate the expression of NFκB in both human and mouse cell lines.

Concurrent with other data, ECR3-4 was the most strongly induced by the pro-inflammatory stimulus in both human and murine cell lines. Fittingly, this was the region that mounted the maximal response for PGE_2 alone and in combination with LPS or IL1 in murine and human cells respectively. In response to PGE_2 treatments, human and murine ECRs appeared to function similarly, something that hasn't necessarily been observed when using other agonists. When isolated, conserved regions 3 and 4 were each induced by PGE_2 alone and show only additive transcriptional potential in cells treated with both *Zfp36* agonists. Similarly MmECR2 responds to the two agonists together with an additive effect on transcriptional induction.

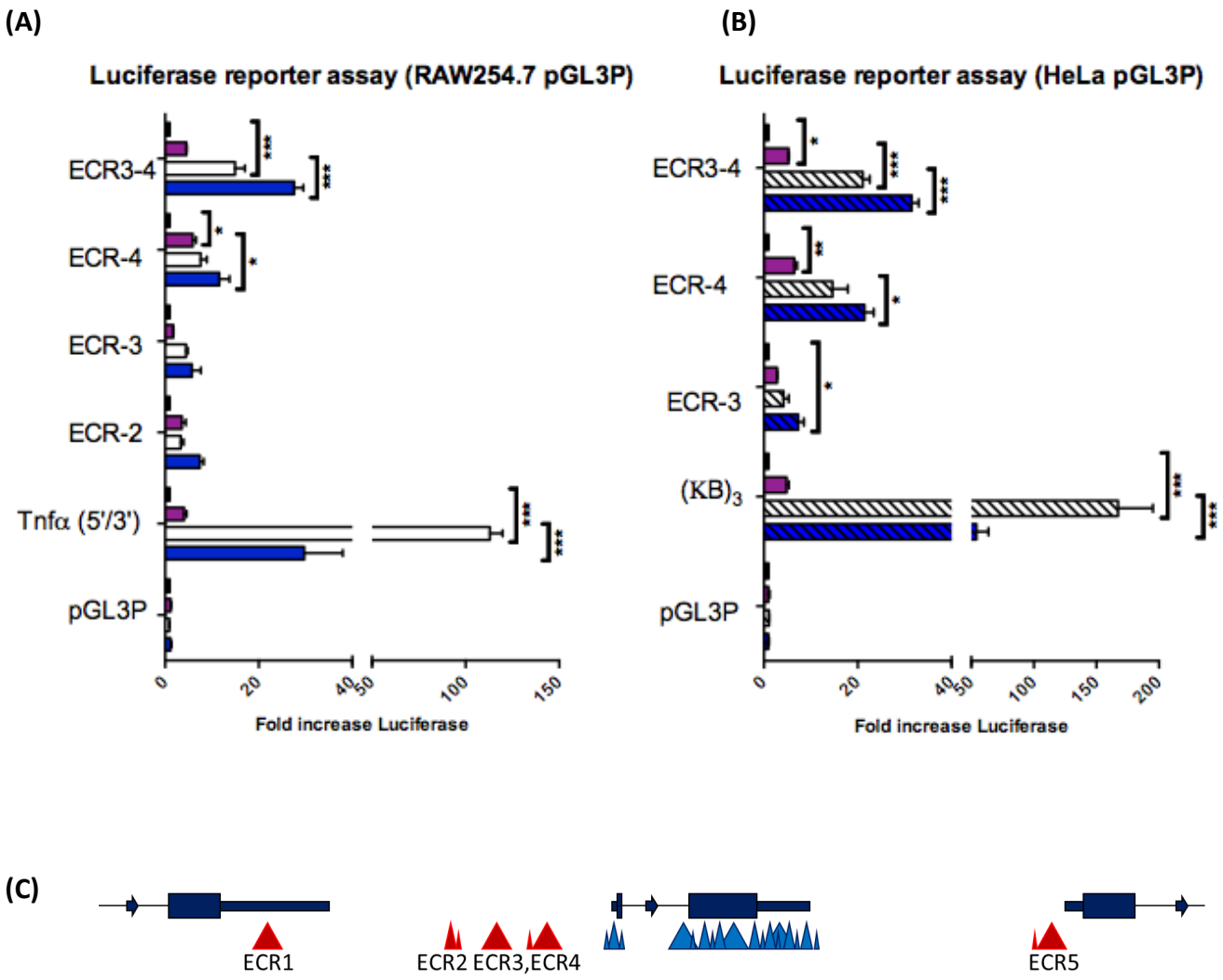


Figure 5.7 PGE2 and LPS co-induction of a luciferase reporter construct containing evolutionarily conserved regions of the Murine and Human *Zfp36* locus

(A) RAW264.7 and **(B)** HeLa cells were transiently transfected with luciferase reporter constructs containing Murine and Human *Zfp36* sequences accordingly. The following day cells were left unstimulated (black bars), activated with (A) LPS 10ng/ml (white bars) or (B) IL1α 1ng/ml (white striped bars), PGE_2 10ng/ml (purple bars) or mutually (A-blue bars/ B-blue striped bars) for an optimum of 6 hours. Following passive lysis, luciferase assays were performed. Data are expressed as mean ± SEM for three separate experiments, each carried out in triplicate for RAW264.6 and HeLa cells. **(C)** is a schematic of the *Zfp36* locus illustrating the location of each ECR.

5.3 Discussion

In the present study, we investigated the effects of PGE_2 on pro- and anti-inflammatory gene expression in primary human and murine macrophages and RAW 264.6 cells. We also studied the role of *Dusp1* in mediating those effects of PGE_2.

The results of this work present PGE_2 as an anti-inflammatory agent with the ability to down-regulate macrophage pro-inflammatory gene expression and cytokine secretion when in combination with LPS. Additionally, in the same experiments, PGE_2 was able to up-regulate three critical feedback node genes at the mRNA level when administered alone and had a cooperative effect when combined with LPS.

These data provide evidence pertaining to a potential mechanism for PGE_2-mediated immune-suppression. Most critically, PGE_2-mediated down-regulation of inflammatory mediators in activated macrophages relied strongly on the expression of *Dusp1*. Concurrently so did the cooperative regulation of *Zfp36* and *Tnfaip3*. Similarly in a recent study, a PDE4 inhibitor named Rolipram was shown to require *Dusp1* in facilitating its anti-inflammatory actions. A handful of other studies have implied PGE_2 mediated up-regulation of *Dusp1* in Fibroblast and epithelial cell lines and mouse peritoneal macrophages (Zhang et al. 2008; Brion et al. 2011; Lee et al. 2012; Korhonen et al. 2013). However, here we have identified this mechanism in primary human and murine macrophages and also quantified the cooperative up-regulation of *Dusp1*, *Zfp36* and *Tnfaip3* by LPS and PGE_2.

The promoter of *Dusp1* is known to contain two cAMP regulatory elements and studies have confirmed that cAMP mediated induction of *Dusp1* is via the PKA/CREB pathway. Agonists of cAMP have been widely successful in the treatment of asthma and their therapeutic actions

rely in part on posttranscriptional control of gene expression. Indeed, in a number of in vitro studies identified that TTP was required for the functional down-regulation of numerous pro-inflammatory mRNAs. However, analyses of sequence elements in the TTP promoter of mouse human and rat have not identified cAMP response element consensus sequences. One study, by Jalonen et al. (2007) suggests that cAMP enhancing compounds might manipulate *Zfp36* transcription through AP2. Required for full serum inducibility of TTP, a number of AP2 binding sites border the *Zfp36* region, particularly within the promoter and intron. Furthermore, the same study highlighted that compounds inducing cAMP-mediated *Zfp36* mRNA expression facilitated nuclear translocation of AP2 but not NFκB. Downstream of EP4, *Zfp36* transcription could also be enhanced by PI3K activation and C/EBPβ mutual recriutment of CBP (CREB) and p300, therefore eliminating the need for proximally located CRE sites. In the second element to this chapter, we show that PGE_2 may initiate transcription via sequences isolated from distally upstream islands of conservation. Up-regulation of luciferase reporters by PGE_2 was mediated by murine ECRs 2 and 4 and human ECR 4. Which suggests that additional cAMP activated regulatory elements lie within these regions. Additionally, the juxtaposition of ECRs 3 and 4 triggered cooperatively induced transcription in cells co-treated with LPS and PGE_2, despite the inability of ECR3 to initiate transcription independently. Sequence analysis identifies conserved sequences for AP2(x2) in ECR3 and for CREB(x1) in both ECR3 and 4, the latter of which encompasses a number of AP2-like DNA motifs also. These important sequences are illustrated in figure 5.8.

Tnfaip3 is a potential target for numerous effectors of inflammatory and apoptotic pathways, each of which presenting with different signalling affinities depending on cell type and stimulus type, effect and duration. In this chapter we show that PGE_2 has divergent

effects on LPS-induced *Tnfaip3* induction depending on stimulus duration. It would appear that the cooperation between LPS and PGE_2 observed at 1 hour in WT BMMs requires *Dusp1*. The synergism observed in WT at 2 hours however is maintained and enhanced in *Dusp1-/-* macrophages, yet PGE_2 dampens the LPS response at 4 hours post stimulation. The transcription of Tnfaip3 is strongly dependent on NFκB (Chapter 3, Figure 3.4), most prominently at the 1-hour time point. As is often the case, it is possible that *Tnfaip3* mRNA up-regulation depends on NFκB associations with accessible coactivators of transcription. The activated macrophage is subject to dynamic alterations in cellular microenvironment and thus the ready availability of various signalling effectors including coactivators and corepressors of transcription. Therefore it is likely that during the time course of LPS-induced *Tnfaip3* expression, NFκB interacts with a number of different transcription factors which together control *Tnfaip3* expression. Lai et al. (2013) identified that C/EBPβ enrichment at the *Tnfaip3* promoter was most significant after 4 hours of LPS stimulation and at this time cooperated most strongly with NFκB. If C/EBPβ availability is dependent on DUSP1 then, putatively, in *Dusp1-/-* cells, PGE_2 could be inhibitory to *Tnfaip3* transcription, which is most prevalent at the 4-hour time point. Another factor to consider is the phosphorylation status and activation of TTP in cells lacking DUSP1. The *Tnfaip3* may be a posttranscriptional target of TTP (Verstrepen et al. 2014; Bakheet et al. 2006; Balkhi et al. 2013) and in the absence of DUSP1 is likely subject to increased degradation due to prolonged TTP activation. The expression if *Zfp36* is biphasic and dips at 2 hours post induction in both WT and *Dusp1-/-* macrophages. We observed cooperative expression of *Tnfaip3* at 2 hours in both WT and KOs and therefore the mechanism at this time point doesn't require DUSP1 expression. It is possible that *Tnfaip3* mRNA stability and therefore expression is increased at 2 hours due to a reduction in TTP expression.

The next step in this chapter would be to carry out a *Zfp36* locus ChIP using antibodies specific for CBP/P300 in macrophages activated with both agonists individually and in conjunction. We expect that such an experiment would enrich for DNA within ECR 4 in the presence of PGE_2 and both ECR3 and 4 in cells stimulated with LPS and PGE_2.

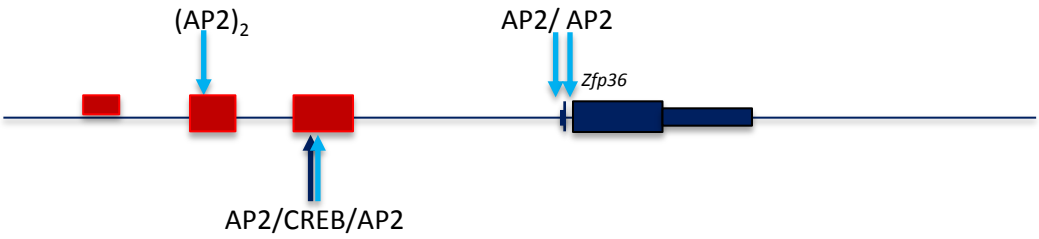


Figure 5.8 Putative cAMP- induced binding sites within the *Zfp36* locus

In addition to previously identified binding motifs for AP2 within the promoter and intron, sequence analysis predicts binding sites for AP2 within ECR3 and possibly ECR4, both of which were transcriptionally responsive to PGE2 stimulation. There are also a putative CREB motifs located in ECRs 3 and 4, the patter of which is surrounded by the aforementioned novel AP2-like binding sequence.

The findings within this chapter are novel and potentially important, regarding therapeutic intervention. In order to continue investigating the biological effect of PGE_2 on FNG expression and truly understand the functional consequences, it would be useful to look at the pattern of protein expression for each TNFAIP3 and DUSP1 as well as TTP. In addition, for the purpose of identifying the signalling pathways involved it might be beneficial to utilise chemical inhibition or even siRNA knockdown of some implicated pathways. Elevation of Dusp1 by cAMP-agonists may be dependent on PKA (Brion et al. 2011; Lee et al. 2012). Similarly PDE4 inhibition might reveal that this pathway also applies to up-regulation of

other FNGs, *Tnfaip3* and *Zfp36*. Some studies support a role for the PI3K/AKT pathway in PGE_2 signalling via EP_4 and ERK1/2 (Fujino et al. 2003; Fujino et al. 2005). *Dusp1* acts primarily on MAPKs p38 and JNK (Abraham & Clark 2006) and therefore this putative mechanism might help to explain the up-regulation of FNG expression at the level of transcription in *Dusp1-/-* macrophages, regardless of mRNA stability.

6.0 EFFECTS OF TGFβ IN INFLAMMATORY RESPONSES OF MACROPHAGES

6.1 Introduction

TGFβ is a multifunctional cytokine and growth factor capable of modulating gene expression via signalling thorough two trans-membrane serine/ threonine receptors, TβRI and TβRII (Shi & Massagué 2003; Kamato et al. 2013). Activation of TβRI may be direct or secondary to TβRII activation and subsequent trans-phosphorylation. Either way, TGFβ receptor stimulation induces signal specific smads 2 and 3 by phosphorylation to form hetero-oligomeric complexes with smad4, triggering translocation to the nucleus (Shi & Massagué 2003; Zi et al. 2012). Once nuclear, smad complexes may activate gene transcription either directly or by associations with additional sequence specific transcription factors. N-terminal MAD homology (MH) domain 1 is primarily responsible for smad DNA binding and direct control of gene regulation whereas MH2 facilitates TGFβ-mediated transactivation and often associates with CBP/P300 (Tan et al. 2004).

In normal epithelia, TGFβ functions through smad dependent and independent pathways to control the expression of numerous genes (Achyut & Yang 2011). Given that many targets of TGFβ are growth-promoting and immunomodulatory, it is important that they are controlled at both the transcriptional and posttranscriptional level. An important function of the TGFβ signalling pathway is the stringent control of these potentially injurious growth factors and cytokines and many of its target genes contain AREs in their 3'UTR (Zavadil et al. 2001; Kang et al. 2003; Kanies et al. 2008). The potent immunosuppressive functions of TGFβ have been highlighted in studies in which smads and/ or TGFβ have been disrupted in mice, resulting in a severe and multifocal inflammatory response in viable pups (Yang et al. 1999; Roberts et al. 2003; Shull et al. 1992; Kulkarni et al. 1993; Levéen et al. 2002).

Originally identified as a target of TGFβ1 and so called TGFβ-activated kinase 1, TAK1 is key to the regulation of numerous biological processes including development, innate immunity, cell survival and carcinogenesis (Roh et al. 2014). TAK1 is now a known downstream target of TGFβR, TNFR1, IL1R, T cell and B cell receptor signalling (Huang et al. n.d.). Deletion of TAK1, and its associated proteins TAK1-binding protein (TAB) 1, 2 and 3 is embryonic lethal, highlighting a critical role for this pathway in development (Komatsu et al. 2002; Sanjo et al. 2003; Shim et al. 2005). The activity of TAK1 is mostly controlled by posttranslational modifications including phosphorylation and ubiquitination, which depend on the upstream pathway that is engaged. TAK1 belongs to a highly conserved protein family of MAP3Ks and acts as an upstream stimulatory molecule to JNK, p38 MAPK and NFκB (Singhirunnusorn et al. 2005). In response to cellular stressors, TAB1 associations increase the kinase activity of TAK1 whereas TAB2 and the closely related TAB3 facilitate interaction with TRAF2 and TRAF6 in response to TNFα and IL1 respectively (Ishitani et al. 2003; Singhirunnusorn et al. 2005). TAK1 is also subject to stimulation by K63 ubiquitination in a TRAF6 dependent manner (Freudlsperger et al. 2013; Chen 2012). Following polyubiquitination, TAK1 becomes an IKK kinase and phosphorylates IKKβ leading to the dissociation, ubiquitination and degradation of IκBα that precedes nuclear translocation of NFκB. Ubiquitinated TAK1 may also phosphorylate MAP2Ks including MKK6/3 and MK74/7 leading to p38 MAPK and JNK activation respectively (Wang et al. 2001; Chen 2012).

A handful of studies have identified the ability of TGFβ to up-regulate *Zfp36* expression in epithelial cells (Blanco et al. 2014; Sohn et al. 2010) and T-cells (Ogawa et al. 2003). Sohn et al. (2010) identified that TTP is frequently down-regulated in hepatocellular carcinoma (HCC) due to methylation at a specific CpG site located within the TGFβ-responsive region of the

Zfp36 promoter. Methylated TRR was found to be associated with a transcription repressor complex which proved sufficient to block TGFβ- mediated and basal levels of Zfp36 induction (Sohn et al. 2010).

Blanco et al. (2014) on the other hand showed that smad-dependent expression of TTP was required for the formation of p-bodies in intestinal epithelial cells stimulated with TGFβ. A smad-responsive element has been identified immediately upstream of the *Zfp36* promoter -550bp from the start site that responds to TGFβ signal transduction in epithelial cells (Sohn et al. 2010). In this study we set out to identify the effects of TGFβ on feedback node gene expression and immunomodulation during an inflammatory response and therefore in macrophages activated by LPS.

6.2 Results

6.2.1 TGFβ inhibits pro-inflammatory mRNA expression in activated primary human and murine macrophages and RAW264.7 cells.

Following on from work by (Ogawa et al. 2003) and the work in this study we carried out stimulation time course experiments in which primary and cell line macrophages were un-stimulated, activated by LPS, TGFβ or the two agonists together. Isolation of RNA was followed by cDNA synthesis and quantitative PCR using a selection of primers targeted at transcripts of interest. We set out to investigate the immunosuppressive effect of TGFβ on pro-inflammatory mediators in macrophages.

Whereas TGFβ had a small inhibitory effect on very low levels of resting TNFα and IL1α mRNA expression, LPS induced both pro-inflammatory mediators strongly (Figure 6.1). LPS induction of TNFα and IL1α was transient and peaked within 1 hour of LPS incubation in all types of macrophage. When treated with LPS and TGFβ in combination, macrophages showed a significant decrease in TNFα and IL1α mRNA that was maintained until pro-inflammatory mRNA expression had dissipated or the last measured time point (8 hours).

Known for its immunosuppressive actions, here we show that administration of exogenous TGFβ is capable of dampening the expression of two key inducers of inflammation in human and murine primary macrophages as well as in RAW264.7 cells. It would be useful as a next step to explore whether this significant effect translates to a functional output at the protein level.

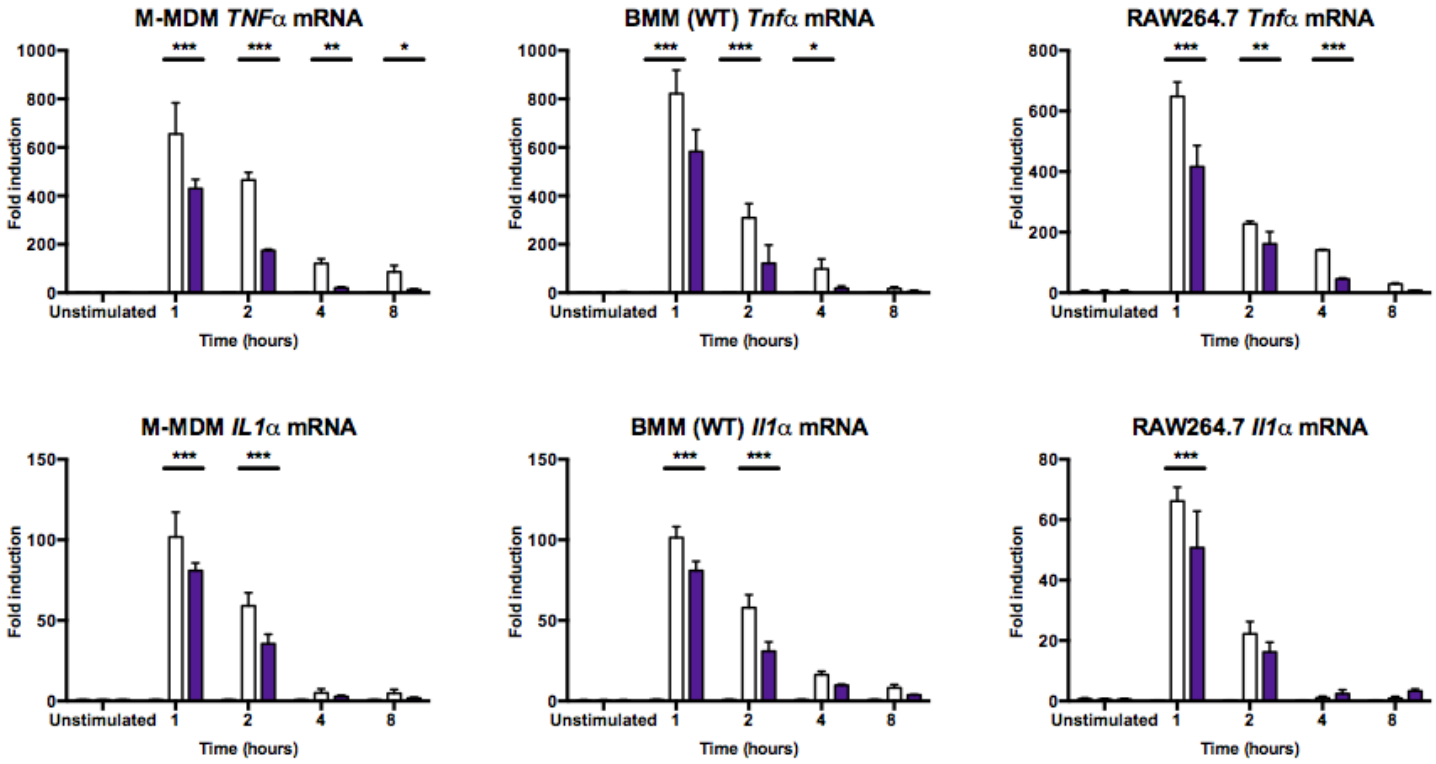


Figure 6.1 Inhibition of TNFα and IL1α gene synthesis by TGFβ in primary macrophages and RAW264.7 cells activated with LPS

Human monocyte-derived and murine bone-marrow derived macrophages (M-MDM and BMM) and RAW264.7 murine macrophage-like cells were seeded at a density of $1x10^6$cells/ml and $5x10^5$cells/ml respectively and either left un-stimulated (black bars), treated with 10ng/ml TGFβ (green bars), 10ng/ml LPS (white bars) or LPS and TGFβ (blue bars) for the times indicated. All data points were normalised to the unstimulated control and show the mean +/- SEM for three and four separate experiments in primary and RAW macrophages respectively. Each QPCR was carried out in triplicate. *** p< 0.001 **p<0.01 *p<0.05

6.2.2 TGFβ and LPS cooperatively regulate feedback node genes DUSP1, ZFP36 and TNFAIP3 expression at the mRNA level

Previous work in this study has highlighted a group of feedback node genes that escape inhibition by inflammation-quenching factors that reduce inflammatory signalling. As seen in 6.2.1, TGFβ reduced the expression of key pro-inflammatory mRNAs. It is possible that TGFβ facilitates up-regulation of critical negative-regulators of inflammation in order to deliver its immunosuppressive functions. Here we quantified the LPS and TGFβ –induced expression of feedback node gene mRNA transcripts in macrophages. As can be seen in Figure 6.2 TGFβ alone was able to induce *Dusp1* in small quantities in primary macrophages. However, when administered in combination with LPS, the effect on *Dusp1* mRNA expression was cooperative suggesting that the two agonists act synergistically to increase *Dusp1* transcription. This effect was mirrored in the expression profile of *Zfp36* mRNA (Figure 6.2). TGFβ plus LPS had a significant inducing effect on *Zfp36* transcription, above that of LPS alone at 1 and 4 hours of co-incubation. TGFβ has previously been described as able to induce *Zfp36* transcription in epithelial cells. The cooperative effect between LPS and TGFβ was less prominent when quantifying *Tnfaip3* mRNA expression (Figure 6.2). However cooperative regulation of *Tnfaip3* was clear in both human primary macrophages and RAW cells at the 1-hour time point.

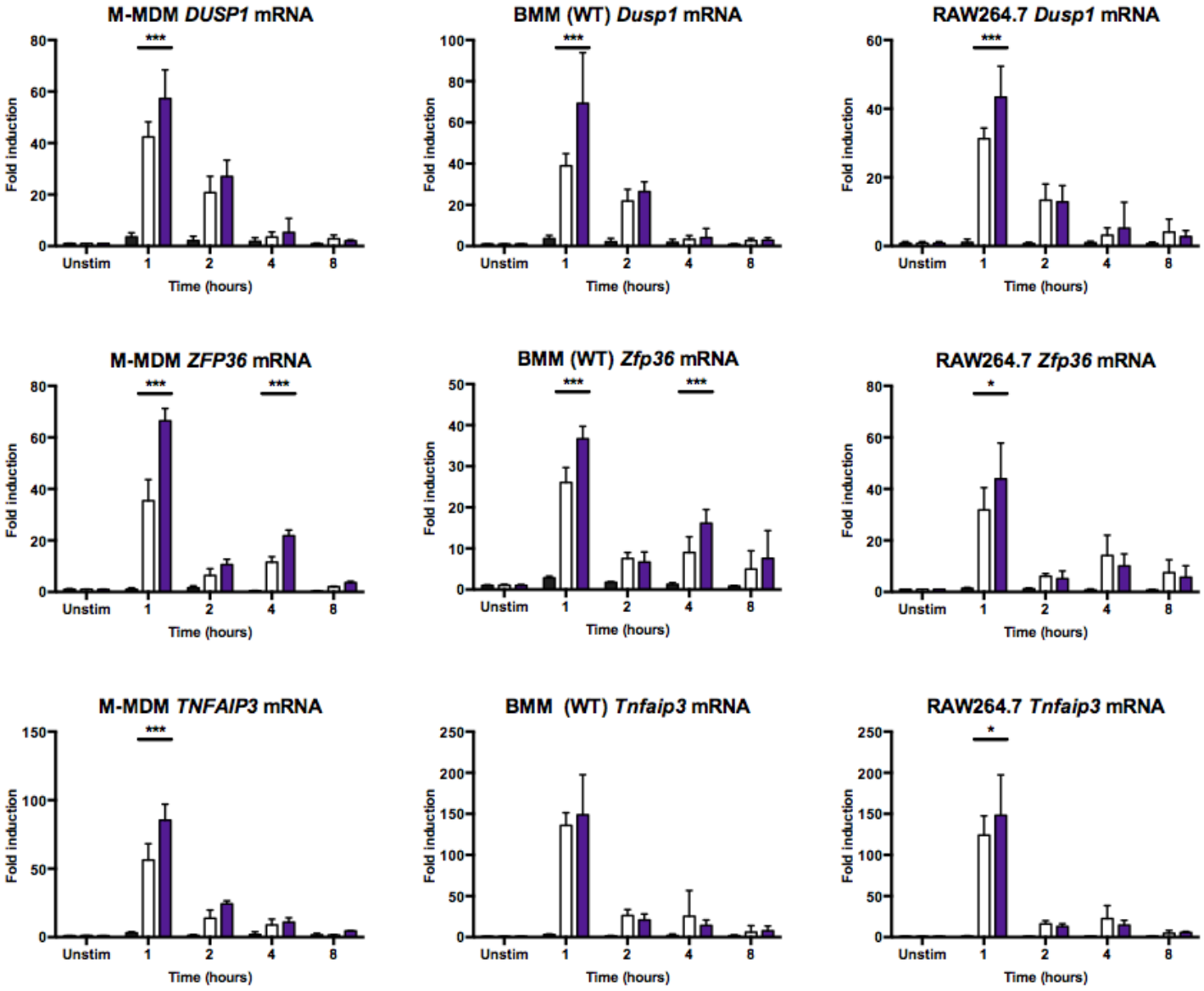


Figure 6.2 Cooperative regulation of feedback node gene expression in macrophages by TGFβ and LPS

In parallel with 6.2.1, the level of feedback node gene mRNA was quantified by QPCR in macrophages untreated or treated with LPS or LPS and TGFβ for the times indicated. All data points were normalised to the unstimulated control and represent the mean ± SEM for three and four separate experiments in primary and RAW macrophages respectively. *** p< 0.001 *p<0.05

6.2.3 TGF activates transcription in a reporter construct containing Zfp36 upstream conserved regions 3 and 4

RAW macrophages and HeLa cells transfected with murine and human reporter constructs were treated with LPS or IL1α, TGFβ or a combination for 8 hours before passive lysis. Luciferase assay reagent was added to lysates and the resulting fluorescence was measured using a luminometer. We have shown previously the capability of LPS and IL1 to up-regulate luciferase transcription via murine and human ECRs upstream of *Zfp36*. Initial observation drew attention to the effect of TGFβ on LPS and IL1 induction of the RAW and HeLa positive control reporters (Figure 6.3). Simultaneous stimulation of RAW or HeLa cells transfected with a TNFα enhancer or $(KB)_3$ elements inhibited luciferase expression by approximately one third and by more than half respectively, when compared to LPS/IL1α treatment alone. There is little recent data highlighting the inhibition of NFκB by TGFβ which makes this observation potentially quite an important one. One study by Arsura et al. (1996) demonstrates that TGFβ significantly down regulates the binding activity of NFκB at 6 hours, with maximal inhibitory effects observed at 9 hours post treatment. The same study highlights the potential of TGFβ to induce Iκβ mRNA synthesis (Arsura et al. 1996). Similarly GCs are also known to up-regulate Iκβ expression as a means of NFκB inhibition (Auphan et al. 1995; Robert I. Scheinman et al. 1995; Castro-Caldas et al. 2003). This notion of NFκB inhibition by TGFβ is somewhat controversial to studies describing TGFβ-mediated up-regulation of NFκB activity via smad independent mechanisms (Gingery et al. 2008).

Taking into consideration the experimental ECR reporters, we show that TGFβ is able to induce luciferase transcription in cells transfected with constructs containing murine and human ECRs 3, 4 and 3-4 (Figure 6.3). However murine ECR2 showed no transcriptional activity in response to TGFβ.

Simultaneous stimulation of RAW or HeLa cells transfected with a TNFα enhancer or $(KB)_3$ elements inhibited luciferase expression by approximately one third and by more than half respectively, when compared to LPS/IL1α activation alone. These results confirm the inhibitory potential of TGFβ and that signalling in the transfected cells remains intact (Figure 6.3).

However, the transcriptional activity directed by *Zfp36* ECRs 3, 4 and ECR 3-4 from both species was significantly enhanced in cells treated with the two agonists simultaneously. The respective increase in luciferase expression as a result of dual stimulation was mostly additive of the two agonists in cells containing constructs 3 and 4. However, HeLa cells containing HsECR3-4, that is a luciferase expression vector containing both ECR3 and 4 juxtaposed, produced a much greater amount of fluorescence in response to co-treatment that was multiplicative of either IL1α or TGFβ alone.

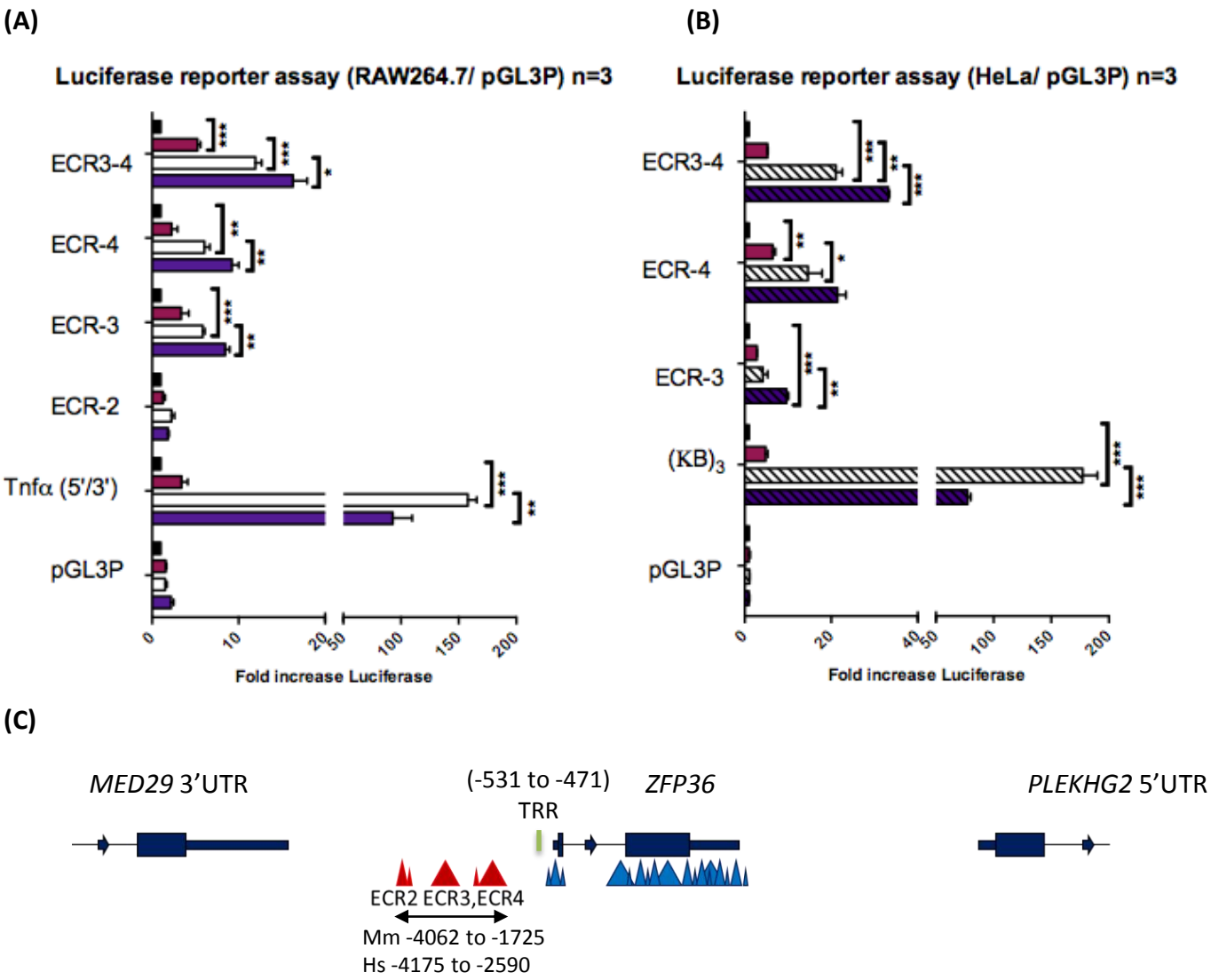


Figure 6.3 Human and murine *Zfp36* ECRs respond to transcriptional regulation by TGFβ and TGFβ plus LPS in HeLa epithelial and RAW macrophage like cells

(A) RAW264.7 and **(B)** HeLa cells were transiently transfected with luciferase reporter constructs. The following day cells were left un-stimulated (black bars); RAW cells activated with 10ng/ml LPS (white bars) and HeLa cells with 1ng/ml IL1α (white-striped bars); 10ng/ml TGFβ (green bars) or in combination (blue bars for RAW and Blue striped bars for HeLa) for an optimum of 8 hours. Following passive lysis, luciferase assays were performed. Data are expressed as mean ± SEM for three separate experiments. Cartoon **(C)** represents murine evolutionary conservation against a human-base genome and the ECRs 2-4 which were cloned for the reporter constructs above are represented here in red (blue is conservation of the Zfp36 coding sequence). Location of the 'TRR' identified by Sohn et al (2010) is shown in green.

6.3 Discussion

In this chapter we showed that TGFβ has the potential to up-regulate the expression of three feedback node genes, *DUSP1*, *ZFP36* and *TNFAIP3*, and cooperates synergistically with the TLR agonist LPS to enhance and extend their expression further. Two groups previously highlighted that TGFβ increases DUSP1 protein in rat vascular smooth muscle and human epithelial cell lines (Tong & Hamel 2007; Mikami et al. 2006). Here we have shown that TGFβ not only increases *DUSP1* mRNA but also enhances and extends the LPS-induced expression of *Dusp1* transcript in primary macrophages and RAW cells. Additionally, TGFβ has been shown to up-regulate A20 protein via tyrosine phosphorylation and cause further inhibition of NFkB signalling (Das et al. 2012). Neither *Tnfaip3* nor *Dusp1* have been investigated for smad binding elements located within their promoter, or indeed, further up-stream. Clearly this area needs some further investigation. Alternatively (and without mutual exclusivity), it is possible that TGFβ increases transcription of these two targets in a smad-independent manner or even by modifying the chromatin environment to facilitate the binding of other co-activators of gene synthesis. TTP is induced by various stimuli, including TGFβ (Lai et al. 1990; Ogawa et al. 2003) and a TGFβ regulatory region (TRR) between -531 and -471 contains four functional smad binding elements (Sohn et al. 2010). However, in a luciferase assay, these promoter-proximal smad sequences were only able to elevate fluorescence 1.5 to 2 times that of the un-stimulated, basal expression. In a previous study, TGFβ raised *Zfp36* mRNA levels more than 4-times basal expression in epithelial cells (Blanco et al. 2014) and 5 to 6-times in primary bone marrow macrophages (Figure 6.2). These data suggest that there are other important sequences required for full TGFβ inducibility of *Zfp36*. Our results indicate that signalling through the TGFβ /smad pathway promotes *Zfp36* transcription via enhancers positioned distally upstream. Although between these highly conserved regions

there are no conserved consensus smad binding elements, sequence analysis predicts smad binding within HsECR4 (+82), MmECR2 (+29) and MmECR3 (+181).

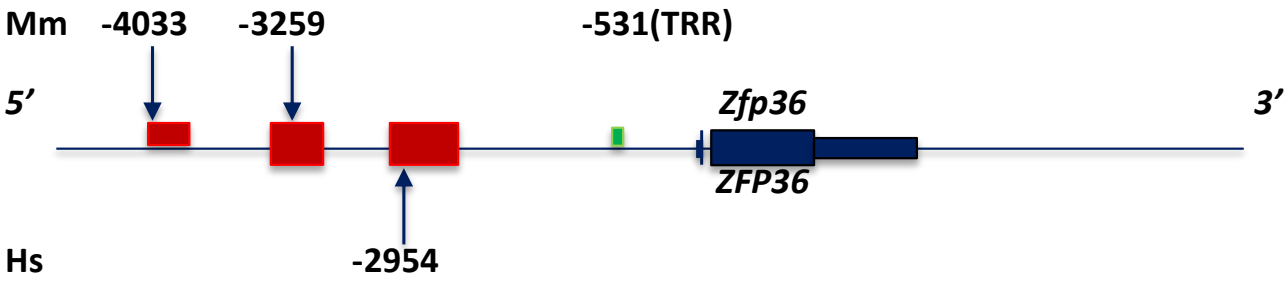


Figure 6.4 Predicted smad binding elements within evolutionarily conserved regions upstream of murine and human ZFP36

The above cartoon demonstrates the location of each predicted smad binding sequence upstream of *Zfp36* in mouse (Mm-above) and human (Hs-below) sequence. The 'TRR' identified by Sohn et al (2010) is in green. There are two putative *Zfp36* upstream smad binding elements in the murine sequence, located within ECRs 2 and 3 at -4033bp and -329bp respectively. The human sequence contains one predicted smad motif within ECR4 at position -2954bp.

Unfortunately it was not possible to carry out ChIP following TGFβ stimulation, due to time constraints. Sohn et al. (2010) used antibodies specific to RNAPII C-terminal phosphorylation status to identify the effect of TGFβ on Zfp36 transcription pre-initiation (un-phosphorylated RNAPII), promoter escape (serine-5 phosphorylation) and elongation (serine-2 phosphorylation) in epithelial cells. They found that RNAPII binding was enhanced at the each stage of *Zfp36* transcription in response to TGFβ and DNA was specifically enriched for in the promoter region and the 3'UTR by the un-phosphorylated, phosphor-ser5 and phosphor-ser2 forms respectively. RNAPII was not found to associate within the TRR domain (Sohn et al. 2010). However, the TRR is associated with H3 and H4 acetylation in response to TGFβ stimulation. More often, it is acetylation of histones within the promoter region of target genes that couples to enhanced transcript synthesis (Clayton et al. 2006). In fact Ross et al. (2006) identified that TGFβ-mediated transcriptional activation requires promoter-located H3 acetylation. We hypothesise that due to the complexity of *Zfp36* transcriptional regulation and the compactness of its locus that the majority of intragenic conserved sequence is required for full inducibility. Therefore we expect that a ChIP of the whole locus using antibodies specific for histone acetylation patterns would enrich for ECR3 and ECR4 in response to TGFβ and TGFβ plus LPS. However ECR2 was not responsive to TGF stimulation and therefore is not likely to play a role in TGF-mediated *Zfp36* up-regulation.

These experiments were undertaken close to the end of the project. However, given the time and resources, I would wish to follow up by examining their biological significance. Firstly it would be valuable to identify protein regulation by TGFβ and pro-inflammatory stimuli. The cooperative effect of TGFβ and LPS was subsequently confirmed at the protein level for TTP in murine primary macrophages by western blot, using an antibody for SAK21b

(data not included). The synergistic effects of co-stimulation on *ZFP36* mRNA production were greatest in human primary macrophages, therefore it would be interesting to identify whether this was also true for TTP protein biosynthesis. Furthermore, provided with the appropriate antibodies, to probe for DUSP1 and TNFAIP3 protein is also key to validation of the biological importance of FNG regulation by TGFβ alone and in the presence of an inflammatory stimulant. Similarly, the inhibitory effect of TGFβ on TNFα and IL1 activation by LPS should also be investigated at the level of protein, this could be done by ELISA. The robust inhibition of NFκB-driven positive control reporter constructs in Figure 6.3 is a potentially novel finding and if also confirmed endogenously and at the level of protein expression could be particularly important. Through activation of TAK1, TGFβ is generally considered to have a positive effect on NFκB activation. However it would appear that TGFβ may have negative effects on NFκB targets, at the level of transcript expression.

7.0 DISCUSSION

7.1 Methodology

This study was conducted to investigate the regulation of expression of three feedback node genes (FNGs) induced by pro-inflammatory stimuli in macrophages, with a particular focus on TTP. Macrophages are central to inflammatory signalling and play a significant role in the pathogenesis of chronic inflammatory syndromes including rheumatoid arthritis, asthma and cancer (Welte & Groneberg 2006; Sabroe & Whyte 2007; Szekanecz & Koch 2007). LPS has been shown to induce the expression of DUSP1, A20 and TTP at the level of mRNA and protein in murine bone marrow derived macrophages (BMMs), the transformed macrophage cell line RAW264.7 and in primary human macrophages. Induction of DUSP1 and TTP expression in the human epithelial HeLa and A549 cell lines however is via stimulation with IL1α. The use of primary murine and, particularly human macrophages strengthens this study in terms of applicability to human physiology. In the majority of endogenous mRNA experiments, the expression patterns observed were recapitulated not only between primary cells and cell lines of equivalent species but also between species. However there were some discrepancies in gene induction between primary human macrophages and the human cell lines and this may have been due to differences in cell type or inflammatory stimulus used i.e. LPS or IL1α. In addition there was some contrast between species in the transcriptional ability of evolutionarily conserved regions. Again however, this may be a stimulus specific effect. THP1 cells are a human leukaemic monocyte line commonly used as a model and also human derived lymphoblast lines HL60 and U937. Perhaps it would be interesting to carry out the reporter analyses in either of these cell types. However the fold induction of luciferase by pro-inflammatory stimulation of *Zfp36*

ECRs was significant, mirrored the endogenous response and yielded reliable results in both HeLa and RAW cells. HEK293s are a human kidney epithelial transformed cell line and are notoriously easy to transfect. This study focused on pro-inflammatory signalling through TLR4 and the IL1R and therefore HEK 293 cells transformed with pUNO-TLR4 and pDUO2-MD2-CD14 plasmids were used for *Zfp36* mini gene reporter assay.

Standard molecular and cellular biology techniques were used to detect and quantify mRNA (real-time quantitative PCR), protein (western blot) and cytokine (ELISA) expression. QPCR is a precise method of DNA transcript quantification and the expression levels of *Cox1*, *Dusp1*, *IL1*, *IL6 TNFα*, *Tnfaip3* and *Zfp36* were measured by this method. As is common, *Gapdh* was used for normalisation. There is tentative evidence to suggest that LPS induces *Gapdh* expression in liver and lung. However in the present study *Gapdh* levels remained constant between treatment group and time period following pro-inflammatory stimulation whereas levels for the induced genes of interest varied by several cycles between treatment groups. Cellular protein expression was detected by western blot, which is an efficient and reliable method. However, it would have been more conclusive to obtain quantitative data from densitometry of the western blots. Sandwich ELISA was used to quantify the expression of cytokines and chemokines which are secreted by macrophages and other cells of the immune system.

7.2 Involvement of NFκB in the regulation of FNG expression

Feedback node genes *Dusp1*, *Tnfaip3* and *Zfp36* are acknowledged for their rapidly up-regulated transcriptional response pro-inflammatory agonists such as LPS. The extent of NFκB involvement in these mechanisms however, is generally ill understood. This study provides evidence that although *Tnfaip3* mRNA synthesis is strongly dependent on NFκB signalling pathways, the expression of both *Dusp1* and *Zfp36* transcripts was only moderately affected by chemical inhibition of NFκB. This result was contrary to findings by Chen et al 2013 and King et al 2009 who showed a strong dependency of TTP mRNA expression on NFκB signalling in the RAW 264.7 and A549 cell lines respectively. *Dusp1* is induced by the stress response, serum and pro-inflammatory stimuli such as IL1 (Li et al. 2001; Lasa et al. 2002; Toh et al. 2004; Charles et al. 1993). In this study, chemical inhibition of NFκB augmented LPS-induction of *Dusp1* mRNA, suggesting that NFκB is inhibitory to *Dusp1* transcription mediated by other transcription factors. Therefore it is likely that a multitude of transcription factors are involved in FNG induction in response to a variety of cellular stimuli and signalling pathways. For example *Dusp1* is subject to up-regulation by heat shock, oxidative stress (Keyse & Emslie 1992) and UV light (Li et al. 2001). Whereas TTP is induced by numerous factors including growth factors (Ogawa et al. 2003; Sohn et al. 2010; Tan & Elowitz 2014), cytokines (Suzuki et al. 2003), agonists of cAMP and even green tea and cinnamon. Indeed, Leppanen et al. (2008; 2010) conducted studies that indicate AP2-dependent as opposed to NFκB dependent mechanisms of TTP induction, in response to pro-inflammatory stimulation.

This study also highlighted the LPS-induced biphasic expression pattern of *Zfp36* transcript in human and murine primary macrophages previously observed in RAW264.7 cells by

Tchen (2004) and, more recently Chen (2013). This observation was taken further in this study and, in an attempt to characterise the mechanism of the second wave of *Zfp36* transcript expression ChIP was carried out in BMMs unstimulated or activated with LPS for 1, 2 and 4 hours. Immunoprecipitation with RelA after 4 hours of LPS stimulation implied that *Zfp36* transcript up-regulation at this time was unlikely to involve NFκB-mediated mechanisms. *Zfp36* is a target of destabilisation by its own protein product, TTP. Transient activation of p38 MAPK during the inflammatory response is inversely proportional to activation of TTP and therefore it is unlikely that the increase in *Zfp36* transcript at 4 hours is due to increased mRNA stability.

Results from this study show strong PGE_2 mediated Zfp36 expression at 4 hours post stimulation. Therefore it may be constructive to immunoprecipitate for cAMP induced transcription factors including CREB or p300 and also C/EBPβ and AP2 which are associated with cAMP signalling. However these data came late in the project and time was too limited for further investigation.

The putative enhancer elements investigated in this study may well be separately important in their actions, for example in response to different stimuli and duration of activation, as well as cell and tissue type. ECR4 contains an NFκB consensus motif and strongly induced a luciferase reporter gene in response to LPS and IL1. Predictably, this construct was inhibited by MLN4924. Whereas ECRs 2 & 3 were able to elicit a transcriptional response following cell stimulation with IL1 or LPS but are unresponsive to MLN4924. These data suggest that sequences within ECR 2 & 3 up-regulate *ZFP36* expression via mechanisms independent of NFκB and are responsible for the induction of *Zfp36* mRNA in the presence of LPS and MLN4924.

All of the FNGs described in this study have been identified as immediate early genes (IEGs) and therefore exist in a state of open chromatin that is accessible to transcription factors and associated transcriptional machinery. Even in un-stimulated primary macrophages, RNAPII is found associated with *Zfp36* upstream elements including ECRs 1 to 4 and within the promoter region. Transcription of TTP is precipitous with pro-inflammatory stimulation and transcripts can be detected as early as 15 minutes post stimulation with LPS (Brooks et al. 2004). After 1 hour of activation we observed RNAPII accumulation within the coding region and downstream, within the 3'UTR of *Zfp36*. Concurrent with mRNA quantification time course data, RNAPII association with the *Zfp36* locus is most significant at 1 hour post stimulation, is reduced at 2 hours and peaks again after 4 hours. In this study we used an antibody directed against the major subunit of RNAPII. An interesting future experiment would be to immunoprecipitate for RNAPII phosphor-serine-5, the posttranslational modification of the major subunit associated with transcriptional elongation. Phospho-serine-2 on the other hand is associated with inactive RNAPII and might be enriched upstream of *Zfp36* in unstimulated macrophages.

The focus on ChIP with primary human cells was to carry out siRNA and identify the effects on RNAPII accumulation at the human *Zfp36* locus however time constraints limited this work. Preliminary experiments confirmed RelA knock down in siRelA treated cells and association of RelA with enhancer regions in control human primary macrophages.

7.3 Endogenous expression of FNGs in response to Anti-inflammatory stimuli

Glucocorticoids are routinely used for the treatment of virtually all chronic inflammatory syndromes (Clark & Belvisi 2012; Hillier 2007) including RA (Kirwan & Power 2007; Gorter et

al. 2010; Hoes et al. 2010) and asthma (Barnes 2006). In addition they may also be prescribed for their immunosuppressive effects in patients undergoing transplantation or their antiproliferative effects in haematological malignancy (Clark & Belvisi 2012). However the mechanisms of glucocorticoid action remain largely unknown. Previous studies have highlighted the induction and importance of DUSP1 in glucocorticoid-mediated anti-inflammatory mechanisms (Clark 2007; Newton & Holden 2007; Owens & Keyse 2007). In *Dusp1-/-* macrophages, the majority of glucocorticoid-mediated anti-inflammatory effects are abrogated. This is partially due to increased pro-inflammatory mRNA stability (Lasa et al. 2001; Lasa et al. 2002; Quante et al. 2008) and in addition the continued activation of NFκB/ AP1 and pro-inflammatory gene expression (Diefenbacher et al. 2008; Bladh et al. 2009; King, Holden, et al. 2009a). Whereas the regulation of A20 mRNA expression has not been investigated in this context, previous study in elucidating the mechanism by which glucocorticoids edit the expression kinetics of *Zfp36* have largely been inconsistent (Jalonen et al. 2005; Bergmann et al. 2004; Smoak & Cidlowski 2006). We hypothesised that dexamethasone would up-regulate FNGs at the level of mRNA. Glucocorticoid stimulation induced expression of each FNG in primary human and murine macrophages as well as in cell lines. Furthermore, this effect was augmented in cells simultaneously activated with LPS or IL1. However in *Dusp1-/-* macrophages the cooperative regulation of *Tnfaip3* and *Zfp36* observed in WT cells was abolished. Indeed, as recognised by previous studies, the anti-inflammatory effects of dexamethasone on Tnfα, Il1 and Il6 were also lost in Dusp1 knock out cells. These data could suggest that glucocorticoids, via DUSP1, utilise the mRNA destabilising effects of TTP and the NFκB inhibitory actions of A20 in order to mediate their full anti-inflammatory function.

Similarly, PGE_2 a cAMP-enhancing compound was able to up-regulate the expression of all three FNGs whilst inhibiting expression of pro-inflammatory cytokines at the level of mRNA and protein secretion. Again, PGE_2 required the expression of Dusp1 to mediate its up-regulatory effects on FNG expression and, interestingly, its anti-inflammatory abilities. In agreement with previous studies (Diefenbacher et al. 2008; Quante et al. 2008; Bladh et al. 2009; King, Holden, et al. 2009b; Hong Yu et al. 2011; H Yu et al. 2011), these data further identify Dusp1 as a central mediator of negative feedback regulation of inflammation that, in all probability, acts via Zfp36 and perhaps also Tnfaip3. Indeed, the anti-inflammatory effects of cAMP-agonists have been shown to require the expression of TTP (Joe et al. 2011; Geyer et al. 2012; Jalonen et al. 2007; Brahma et al. 2012).

As aforementioned, glucocorticoids are the conventional treatment for asthma patients. However, glucocorticoid therapy is often ineffective in the treatment of pronounced inflammation of the airway, or indeed in COPD. In such cases often a combination therapy of glucocorticoids and long-acting β_2-adrenoceptor agonists (LABAs) or the PDE4-inhibitor Roflumilast is administered. This combination increases bronchodilation at the same time as promoting glucocorticoid effects in a cAMP-dependent manner. Part of this mechanism is to do with the additive up-regulation of DUSP1 mediated by the two agonists and an enhanced and extended DUSP1 expression profile is key to augmenting the signalling pathways that induce and activate A20 and TTP (as represented in this thesis as well as in Smallie et al. 2015; Ross et al. 2015).

TGFβ induced Zfp36 mRNA expression in human and murine primary macrophages and augmented the effects of LPS on all three FNGs. As well as wound healing, TGFβ is also associated with the development of cancer. TTP and A20 are reputed tumour suppressors and may play roles in TGFβ signalling. Indeed, a mutation in the TTP promoter that confers insensitivity to the anti-proliferative effects of TGFβ is associated with HCC (Sohn et al. 2010). A20 on the other hand negatively regulates TAK-1 mediated NFκB activation through ubiquitination. MAPKs are activated via TAK1 Ras and Rho A proteins downstream of TGFβ signalling (Ding et al. 2014; X.-L. Yuan et al. 2011; Gui et al. 2012; Yamaguchi et al. 1995). Therefore through induction of DUSP1 and A20, LPS and TGFβ form an anti-inflammatory and potentially anti-tumourogenic negative feedback loop.

It is clear the DUSP1 is central to the anti-inflammatory and therapeutic effects of numerous compounds. In addition it would appear that DUSP1 mediates its effect not only by inhibition of MAPK signalling but via activation of TTP and possibly A20. However what is most intriguing is the mechanism of cooperative up-regulation of each of these FNGs.

7.4 Novel putative enhancer regions and maintained and composite transcription factor binding sites

Although the majority of transcriptional studies investigating Zfp36 have focused on the promoter region, here we have potentially identified a number of novel transcription factor binding motifs and putative enhancer sequences located distally upstream of the Zfp36 sequence. The evolutionarily conserved regions described here were responsive to pro-inflammatory stimuli in both human and murine cells and are likely to be involved in Zfp36 induction in vivo. The compound ECR 3-4 was transcriptionally responsive to anti-inflammatory stimulation by dexamethasone, TGFβ and PGE_2 in both human and murine cells. Furthermore co-stimulation with pro-inflammatory and anti-inflammatory stimuli was able to cooperatively induce transcription from ECR3-4. ECR2 on the other hand did not respond to either agonist alone, however was cooperatively activated by co-stimulation with inflammatory agonist and either dexamethasone or PGE_2. ECR2 contains a GR-like motif which is occupied in the presence of dex. During combined treatment with dex, this motif was bound by both GR and RelA in primary murine macrophages. Similarly, ECR 4, the promoter and intron were all found associated with both GR and RelA during co-treatment. Of these composite sites, only ECR4 contains an NFκB binding motif. These data suggest that mutual binding of NFκB and GR at novel, context dependent sites induces Zfp36 transcription in a cooperative manner. This kind of context-dependent regulation of gene expression in response to GC and LPS co-stimulation has been observed in RAW cells at an upstream region of TNFAIP3 (Altonsy et al. 2014). Both ECR1 and ECR5 contain glucocorticoid elements and are associated with GR under conditions of dexamethasone, as is the 3'UTR.

A TGFβ responsive region (TRR) was identified upstream of *Zfp36* and mutations to this CpG-rich sequence associate with HCC (Sohn et al. 2010). Additionally, the promoter region of *Zfp36* contains a functional smad element. In this study we identified two TGFβ-driven transcriptionally responsive sequences further upstream of these sites, within ECR3 and 4 which responded to TGFβ individually and were cooperatively activated by LPS and TGFβ co-stimulation. Sequence analyses of the *Zfp36* locus do not highlight putative smad motifs within these evolutionarily conserved regions. However, as can be seen from the ChIP with GR in dex and lps+dex activated macrophages, smad/ TGFβ-induced transcription factor binding *in vivo* cannot be ruled out. It would be of great benefit to carry out chromatin immunoprecipitation of the *Zfp36* locus with antibodies against smads2/3 in order to confirm in vivo up-regulation of *Zfp36* in response to TGFβ and combination treatment of TGFβ and pro-inflammatory stimulus LPS. Similarly, this would be a beneficial experiment to do with regards to PGE_2-mediated regulation of *Zfp36*.

A number of studies have identified consensus motifs for the cAMP-responsive transcription factor AP2 in the TTP promoter (DuBois et al. 1990; Heximer & Forsdyke 1993; Lai et al. 1995; Kaneda et al. 2000) and intron (Lai et al. 1998). Deletion of the AP2 site within the promoter significantly reduced the serum inducibility of TTP in NIH3T3 cells (Lai et al. 1995). Inducers of cAMP-signalling pathways include β_2 agonists, forskolin and Db-cAMP which have been shown to upregulate TTP mRNA and protein expression in 3T3 fibroblasts, rat secondary astrocytes and PC12 pheochromocytoma cells (DuBois et al. 1990; Arenander et al. 1989; Kaneda et al. 1992). Transcription factor AP2 is activated downstream of cAMP and numerous studies have identified the presence of AP2 consensus motifs within the TTP

promoter (DuBois et al. 1990; Heximer & Forsdyke 1993; Lai et al. 1995; Kaneda et al. 2000) and intron (Lai et al. 1998). Deletion of the promoter but not the intron AP2 consensus sequence significantly reduced serum responsiveness of *Zfp36* expression. Forskolin induces the nuclear translocation of AP2 and this has been postulated as a mechanism for cAMP-mediated TTP up-regulation. In addition to these factors and PGE_2

Given the evidence supporting cAMP up-regulation of and functional dependency on Dusp1 it would also be very interesting to investigate the in vivo interactions between C/EBP, P300 & other cAMP associated transcription factors and the Dusp1 locus.

7.5 Summary and Conclusions

Feedback node genes (FNGs) are essential for negative feedback control of inflammatory responses and by definition are controlled by both pro- and anti-inflammatory stimuli. In this study, I have shown that the pro-inflammatory induction of three FNGs is cooperatively induced by anti-inflammatory agonists dexamethasone, PGE_2 and TGFβ. In addition, experiments in primary mouse knock-out macrophages suggest that Dusp1 may be required for the anti-inflammatory effects of PGE_2 and indeed other anti-inflammatory agents.

Finally, I identified three novel putative enhancer sites upstream of the *Zfp36* locus that also demonstrated cooperative transcriptional regulation by various combinations of pro- and anti-inflammatory agonists (Figure 7.1). Further to this, Chromatin immunoprecipitation highlighted dynamic remodelling of the *Zfp36* locus in response to pro-inflammatory stimuli and the anti-inflammatory agonist dexamethasone.

There is increasing evidence to suggest that modulation of the expression of FNGs could be a powerful therapeutic strategy. Therefore it is important to gain more knowledge of the mechanisms involved in regulation of anti-inflammatory feedback node genes such as DUSP1, TTP and A20. Not only for the purpose of understanding current therapies but also to develop the potential for novel gene-targeted techniques.

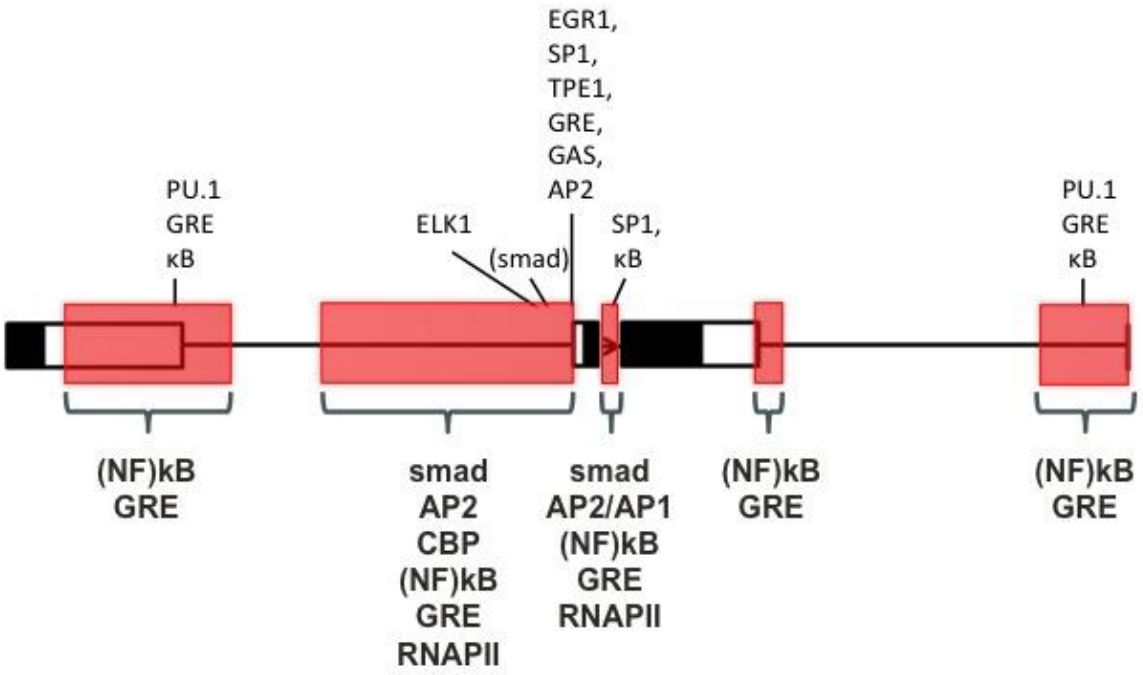


Figure 7.1 Novel regulatory elements and super-enhancer sequences involved in the complex regulation of Zfp36

A schematic of the TTP gene locus showing putative regulatory regions highlighted by previous work (shown above) and those functional elements identified in this study (shown below). The red bars represent the evoulutionarily-conserved regions, exhibiting super-enhance potential.

9.0 REFERENCES

Abraham, S.M. et al., 2006. Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. *The Journal of experimental medicine*, 203(8), pp.1883–1889.

Abraham, S.M. & Clark, A.R., 2006. Dual-specificity phosphatase 1: a critical regulator of innate immune responses. *Biochemical Society transactions*, 34(Pt 6), pp.1018–23.

Achyut, B.R. & Yang, L., 2011. Transforming growth factor-β in the gastrointestinal and hepatic tumor microenvironment. *Gastroenterology*, 141(4), pp.1167–78.

Adamik, J. et al., 2013. Distinct mechanisms for induction and tolerance regulate the immediate early genes encoding interleukin 1β and tumor necrosis factor α. *PloS one*, 8(8), p.e70622.

Aggarwal, B.B., 2003. Signalling pathways of the TNF superfamily: a double-edged sword. *Nature reviews. Immunology*, 3(9), pp.745–56.

Akdis, M. et al., 2011. Interleukins, from 1 to 37, and interferon-γ: receptors, functions, and roles in diseases. *The Journal of allergy and clinical immunology*, 127(3), pp.701–21.e1–70.

Alexopoulou, L. et al., 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413(6857), pp.732–8.

Alfranca, A. et al., 2006. Prostanoid signal transduction and gene expression in the endothelium: role in cardiovascular diseases. *Cardiovascular research*, 70(3), pp.446–56.

Allocca, M. et al., 2013. Anti-IL-6 treatment for inflammatory bowel diseases: next cytokine, next target. *Current drug targets*, 14(12), pp.1508–21.

Alonzi, T. et al., 1998. Interleukin 6 Is Required for the Development of Collagen-induced Arthritis. *Journal of Experimental Medicine*, 187(4), pp.461–468.

Altonsy, M.O. et al., 2014. Context-dependent cooperation between nuclear factor κB (NF-κB) and the glucocorticoid receptor at a TNFAIP3 intronic enhancer: a mechanism to maintain negative feedback control of inflammation. *The Journal of biological chemistry*, 289(12), pp.8231–9.

Amano, Y., Lee, S.W. & Allison, A.C., 1993. Inhibition by glucocorticoids of the formation of interleukin-1 alpha, interleukin-1 beta, and interleukin-6: mediation by decreased mRNA stability. *Molecular pharmacology*, 43(2), pp.176–82.

Amer, M. et al., Use of nonsteroidal anti-inflammatory drugs in patients with cardiovascular disease: a cautionary tale. *Cardiology in review*, 18(4), pp.204–12.

Anderson, P. & Kedersha, N., 2006. RNA granules. *The Journal of cell biology*, 172(6), pp.803–8.

Antoniv, T.T. & Ivashkiv, L.B., 2006. Dysregulation of interleukin-10-dependent gene expression in rheumatoid arthritis synovial macrophages. *Arthritis and rheumatism*, 54(9), pp.2711–21.

Arango Duque, G. & Descoteaux, A., 2014. Macrophage cytokines: involvement in immunity and infectious diseases. *Frontiers in immunology*, 5, p.491.

Arenander, A.T. et al., 1989. TIS gene expression in cultured rat astrocytes: induction by mitogens and stellation agents. *Journal of neuroscience research*, 23(3), pp.247–56.

Aronoff, D.M. et al., 2006. Short communication: differences between macrophages and dendritic cells in the cyclic AMP-dependent regulation of lipopolysaccharide-induced cytokine and chemokine synthesis. *Journal of interferon &*

cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 26(11), pp.827–33.

Arsura, M., Wu, M. & Sonenshein, G.E., 1996. TGF beta 1 inhibits NF-kappa B/Rel activity inducing apoptosis of B cells: transcriptional activation of I kappa B alpha. *Immunity*, 5(1), pp.31–40.

Arthur, J.S.C., 2008. MSK activation and physiological roles. *Frontiers in bioscience : a journal and virtual library*, 13, pp.5866–79.

Arthur, J.S.C. & Ley, S.C., 2013. Mitogen-activated protein kinases in innate immunity. *Nature reviews. Immunology*, 13(9), pp.679–92.

Auphan, N. et al., 1995. Immunosuppression by glucocorticoids: Inhibition of NF-κB activity through induction of IκB synthesis. *Science*, 270(5234), pp.286–290.

Ayroldi, E. et al., 2002. Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Molecular and cellular biology*, 22(22), pp.7929–41.

Babon, J.J., Varghese, L.N. & Nicola, N.A., 2014. Inhibition of IL-6 family cytokines by SOCS3. *Seminars in immunology*, 26(1), pp.13–9.

Bakheet, T., 2001. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Research*, 29(1), pp.246–254.

Bakheet, T., Williams, B.R.G. & Khabar, K.S.A., 2006. ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic acids research*, 34(Database issue), pp.D111–4.

Balkhi, M.Y. et al., 2013. miR-29 acts as a decoy in sarcomas to protect the tumor suppressor A20 mRNA from degradation by HuR. *Science signaling*, 6(286), p.ra63.

Balkwill, F. & Mantovani, A., 2001. Inflammation and cancer: back to Virchow? *Lancet*, 357(9255), pp.539–45.

Ball, E.M.A. et al., 2014. Plasma IL-6 levels correlate with clinical and ultrasound measures of arthritis in patients with systemic lupus erythematosus. *Lupus*, 23(1), pp.46–56.

Banchereau, J. et al., 2000. Immunobiology of dendritic cells. *Annual review of immunology*, 18, pp.767–811.

Bao, J. et al., 2014. Good response to infliximab in rheumatoid arthritis following failure of interleukin-1 receptor antagonist. *International journal of rheumatic diseases*.

Barbalat, R. et al., 2009. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nature immunology*, 10(11), pp.1200–7.

Barnes, M.J. & Powrie, F., 2009a. Hybrid Treg cells: steel frames and plastic exteriors. *Nature immunology*, 10(6), pp.563–4.

Barnes, M.J. & Powrie, F., 2009b. Regulatory T cells reinforce intestinal homeostasis. *Immunity*, 31(3), pp.401–11.

Barnes, N.C., 2006. Reducing oral steroids with inhaled steroids: is that all that can be achieved? *Chest*, 129(5), pp.1124–5.

Barnett, M.P.G. et al., 2010. Changes in colon gene expression associated with increased colon inflammation in interleukin-10 gene-deficient mice inoculated with Enterococcus species. *BMC immunology*, 11, p.39.

Baron, M.H., Isern, J. & Fraser, S.T., 2012. The embryonic origins of erythropoiesis in mammals. *Blood*, 119(21), pp.4828–37.

Barthel, R. & Goldfeld, A.E., 2003. T cell-specific expression of the human TNF-alpha gene involves a functional and highly conserved chromatin signature in intron 3. *Journal of immunology (Baltimore, Md. : 1950)*, 171(7), pp.3612–9.

Beck, P.L. & Wallace, J.L., 1997. Cytokines in inflammatory bowel disease. *Mediators of inflammation*, 6(2), pp.95–103.

Beebe, A.M., Cua, D.J. & de Waal Malefyt, R., 2002. The role of interleukin-10 in autoimmune disease: systemic lupus erythematosus (SLE) and multiple sclerosis (MS). *Cytokine & growth factor reviews*, 13(4-5), pp.403–12.

Beisang, D. & Bohjanen, P.R., Perspectives on the ARE as it turns 25 years old. *Wiley interdisciplinary reviews. RNA*, 3(5), pp.719–31.

Bellail, A.C. et al., 2012. A20 ubiquitin ligase-mediated polyubiquitination of RIP1 inhibits caspase-8 cleavage and TRAIL-induced apoptosis in glioblastoma. *Cancer discovery*, 2(2), pp.140–55.

Bellantuono, I., 2004. Haemopoietic stem cells. *The international journal of biochemistry & cell biology*, 36(4), pp.607–20.

Vanden Berghe, W. et al., 2006. Keeping up NF-kappaB appearances: epigenetic control of immunity or inflammation-triggered epigenetics. *Biochemical pharmacology*, 72(9), pp.1114–1131.

Bergmann, M.W. et al., 2004. Nuclear factor-kappaB does not mediate the inhibitory effects of dexamethasone on granulocyte-macrophage colony-stimulating factor expression. *Immunology*, 111(4), pp.430–434.

Bertrand, M.J.M. et al., 2008. cIAP1 and cIAP2 Facilitate Cancer Cell Survival by Functioning as E3 Ligases that Promote RIP1 Ubiquitination. *Molecular Cell*, 30(6), pp.689–700.

Beutler, B., 2004. Innate immunity: an overview. *Molecular immunology*, 40(12), pp.845–59.

Bhattacharyya, S.N. et al., 2006. Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harbor symposia on quantitative biology*, 71, pp.513–21.

Biddie, S.C. et al., 2011. Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding. *Molecular cell*, 43(1), pp.145–155.

Bladh, L.G. et al., 2009. Inhibition of extracellular signal-regulated kinase (ERK) signaling participates in repression of nuclear factor (NF)-kappaB activity by glucocorticoids. *Biochimica et biophysica acta*, 1793(3), pp.439–446.

Blanco, F.F. et al., 2014. Transforming growth factor β regulates P-body formation through induction of the mRNA decay factor tristetraprolin. *Molecular and cellular biology*, 34(2), pp.180–95.

Bogdarina, I.G., King, P.J. & Clark, A.J.L., 2009. Characterization of the angiotensin (AT1b) receptor promoter and its regulation by glucocorticoids. *Journal of molecular endocrinology*, 43(2), pp.73–80.

Boone, D.L. et al., 2004. The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature immunology*, 5(10), pp.1052–60.

De Bosscher, K., 2010. Selective Glucocorticoid Receptor modulators. *The Journal of steroid biochemistry and molecular biology*, 120(2-3), pp.96–104.

Boulanger, M.J. et al., 2003. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. *Science (New York, N.Y.)*, 300(5628), pp.2101–4.

Bowie, A. & O'Neill, L.A., 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *Journal of leukocyte biology*, 67(4), pp.508–14.

Boyle, A.P. et al., 2011. High-resolution genome-wide in vivo footprinting of diverse transcription factors in human cells. *Genome research*, 21(3), pp.456–64.

Bradley, J.R., 2008. TNF-mediated inflammatory disease. *The Journal of pathology*, 214(2), pp.149–60.

Brahma, P.K. et al., 2012. The mRNA-binding protein Zfp36 is upregulated by β-adrenergic stimulation and represses IL-6 production in 3T3-L1 adipocytes. *Obesity (Silver Spring, Md.)*, 20(1), pp.40–7.

Brennan, F.M. & Feldmann, M., 1996. Cytokines in autoimmunity. *Current opinion in immunology*, 8(6), pp.872–7.

Brion, L. et al., 2011. MAPK Phosphatase-1 (MKP-1) Expression Is Up-Regulated by hCG/cAMP and Modulates Steroidogenesis in MA-10 Leydig Cells. *Endocrinology*, 152(7), pp.2665–2677.

Vanden Broeck, D., Horvath, C. & De Wolf, M.J.S., 2007. Vibrio cholerae: cholera toxin. *The international journal of biochemistry & cell biology*, 39(10), pp.1771–5.

Brook, M. et al., 2006. Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. *Molecular and cellular biology*, 26(6), pp.2408–2418.

Brook, M. et al., 2000. Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. *FEBS letters*, 483(1), pp.57–61.

Brooks, S.A. & Blackshear, P.J., 2013. Tristetraprolin (TTP): Interactions with mRNA and proteins, and current thoughts on mechanisms of action. *Biochimica et biophysica acta*, 1829(6-7), pp.666–79.

Brooks, S.A., Connolly, J.E. & Rigby, W.F.C., 2004. The Role of mRNA Turnover in the Regulation of Tristetraprolin Expression: Evidence for an Extracellular Signal-Regulated Kinase-Specific, AU-Rich Element-Dependent, Autoregulatory Pathway. *Journal of immunology*, 172(12), pp.7263–7271.

Brostjan, C. et al., 1997. Glucocorticoids inhibit E-selectin expression by targeting NF-kappaB and not ATF/c-Jun. *Journal of immunology (Baltimore, Md.: 1950)*, 158(8), pp.3836–44.

Brunet, A. et al., 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *The EMBO journal*, 18(3), pp.664–74.

Büchler, G. et al., 2012. Strain-specific colitis susceptibility in IL10-deficient mice depends on complex gut microbiota-host interactions. *Inflammatory bowel diseases*, 18(5), pp.943–54.

Buckingham, J.C., 2006. Glucocorticoids: exemplars of multi-tasking. *British journal of pharmacology*, 147 Suppl , pp.S258–68.

Burdelya, L.G. et al., 2008. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science (New York, N.Y.)*, 320(5873), pp.226–30.

Buss, H. et al., 2004. Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity. *The Journal of biological chemistry*, 279(48), pp.49571–4.

Cabal-Hierro, L. & Lazo, P.S., 2012. Signal transduction by tumor necrosis factor receptors. *Cellular signalling*, 24(6), pp.1297–305.

Caldenhoven, E. et al., 1995. Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Molecular endocrinology (Baltimore, Md.)*, 9(4), pp.401–12.

Cantor, H. & Boyse, E.A., 1977. Regulation of Cellular and Humoral Immune Responses by T-cell Subclasses. *Cold Spring Harbor Symposia on Quantitative Biology*, 41, pp.23–32.

Cao, H. et al., 2006. Identification of the anti-inflammatory protein tristetraprolin as a hyperphosphorylated protein by mass spectrometry and site-directed mutagenesis. *The Biochemical journal*, 394(Pt 1), pp.285–297.

Cao, H., Deterding, L.J. & Blackshear, P.J., 2014. Identification of a major phosphopeptide in human tristetraprolin by phosphopeptide mapping and mass spectrometry. *PloS one*, 9(7), p.e100977.

Cao, H., Dzineku, F. & Blackshear, P.J., 2003. Expression and purification of recombinant tristetraprolin that can bind to tumor necrosis factor-alpha mRNA and serve as a substrate for mitogen-activated protein kinases. *Archives of biochemistry and biophysics*, 412(1), pp.106–120.

Cao, H. & Lin, R., 2009. Quantitative evaluation of His-tag purification and immunoprecipitation of tristetraprolin and its mutant proteins from transfected human cells. *Biotechnology progress*, 25(2), pp.461–467.

Carayol, N. et al., 2006. A dominant function of IKK/NF-kappaB signaling in global lipopolysaccharide-induced gene expression. *The Journal of biological chemistry*, 281(41), pp.31142–31151.

Carayol, N. & Wang, C.Y., 2006. IKKalpha stabilizes cytosolic beta-catenin by inhibiting both canonical and non-canonical degradation pathways. *Cellular signalling*, 18(11), pp.1941–1946.

Carballo, E. et al., 2001. Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway. *The Journal of biological chemistry*, 276(45), pp.42580–42587.

Carballo, E., Lai, W.S. & Blackshear, P.J., 2000. Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood*, 95(6), pp.1891–9.

Carballo, E., Lai, W.S. & Blackshear, P.J., 1998. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science (New York, N.Y.)*, 281(5379), pp.1001–5.

Carey, V.J. & Gentleman, R., 2009. Interpreting genetics of gene expression: integrative architecture in Bioconductor. *Pacific Symposium on Biocomputing. Pacific Symposium on Biocomputing*, pp.380–90.

Cartmell, T. et al., 2001. Role of endogenous interleukin-1 receptor antagonist in regulating fever induced by localised inflammation in the rat. *The Journal of physiology*, 531(Pt 1), pp.171–80.

Castro-Caldas, M. et al., 2003. Dexamethasone prevents interleukin-1beta-induced nuclear factor-kappaB activation by upregulating IkappaB-alpha synthesis, in lymphoblastic cells. *Mediators of inflammation*, 12(1), pp.37–46.

Catrysse, L. et al., 2014. A20 in inflammation and autoimmunity. *Trends in immunology*, 35(1), pp.22–31.

De Cesare, D. & Sassone-Corsi, P., 2000. Transcriptional regulation by cyclic AMP-responsive factors. *Progress in nucleic acid research and molecular biology*, 64, pp.343–69.

Chang, F.-M. et al., 2012. Inhibition of Neddylation Represses Lipopolysaccharide-induced Proinflammatory Cytokine Production in Macrophage Cells. *Journal of Biological Chemistry*, 287(42), pp.35756–35767.

Charles, C.H. et al., 1993. The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase. *Proceedings of the National Academy of Sciences of the United States of America*, 90(11), pp.5292–6.

Chauveau, A. et al., 2010. Membrane nanotubes facilitate long-distance interactions between natural killer cells and target cells. *Proceedings of the National Academy of Sciences of the United States of America*, 107(12), pp.5545–50.

Cheadle, C. et al., 2005. Stability regulation of mRNA and the control of gene expression. *Annals of the New York Academy of Sciences*, 1058, pp.196–204.

Chen, P. et al., 2002. Restraint of proinflammatory cytokine biosynthesis by mitogen-activated protein kinase phosphatase-1 in lipopolysaccharide-stimulated macrophages. *Journal of immunology (Baltimore, Md.: 1950)*, 169(11), pp.6408–16.

Chen, P.-M. et al., 2014. Induction of immunomodulatory monocytes by human mesenchymal stem cell-derived hepatocyte growth factor through ERK1/2. *Journal of leukocyte biology*, 96(2), pp.295–303.

Chen, Y.L. et al., 2013. Transcriptional regulation of tristetraprolin by NF-kappaB signaling in LPS-stimulated macrophages. *Molecular biology reports*, 40(4), pp.2867–2877.

Chen, Z., Marsden, P.A. & Gorczynski, R.M., 2009. Role of a distal enhancer in the transcriptional responsiveness of the human CD200 gene to interferon-gamma and tumor necrosis factor-alpha. *Molecular immunology*, 46(10), pp.1951–63.

Chen, Z.J., 2012. Ubiquitination in signaling to and activation of IKK. *Immunological reviews*, 246(1), pp.95–106.

Cherwinski, H.M. et al., 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *The Journal of experimental medicine*, 166(5), pp.1229–44.

Chi, H. et al., 2006. Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, 103(7), pp.2274–9.

Chichlowski, M. et al., 2010. Role of Mast Cells in Inflammatory Bowel Disease and Inflammation-Associated Colorectal Neoplasia in IL-10-Deficient Mice D. Unutmaz, ed. *PLoS ONE*, 5(8), p.e12220.

Chrestensen, C.A. et al., 2004. MAPKAP kinase 2 phosphorylates tristetraprolin on in vivo sites including Ser178, a site required for 14-3-3 binding. *The Journal of biological chemistry*, 279(11), pp.10176–84.

Christ, M. et al., 1994. Immune dysregulation in TGF-beta 1-deficient mice. *Journal of immunology (Baltimore, Md.: 1950)*, 153(5), pp.1936–46.

Chu, Y. et al., 1996. The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. *The Journal of biological chemistry*, 271(11), pp.6497–501.

Clark, A.R., 2007. Anti-inflammatory functions of glucocorticoid-induced genes. *Molecular and cellular endocrinology*, 275(1-2), pp.79–97.

Clark, A.R. & Belvisi, M.G., 2012. Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor. *Pharmacology & therapeutics*, 134(1), pp.54–67.

Clark, A.R. & Dean, J.L., 2012. The p38 MAPK Pathway in Rheumatoid Arthritis: A Sideways Look. *The open rheumatology journal*, 6, pp.209–219.

Clark, A.R., Dean, J.L. & Saklatvala, J., 2003. Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. *FEBS letters*, 546(1), pp.37–44.

Clarke, D.L. et al., 2004. Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which PGE2 inhibits the release of GM-CSF. *British journal of pharmacology*, 141(7), pp.1141–50.

Clayton, A.L., Hazzalin, C.A. & Mahadevan, L.C., 2006. Enhanced histone acetylation and transcription: a dynamic perspective. *Molecular cell*, 23(3), pp.289–96.

Clement, S.L. et al., 2011. Phosphorylation of tristetraprolin by MK2 impairs AU-rich element mRNA decay by preventing deadenylase recruitment. *Molecular and Cellular Biology*, 31(2), pp.256–266.

Cruvinel, W. de M. et al., 2010. Immune system - part I. Fundamentals of innate immunity with emphasis on molecular and cellular mechanisms of inflammatory response. *Revista brasileira de reumatologia*, 50(4), pp.434–61.

Csaba, G., 2014. Immunoendocrinology: faulty hormonal imprinting in the immune system. *Acta microbiologica et immunologica Hungarica*, 61(2), pp.89–106.

Cuenda, A. et al., 1997. Activation of the novel MAP kinase homologue SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6). *Biochemical Society transactions*, 25(4), p.S569.

Cuenda, A. et al., 1996. Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress- and cytokine-stimulated monocytes and epithelial cells. *The EMBO journal*, 15(16), pp.4156–64.

Cullen, S.P. & Martin, S.J., 2015. Fas and TRAIL “death receptors” as initiators of inflammation: Implications for cancer. *Seminars in cell & developmental biology*.

Dahl, R. et al., 2003. Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nature immunology*, 4(10), pp.1029–36.

Dai, C. & Holland, E.C., 2003. Astrocyte differentiation states and glioma formation. *Cancer journal (Sudbury, Mass.)*, 9(2), pp.72–81.

Das, S. et al., 2012. TGF-β1 re-programs TLR4 signaling in L. donovani infection: enhancement of SHP-1 and ubiquitin-editing enzyme A20. *Immunology and cell biology*, 90(6), pp.640–54.

Davies, C. & Tournier, C., 2012. Exploring the function of the JNK (c-Jun N-terminal kinase) signalling pathway in physiological and pathological processes to design novel therapeutic strategies. *Biochemical Society transactions*, 40(1), pp.85–9.

Davis, R.J., 1995. Transcriptional regulation by MAP kinases. *Molecular reproduction and development*, 42(4), pp.459–67.

Davoine, F. & Lacy, P., 2014. Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Frontiers in immunology*, 5, p.570.

Deleault, K.M., Skinner, S.J. & Brooks, S.A., 2008. Tristetraprolin regulates TNF TNF-alpha mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. *Molecular immunology*, 45(1), pp.13–24.

Dennler, S., Goumans, M.-J. & ten Dijke, P., 2002. Transforming growth factor beta signal transduction. *Journal of leukocyte biology*, 71(5), pp.731–40.

Denys, A. et al., 2002. Evidence for a dual mechanism for IL-10 suppression of TNF-alpha production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-kappa B in primary human macrophages. *Journal of immunology (Baltimore, Md.: 1950)*, 168(10), pp.4837–45.

Dérijard, B. et al., 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science (New York, N.Y.)*, 267(5198), pp.682–5.

Díaz-Muñoz, M.D. et al., 2012. Involvement of PGE2 and the cAMP signalling pathway in the up-regulation of COX-2 and mPGES-1 expression in LPS-activated macrophages. *The Biochemical journal*, 443(2), pp.451–61.

Diefenbacher, M. et al., 2008. Restriction to Fos family members of Trip6-dependent coactivation and glucocorticoid receptor-dependent trans-repression of activator protein-1. *Molecular endocrinology (Baltimore, Md.)*, 22(8), pp.1767–80.

Dinarello, C.A., 2009. Interleukin-1beta and the autoinflammatory diseases. *The New England journal of medicine*, 360(23), pp.2467–70.

Ding, Y. et al., 2014. Induction of TGF-β and IL-10 production in dendritic cells using astilbin to inhibit dextran sulfate sodium-induced colitis. *Biochemical and biophysical research communications*, 446(2), pp.529–34.

Dobrovolna, J. et al., 2012. Glucocorticoid-dependent phosphorylation of the transcriptional coregulator GRIP1. *Molecular and cellular biology*, 32(4), pp.730–9.

Domínguez, P.M. & Ardavín, C., 2010. Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. *Immunological reviews*, 234(1), pp.90–104.

Dostert, A. & Heinzel, T., 2004. Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Current pharmaceutical design*, 10(23), pp.2807–16.

Duan, W. & Wong, W.S.F., 2006. Targeting mitogen-activated protein kinases for asthma. *Current drug targets*, 7(6), pp.691–8.

DuBois, R.N. et al., 1990. A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. *The Journal of biological chemistry*, 265(31), pp.19185–19191.

Dunn, S.M. et al., 1994. Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood*, 83(9), pp.2469–79.

Duran, A., Diaz-Meco, M.T. & Moscat, J., 2003. Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation. *The EMBO journal*, 22(15), pp.3910–8.

Düwel, M. et al., 2009. A20 negatively regulates T cell receptor signaling to NF-kappaB by cleaving Malt1 ubiquitin chains. *Journal of immunology (Baltimore, Md.: 1950)*, 182(12), pp.7718–28.

Ebisuya, M., Kondoh, K. & Nishida, E., 2005. The duration, magnitude and compartmentalization of ERK MAP kinase activity: mechanisms for providing signaling specificity. *Journal of cell science*, 118(Pt 14), pp.2997–3002.

Eisele, G. & Weller, M., 2013. Targeting apoptosis pathways in glioblastoma. *Cancer letters*, 332(2), pp.335–45.

Engelbrecht, Y. et al., 2003. Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. *Endocrinology*, 144(2), pp.412–22.

Essafi-Benkhadir, K., Pouysségur, J. & Pagès, G., 2010. Implication of the ERK pathway on the post-transcriptional regulation of VEGF mRNA stability. *Methods in molecular biology (Clifton, N.J.)*, 661, pp.451–69.

Esser, C. & Radbruch, A., 1990. Immunoglobulin class switching: molecular and cellular analysis. *Annual review of immunology*, 8, pp.717–35.

Eulenfeld, R. et al., Interleukin-6 signalling: more than Jaks and STATs. *European journal of cell biology*, 91(6-7), pp.486–95.

Fabian, M.R. et al., 2013. Structural basis for the recruitment of the human CCR4-NOT deadenylase complex by tristetraprolin. *Nature structural & molecular biology*, 20(6), pp.735–9.

Fan, Y. et al., 2011. The association between BANK1 and TNFAIP3 gene polymorphisms and systemic lupus erythematosus: a meta-analysis. *International journal of immunogenetics*, 38(2), pp.151–9.

Fantuzzi, G. & Dinarello, C.A., 1996. The inflammatory response in interleukin-1 beta-deficient mice: comparison with other cytokine-related knock-out mice. *Journal of leukocyte biology*, 59(4), pp.489–93.

Della Fazia, M.A., Servillo, G. & Sassone-Corsi, P., 1997. Cyclic AMP signalling and cellular proliferation: regulation of CREB and CREM. *FEBS letters*, 410(1), pp.22–4.

Feldmann, M., The cytokine network in rheumatoid arthritis: definition of TNF alpha as a therapeutic target. *Journal of the Royal College of Physicians of London*, 30(6), pp.560–70.

Feldmann, M., Brennan, F.M. & Maini, R., 2009. Cytokines in Autoimmune Disorders.

Feldmeyer, L. et al., 2010. Interleukin-1, inflammasomes and the skin. *European journal of cell biology*, 89(9), pp.638–44.

Felix, R. et al., 1994. Role of colony-stimulating factor-1 in bone metabolism. *Journal of cellular biochemistry*, 55(3), pp.340–9.

Feng, J. et al., 1997. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. *Molecular and cellular biology*, 17(5), pp.2497–501.

Fenger-Grøn, M. et al., 2005. Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Molecular cell*, 20(6), pp.905–15.

Ferber, I.A. et al., 1996. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *Journal of immunology (Baltimore, Md.: 1950)*, 156(1), pp.5–7.

Fernandes, D. et al., 1999. Glucocorticoids inhibit proliferation, cyclin D1 expression, and retinoblastoma protein phosphorylation, but not activity of the extracellular-regulated kinases in human cultured airway smooth muscle. *American journal of respiratory cell and molecular biology*, 21(1), pp.77–88.

Figgett, W.A. et al., 2014. Roles of ligands from the TNF superfamily in B cell development, function, and regulation. *Seminars in immunology*, 26(3), pp.191–202.

Finbloom, D.S. & Winestock, K.D., 1995. IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. *Journal of immunology (Baltimore, Md.: 1950)*, 155(3), pp.1079–90.

Flammer, J.R. & Rogatsky, I., 2011. Minireview: Glucocorticoids in autoimmunity: unexpected targets and mechanisms. *Molecular endocrinology (Baltimore, Md.)*, 25(7), pp.1075–86.

Florkowska, M. et al., 2012. EGF activates TTP expression by activation of ELK-1 and EGR-1 transcription factors. *BMC molecular biology*, 13, p.8.

Frazier, W.J. et al., 2009. Increased inflammation, impaired bacterial clearance, and metabolic disruption after gram-negative sepsis in Mkp-1-deficient mice. *Journal of immunology (Baltimore, Md.: 1950)*, 183(11), pp.7411–9.

Freudlsperger, C. et al., 2013. TGF-β and NF-κB signal pathway cross-talk is mediated through TAK1 and SMAD7 in a subset of head and neck cancers. *Oncogene*, 32(12), pp.1549–59.

Frischmeyer, P.A. et al., 2002. An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. *Science (New York, N.Y.)*, 295(5563), pp.2258–61.

Fujino, H., Salvi, S. & Regan, J.W., 2005. Differential regulation of phosphorylation of the cAMP response element-binding protein after activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *Molecular pharmacology*, 68(1), pp.251–9.

Fujino, H., Xu, W. & Regan, J.W., 2003. Prostaglandin E2 induced functional expression of early growth response factor-1 by EP4, but not EP2, prostanoid receptors via the phosphatidylinositol 3-kinase and extracellular signal-regulated kinases. *The Journal of biological chemistry*, 278(14), pp.12151–6.

Gaba, A. et al., 2012. Cutting edge: IL-10-mediated tristetraprolin induction is part of a feedback loop that controls macrophage STAT3 activation and cytokine production. *Journal of immunology*, 189(5), pp.2089–93.

Gabay, C., Lamacchia, C. & Palmer, G., 2010. IL-1 pathways in inflammation and human diseases. *Nature reviews. Rheumatology*, 6(4), pp.232–41.

Galluzzi, L. et al., 2012. Molecular mechanisms of cisplatin resistance. *Oncogene*, 31(15), pp.1869–83.

Gantier, M.P. et al., 2012. A miR-19 regulon that controls NF-κB signaling. *Nucleic acids research*, 40(16), pp.8048–58.

Garaude, J. & Blander, J.M., 2012. “Flagellated” cancer cells propel anti-tumor immunity. *Oncoimmunology*, 1(6), pp.940–942.

Garlanda, C., Dinarello, C.A. & Mantovani, A., 2013. The interleukin-1 family: back to the future. *Immunity*, 39(6), pp.1003–18.

Garneau, N.L., Wilusz, J. & Wilusz, C.J., 2007. The highways and byways of mRNA decay. *Nature reviews. Molecular cell biology*, 8(2), pp.113–26.

Gasparoto, T.H. et al., 2012. Inflammatory events during murine squamous cell carcinoma development. *Journal of Inflammation*, 9(1), p.46.

Gasperini, S. et al., Interleukin-10 and cAMP-elevating agents cooperate to induce suppressor of cytokine signaling-3 via a protein kinase A-independent signal. *European cytokine network*, 13(1), pp.47–53.

Gaur, U. & Aggarwal, B.B., 2003. Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochemical pharmacology*, 66(8), pp.1403–8.

Gautier, E.L. et al., 2012. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nature immunology*, 13(11), pp.1118–28.

Gekas, C. et al., 2005. The placenta is a niche for hematopoietic stem cells. *Developmental cell*, 8(3), pp.365–75.

Germain, R.N., 2002. T-cell development and the CD4-CD8 lineage decision. *Nature reviews. Immunology*, 2(5), pp.309–22.

Germain, R.N., Stefanova, I. & Dorfman, J., 2002. Self-recognition and the regulation of CD4+ T cell survival. *Advances in experimental medicine and biology*, 512, pp.97–105.

Geyer, B.C. et al., 2012. Nicotinic stimulation induces Tristetraprolin over-production and attenuates inflammation in muscle. *Biochimica et biophysica acta*, 1823(2), pp.368–78.

Ghisletti, S. et al., 2010. Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity*, 32(3), pp.317–328.

Ghosh, S. & Baltimore, D., 1990. Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature*, 344(6267), pp.678–82.

Giembycz, M.A. et al., 2009. A Holy Grail of asthma management: toward understanding how long-acting β2-adrenoceptor agonists enhance the clinical efficacy of inhaled corticosteroids. *British Journal of Pharmacology*, 153(6), pp.1090–1104.

Gille, J. et al., 2001. Decreased mRNA stability as a mechanism of glucocorticoid-mediated inhibition of vascular endothelial growth factor gene expression by cultured keratinocytes. *The Journal of investigative dermatology*, 117(6), pp.1581–7.

Gingerich, T.J., Feige, J.-J. & LaMarre, J., 2004. AU-rich elements and the control of gene expression through regulated mRNA stability. *Animal health research reviews / Conference of Research Workers in Animal Diseases*, 5(1), pp.49–63.

Gingery, A. et al., 2008. TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival. *Experimental cell research*, 314(15), pp.2725–38.

Ginhoux, F. et al., 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science (New York, N.Y.)*, 330(6005), pp.841–5.

Ginhoux, F. et al., 2009. The origin and development of nonlymphoid tissue CD103+ DCs. *The Journal of experimental medicine*, 206(13), pp.3115–30.

Glass, C.K. & Saijo, K., 2010. Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. *Nature reviews. Immunology*, 10(5), pp.365–76.

Glocker, E.-O. et al., 2009. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *The New England journal of medicine*, 361(21), pp.2033–45.

Godin, I. & Cumano, A., 2005. Of birds and mice: hematopoietic stem cell development. *The International journal of developmental biology*, 49(2-3), pp.251–7.

Gomez, P.F. et al., 2005. Resolution of Inflammation: Prostaglandin E2 Dissociates Nuclear Trafficking of Individual NF- B Subunits (p65, p50) in Stimulated Rheumatoid Synovial Fibroblasts. *The Journal of Immunology*, 175(10), pp.6924–6930.

Gomperts, M., Pascall, J.C. & Brown, K.D., 1990. The nucleotide sequence of a cDNA encoding an EGF-inducible gene indicates the existence of a new family of mitogen-induced genes. *Oncogene*, 5(7), pp.1081–3.

Gordon, S., 2003. Alternative activation of macrophages. *Nature reviews. Immunology*, 3(1), pp.23–35.

Gorter, S.L. et al., 2010. Current evidence for the management of rheumatoid arthritis with glucocorticoids: a systematic literature review informing the EULAR recommendations for the management of rheumatoid arthritis. *Annals of the rheumatic diseases*, 69(6), pp.1010–4.

Grad, I. & Picard, D., 2007. The glucocorticoid responses are shaped by molecular chaperones. *Molecular and cellular endocrinology*, 275(1-2), pp.2–12.

Gronemeyer, H. & Bourguet, W., 2009. Allosteric effects govern nuclear receptor action: DNA appears as a player. *Science signaling*, 2(73), p.pe34.

Grzechnik, P., Tan-Wong, S.M. & Proudfoot, N.J., 2014. Terminate and make a loop: regulation of transcriptional directionality. *Trends in biochemical sciences*, 39(7), pp.319–327.

Gui, T. et al., 2012. The Roles of Mitogen-Activated Protein Kinase Pathways in TGF-β-Induced Epithelial-Mesenchymal Transition. *Journal of signal transduction*, 2012, p.289243.

Hakim, O. et al., 2009. Glucocorticoid receptor activation of the Ciz1-Lcn2 locus by long range interactions. *The Journal of biological chemistry*, 284(10), pp.6048–52.

Hale, L.P. & Greer, P.K., 2012. A novel murine model of inflammatory bowel disease and inflammation-associated colon cancer with ulcerative colitis-like features. *PloS one*, 7(7), p.e41797.

Hameedaldeen, A. et al., 2014. FOXO1, TGF-β regulation and wound healing. *International journal of molecular sciences*, 15(9), pp.16257–69.

Hammer, M. et al., 2005. Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. *European journal of immunology*, 35(10), pp.2991–3001.

Hammer, M. et al., 2006. Dual specificity phosphatase 1 (DUSP1) regulates a subset of LPS-induced genes and protects mice from lethal endotoxin shock. *The Journal of experimental medicine*, 203(1), pp.15–20.

Hansen, J.J., Holt, L. & Sartor, R.B., 2009. Gene expression patterns in experimental colitis in IL-10-deficient mice. *Inflammatory bowel diseases*, 15(6), pp.890–9.

Hao, S. & Baltimore, D., 2009. The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. *Nature immunology*, 10(3), pp.281–288.

Hargreaves, D.C., Horng, T. & Medzhitov, R., 2009. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell*, 138(1), pp.129–145.

Harigaya, Y. et al., 2010. Identification and analysis of the interaction between Edc3 and Dcp2 in Saccharomyces cerevisiae. *Molecular and cellular biology*, 30(6), pp.1446–56.

Harizi, H., Norbert, G. & Hedi, H., 2004. Inhibition of IL-6, TNF-alpha, and cyclooxygenase-2 protein expression by prostaglandin E2-induced IL-10 in bone marrow-derived dendritic cells. *Cellular immunology*, 228(2), pp.99–109.

Hata, A.N. & Breyer, R.M., 2004. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. *Pharmacology & therapeutics*, 103(2), pp.147–66.

Hayden, M.S. & Ghosh, S., 2014. Regulation of NF-κB by TNF family cytokines. *Seminars in immunology*, 26(3), pp.253–66.

Healy, S. et al., 2012. Histone H3 phosphorylation, immediate-early gene expression, and the nucleosomal response: a historical perspective. *Biochemistry and cell biology = Biochimie et biologie cellulaire*, 90(1), pp.39–54.

Hemmann, U. et al., 1996. Differential activation of acute phase response factor/Stat3 and Stat1 via the cytoplasmic domain of the interleukin 6 signal transducer gp130. II. Src homology SH2 domains define the specificity of stat factor activation. *The Journal of biological chemistry*, 271(22), pp.12999–3007.

Henkel, T. et al., 1993. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature*, 365(6442), pp.182–5.

Heximer, S.P. & Forsdyke, D.R., 1993. A human putative lymphocyte G0/G1 switch gene homologous to a rodent gene encoding a zinc-binding potential transcription factor. *DNA and cell biology*, 12(1), pp.73–88.

Heyninck, K. et al., Inhibition of tumor necrosis factor-induced necrotic cell death by the zinc finger protein A20. *Anticancer research*, 19(4B), pp.2863–8.

Higaki, T. et al., 2012. COX/PGE(2) axis critically regulates effects of LPS on eosinophilia-associated cytokine production in nasal polyps. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*, 42(8), pp.1217–26.

Hillier, S.G., 2007. Diamonds are forever: the cortisone legacy. *The Journal of endocrinology*, 195(1), pp.1–6.

Hinz, M., Arslan, S.Ç. & Scheidereit, C., 2012. It takes two to tango: IκBs, the multifunctional partners of NF-κB. *Immunological reviews*, 246(1), pp.59–76.

Hishikari, K. et al., 2009. Pharmacological activation of the prostaglandin E2 receptor EP4 improves cardiac function after myocardial ischaemia/reperfusion injury. *Cardiovascular research*, 81(1), pp.123–32.

Hitotsumatsu, O. et al., 2008. The ubiquitin-editing enzyme A20 restricts nucleotide-binding oligomerization domain containing 2-triggered signals. *Immunity*, 28(3), pp.381–90.

Hitti, E. & Khabar, K.S.A., 2012. Sequence variations affecting AU-rich element function and disease. *Frontiers in bioscience (Landmark edition)*, 17, pp.1846–60.

Hochrainer, K., Racchumi, G. & Anrather, J., 2013. Site-specific phosphorylation of the p65 protein subunit mediates selective gene expression by differential NF-κB and RNA polymerase II promoter recruitment. *The Journal of biological chemistry*, 288(1), pp.285–93.

Hoes, J.N. et al., 2010. Current view of glucocorticoid co-therapy with DMARDs in rheumatoid arthritis. *Nature reviews. Rheumatology*, 6(12), pp.693–702.

Hoes, J.N. et al., 2007. EULAR evidence-based recommendations on the management of systemic glucocorticoid therapy in rheumatic diseases. *Annals of the rheumatic diseases*, 66(12), pp.1560–7.

Hofbauer, L.C. & Rauner, M., 2009. Minireview: live and let die: molecular effects of glucocorticoids on bone cells. *Molecular endocrinology (Baltimore, Md.)*, 23(10), pp.1525–31.

Hofmann, T.G. & Schmitz, M.L., 2002. The promoter context determines mutual repression or synergism between NF-kappaB and the glucocorticoid receptor. *Biological chemistry*, 383(12), pp.1947–51.

Holden, N.S. et al., 2010. Enhancement of inflammatory mediator release by beta(2)-adrenoceptor agonists in airway epithelial cells is reversed by glucocorticoid action. *British Journal of Pharmacology*, 160(2), pp.410–420.

Hollien, J. & Weissman, J.S., 2006. Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response. *Science (New York, N.Y.)*, 313(5783), pp.104–7.

Honda, A. et al., 1993. Cloning and expression of a cDNA for mouse prostaglandin E receptor EP2 subtype. *The Journal of biological chemistry*, 268(11), pp.7759–62.

Honda, T. et al., 2006. Prostacyclin-IP signaling and prostaglandin E2-EP2/EP4 signaling both mediate joint inflammation in mouse collagen-induced arthritis. *The Journal of experimental medicine*, 203(2), pp.325–35.

Hovden, A.-O. et al., 2012. The bacterial preparation OK432 induces IL-12p70 secretion in human dendritic cells in a TLR3 dependent manner. *PloS one*, 7(2), p.e31217.

Hu, J. et al., 2015. IL-1RA gene-transfected bone marrow-derived mesenchymal stem cells in APA microcapsules could alleviate rheumatoid arthritis. *International journal of clinical and experimental medicine*, 8(1), pp.706–13.

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

Huang, C.-Y. & Tan, T.-H., 2012. DUSPs, to MAP kinases and beyond. *Cell & Bioscience*, 2(1), p.24.

Huang, G. et al., 2011. Downregulation of B lymphocyte stimulator expression by curcumin in B lymphocyte via suppressing nuclear translocation of NF-κB. *European Journal of Pharmacology*, 650(1), pp.451–457.

Huang, H.-L. et al., Targeting of TGF-β-activated protein kinase 1 inhibits chemokine (C-C motif) receptor 7 expression, tumor growth and metastasis in breast cancer. *Oncotarget*, 6(2), pp.995–1007.

Iha, H. et al., 2008. Inflammatory cardiac valvulitis in TAX1BP1-deficient mice through selective NF-kappaB activation. *The EMBO journal*, 27(4), pp.629–41.

Infante-Duarte, C. et al., 2000. Microbial lipopeptides induce the production of IL-17 in Th cells. *Journal of immunology (Baltimore, Md.: 1950)*, 165(11), pp.6107–15.

Ishitani, T. et al., 2003. Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. *The EMBO journal*, 22(23), pp.6277–88.

Ishmael, F.T. et al., 2008. Role of the RNA-binding protein tristetraprolin in glucocorticoid-mediated gene regulation. *Journal of immunology*, 180(12), pp.8342–8353.

Issa, R. et al., 2007. Corticosteroid inhibition of growth-related oncogene protein-alpha via mitogen-activated kinase phosphatase-1 in airway smooth muscle cells. *Journal of immunology (Baltimore, Md.: 1950)*, 178(11), pp.7366–75.

Itakura, E. et al., 2011. IL-10 expression by primary tumor cells correlates with melanoma progression from radial to vertical growth phase and development of metastatic competence. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*, 24(6), pp.801–9.

Itoh, K. et al., 1995. [Tumor-rejection antigens expressed on human squamous cell carcinoma]. *Human cell*, 8(4), pp.149–54.

Iwasaki, A. & Medzhitov, R., 2015. Control of adaptive immunity by the innate immune system. *Nature Immunology*, 16(4), pp.343–353.

Iwasaki, A. & Medzhitov, R., 2010. Regulation of adaptive immunity by the innate immune system. *Science (New York, N.Y.)*, 327(5963), pp.291–5.

Jalonen, U. et al., 2005. Inhibition of tristetraprolin expression by dexamethasone in activated macrophages. *Biochemical pharmacology*, 69(5), pp.733–740.

Jalonen, U. et al., 2007. Salbutamol increases tristetraprolin expression in macrophages. *Life sciences*, 81(25-26), pp.1651–1658.

Jesus, A.A. & Goldbach-Mansky, R., 2014. IL-1 blockade in autoinflammatory syndromes. *Annual review of medicine*, 65, pp.223–44.

Ji, Y.-I. et al., 2013. Expression patterns of Thymosin β4 and cancer stem cell marker CD133 in ovarian cancers. *Pathology oncology research: POR*, 19(2), pp.237–45.

Jiang, Y. & Gong, X.W., 2000. [Regulation of inflammatory responses by MAPK signal transduction pathways]. *Sheng li xue bao: [Acta physiologica Sinica]*, 52(4), pp.267–71.

Jima, D.D. et al., 2010. Deep sequencing of the small RNA transcriptome of normal and malignant human B cells identifies hundreds of novel microRNAs. *Blood*, 116(23), pp.e118–27.

Jin, Z. et al., 2009. Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell*, 137(4), pp.721–35.

Jinesh G, G. & Kamat, A.M., 2012. Redirecting neutrophils against bladder cancer cells by BCG and Smac mimetic combination. *Oncoimmunology*, 1(7), pp.1161–1162.

Joanny, E. et al., 2012. Anti-inflammatory effects of selective glucocorticoid receptor modulators are partially dependent on up-regulation of dual specificity phosphatase 1. *British journal of pharmacology*, 165(4b), pp.1124–1136.

Joe, Y. et al., 2011. Tristetraprolin mediates anti-inflammatory effects of nicotine in lipopolysaccharide-stimulated macrophages. *The Journal of biological chemistry*, 286(28), pp.24735–42.

Johansson-Haque, K., Palanichamy, E. & Okret, S., 2008. Stimulation of MAPK-phosphatase 1 gene expression by glucocorticoids occurs through a tethering mechanism involving C/EBP. *Journal of molecular endocrinology*, 41(4), pp.239–249.

John, S. et al., 2008. Interaction of the glucocorticoid receptor with the chromatin landscape. *Molecular cell*, 29(5), pp.611–624.

Jonkers, I. & Lis, J.T., 2015. Getting up to speed with transcription elongation by RNA polymerase II. *Nature reviews. Molecular cell biology*, 16(3), pp.167–177.

Juhász, K., Buzás, K. & Duda, E., 2013. Importance of reverse signaling of the TNF superfamily in immune regulation. *Expert review of clinical immunology*, 9(4), pp.335–48.

Kaech, S.M. & Ahmed, R., 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naïve cells. *Nature immunology*, 2(5), pp.415–22.

Kaech, S.M., Wherry, E.J. & Ahmed, R., 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nature reviews. Immunology*, 2(4), pp.251–62.

Kajdaniuk, D. et al., 2013. Transforming growth factor β1 (TGFβ1) in physiology and pathology. *Endokrynologia Polska*, 64(5), pp.384–96.

Kalinski, P., 2012. Regulation of immune responses by prostaglandin E2. *Journal of immunology (Baltimore, Md.□: 1950)*, 188(1), pp.21–8.

Kamanova, J. et al., 2008. Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. *Journal of immunology (Baltimore, Md.□: 1950)*, 181(8), pp.5587–97.

Kamato, D. et al., 2013. Transforming growth factor-β signalling: role and consequences of Smad linker region phosphorylation. *Cellular signalling*, 25(10), pp.2017–24.

Kanamaru, Y. et al., 2001. Blockade of TGF-beta signaling in T cells prevents the development of experimental glomerulonephritis. *Journal of immunology (Baltimore, Md.□: 1950)*, 166(4), pp.2818–23.

Kaneda, N. et al., 2000. Cloning and characterization of the rat TIS11 gene. *Molecular and cellular biochemistry*, 213(1-2), pp.119–26.

Kaneda, N. et al., 1992. Sequence of a rat TIS11 cDNA, an immediate early gene induced by growth factors and phorbol esters. *Gene*, 118(2), pp.289–91.

Kang, B.N. et al., 2008. Glucocorticoid regulation of CD38 expression in human airway smooth muscle cells: role of dual specificity phosphatase 1. *American journal of physiology. Lung cellular and molecular physiology*, 295(1), pp.L186–93.

Kang, Y., Chen, C.-R. & Massagué, J., 2003. A self-enabling TGFbeta response coupled to stress signaling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. *Molecular cell*, 11(4), pp.915–26.

Kanies, C.L. et al., 2008. Oncogenic Ras and transforming growth factor-beta synergistically regulate AU-rich element-containing mRNAs during epithelial to mesenchymal transition. *Molecular cancer research□: MCR*, 6(7), pp.1124–36.

Kanis, J.A. et al., 2007. Glucocorticoid-induced osteoporosis: a systematic review and cost-utility analysis. *Health technology assessment (Winchester, England)*, 11(7), pp.iii–iv, ix–xi, 1–231.

Kanji, S., Pompili, V.J. & Das, H., 2011. Plasticity and maintenance of hematopoietic stem cells during development. *Recent patents on biotechnology*, 5(1), pp.40–53.

Karin, M., 1998. New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? *Cell*, 93(4), pp.487–90.

Karmakar, S., Jin, Y. & Nagaich, A.K., 2013. Interaction of glucocorticoid receptor (GR) with estrogen receptor (ER) α and activator protein 1 (AP1) in dexamethasone-mediated interference of ERα activity. *The Journal of biological chemistry*, 288(33), pp.24020–34.

Kassel, O. et al., 2001. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *The EMBO journal*, 20(24), pp.7108–16.

Kassel, O. & Herrlich, P., 2007. Crosstalk between the glucocorticoid receptor and other transcription factors: molecular aspects. *Molecular and cellular endocrinology*, 275(1-2), pp.13–29.

Kawahara, K. et al., 2014. Prostaglandin E2-induced inflammation: Relevance of prostaglandin E receptors. *Biochimica et biophysica acta*.

Kawasaki, A. et al., 2010. Association of TNFAIP3 interacting protein 1, TNIP1 with systemic lupus erythematosus in a Japanese population: a case-control association study. *Arthritis research & therapy*, 12(5), p.R174.

Kellis, M. et al., 2014. Defining functional DNA elements in the human genome. *Proceedings of the National Academy of Sciences of the United States of America*, 111(17), pp.6131–8.

Keyse, S.M., 2000. Protein phosphatases and the regulation of mitogen-activated protein kinase signalling. *Current opinion in cell biology*, 12(2), pp.186–92.

Keyse, S.M. & Emslie, E.A., 1992. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature*, 359(6396), pp.644–7.

Khabar, K.S.A., 2010. Post-transcriptional control during chronic inflammation and cancer: a focus on AU-rich elements. *Cellular and molecular life sciences□: CMLS*, 67(17), pp.2937–55.

Kierdorf, K. et al., 2013. Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nature neuroscience*, 16(3), pp.273–80.

Kikuchi, H. et al., 2014. Involvement of histone H3 phosphorylation via the activation of p38 MAPK pathway and intracellular redox status in cytotoxicity of HL-60 cells induced by Vitex agnus-castus fruit extract. *International journal of oncology*, 45(2), pp.843–52.

Kim, S. et al., 2014. Self-priming determines high type I IFN production by plasmacytoid dendritic cells. *European journal of immunology*, 44(3), pp.807–18.

Kim, S.-W. et al., 2012. MicroRNAs miR-125a and miR-125b constitutively activate the NF-κB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). *Proceedings of the National Academy of Sciences of the United States of America*, 109(20), pp.7865–70.

Kim, T.H. & Lee, H.K., 2014. Differential roles of lung dendritic cell subsets against respiratory virus infection. *Immune network*, 14(3), pp.128–37.

King, E.M. et al., 2013. Glucocorticoid repression of inflammatory gene expression shows differential responsiveness by transactivation- and transrepression-dependent mechanisms. *PloS one*, 8(1), p.e53936.

King, E.M., Holden, N.S., et al., 2009a. Inhibition of NF-kappaB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. *The Journal of Biological Chemistry*, 284(39), pp.26803–26815.

King, E.M., Holden, N.S., et al., 2009b. Inhibition of NF-κB-dependent Transcription by MKP-1. *The Journal of Biological Chemistry*, 284, pp.26803–26815.

King, E.M., Kaur, M., et al., 2009. Regulation of tristetraprolin expression by interleukin-1 beta and dexamethasone in human pulmonary epithelial cells: roles for nuclear factor-kappa B and p38 mitogen-activated protein kinase. *The Journal of pharmacology and experimental therapeutics*, 330(2), pp.575–585.

Kirwan, J. & Power, L., 2007. Glucocorticoids: action and new therapeutic insights in rheumatoid arthritis. *Current opinion in rheumatology*, 19(3), pp.233–7.

Kirwan, J.R. et al., 2007. Effects of glucocorticoids on radiological progression in rheumatoid arthritis. *The Cochrane database of systematic reviews*, (1), p.CD006356.

Kishimoto, T., 1992. Interleukin-6 and its receptor in autoimmunity. *Journal of autoimmunity*, 5 Suppl A, pp.123–32.

Kleinert, H. et al., 1996. Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-kappa B. *Molecular pharmacology*, 49(1), pp.15–21.

De Klerck, B. et al., 2004. Enhanced osteoclast development in collagen-induced arthritis in interferon-gamma receptor knock-out mice as related to increased splenic CD11b+ myelopoiesis. *Arthritis research & therapy*, 6(3), pp.R220–31.

Komatsu, Y. et al., 2002. Targeted disruption of the Tab1 gene causes embryonic lethality and defects in cardiovascular and lung morphogenesis. *Mechanisms of development*, 119(2), pp.239–49.

Kontoyiannis, D. et al., 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity*, 10(3), pp.387–98.

Kontoyiannis, D. et al., 2001. Interleukin-10 targets p38 MAPK to modulate ARE-dependent TNF mRNA translation and limit intestinal pathology. *The EMBO journal*, 20(14), pp.3760–70.

Konya, V. et al., 2013. E-type prostanoid receptor 4 (EP4) in disease and therapy. *Pharmacology & therapeutics*, 138(3), pp.485–502.

Korhonen, R. et al., 2013. Attenuation of TNF production and experimentally induced inflammation by PDE4 inhibitor rolipram is mediated by MAPK phosphatase-1. *British journal of pharmacology*, 169(7), pp.1525–36.

Kotlyarov, A. et al., 1999. MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. *Nature cell biology*, 1(2), pp.94–7.

Kratochvill, F. et al., 2011. Tristetraprolin-driven regulatory circuit controls quality and timing of mRNA decay in inflammation. *Molecular systems biology*, 7, p.560.

Kretzschmar, M. et al., 1997. The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes & development*, 11(8), pp.984–95.

Kretzschmar, M. & Massagué, J., 1998. SMADs: mediators and regulators of TGF-beta signaling. *Current opinion in genetics & development*, 8(1), pp.103–11.

Kroenke, M.A. et al., 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *The Journal of experimental medicine*, 205(7), pp.1535–41.

Krüger-Krasagakes, S. et al., 1994. Expression of interleukin 10 in human melanoma. *British journal of cancer*, 70(6), pp.1182–5.

Kuhn, K.A. & Stappenbeck, T.S., 2013. Peripheral education of the immune system by the colonic microbiota. *Seminars in immunology*, 25(5), pp.364–9.

Kühn, R. et al., 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, 75(2), pp.263–74.

Kulkarni, A.B. et al., 1993. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proceedings of the National Academy of Sciences of the United States of America*, 90(2), pp.770–4.

Kumaravelu, P. et al., 2002. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development (Cambridge, England)*, 129(21), pp.4891–9.

Lai, T.-Y. et al., 2013. Transcription of Tnfaip3 is regulated by NF-κB and p38 via C/EBPβ in activated macrophages. *PloS one*, 8(9), p.e73153.

Lai, W.S. et al., 1995. Promoter analysis of Zfp-36, the mitogen-inducible gene encoding the zinc finger protein tristetraprolin. *The Journal of biological chemistry*, 270(42), pp.25266–25272.

Lai, W.S., Stumpo, D.J. & Blackshear, P.J., 1990. Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. *The Journal of biological chemistry*, 265(27), pp.16556–16563.

Lai, W.S., Thompson, M.J. & Blackshear, P.J., 1998. Characteristics of the intron involvement in the mitogen-induced expression of Zfp-36. *The Journal of biological chemistry*, 273(1), pp.506–17.

Landskron, G. et al., 2014. Chronic inflammation and cytokines in the tumor microenvironment. *Journal of immunology research*, 2014, p.149185.

Lannan, E.A. et al., 2012. Proinflammatory actions of glucocorticoids: glucocorticoids and TNFα coregulate gene expression in vitro and in vivo. *Endocrinology*, 153(8), pp.3701–12.

Lasa, M. et al., 2002. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Molecular and cellular biology*, 22(22), pp.7802–7811.

Lasa, M. et al., 2001. Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. *Molecular and cellular biology*, 21(3), pp.771–780.

Lee, E.G. et al., 2000. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. *Science (New York, N.Y.)*, 289(5488), pp.2350–4.

Lee, J. et al., 2012. Phosphodiesterase 4B Mediates Extracellular Signal-regulated Kinase-dependent Up-regulation of Mucin MUC5AC Protein by Streptococcus pneumoniae by Inhibiting cAMP-protein Kinase A-dependent MKP-1 Phosphatase Pathway. *Journal of Biological Chemistry*, 287(27), pp.22799–22811.

Lee, J.C. et al., 2000. Inhibition of p38 MAP kinase as a therapeutic strategy. *Immunopharmacology*, 47(2-3), pp.185–201.

Lee, S.W. et al., 1988. Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA. *Proceedings of the National Academy of Sciences of the United States of America*, 85(4), pp.1204–8.

Lee, Y.H. et al., 1997. The ability of C/EBP beta but not C/EBP alpha to synergize with an Sp1 protein is specified by the leucine zipper and activation domain. *Molecular and cellular biology*, 17(4), pp.2038–47.

Lefstin, J.A., Thomas, J.R. & Yamamoto, K.R., 1994. Influence of a steroid receptor DNA-binding domain on transcriptional regulatory functions. *Genes & development*, 8(23), pp.2842–56.

Lekstrom-Himes, J. & Xanthopoulos, K.G., 1998. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *The Journal of biological chemistry*, 273(44), pp.28545–8.

Leppanen, T. et al., 2008. Inhibition of protein kinase C beta II downregulates tristetraprolin expression in activated macrophages. *Inflammation research□: official journal of the European Histamine Research Society ... [et al.]*, 57(5), pp.230–240.

Leppanen, T. et al., 2010. Inhibition of protein kinase Cdelta reduces tristetraprolin expression by destabilizing its mRNA in activated macrophages. *European journal of pharmacology*, 628(1-3), pp.220–225.

Levéen, P. et al., 2002. Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood*, 100(2), pp.560–8.

Li, D. et al., 2012. Down-regulation of A20 mRNA expression in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Journal of clinical immunology*, 32(6), pp.1287–91.

Li, J. et al., 2006. Conditional deletion of the colony stimulating factor-1 receptor (c-fms proto-oncogene) in mice. *Genesis (New York, N.Y.□: 2000)*, 44(7), pp.328–35.

Li, J. et al., 2001. Transcriptional induction of MKP-1 in response to stress is associated with histone H3 phosphorylation-acetylation. *Molecular and cellular biology*, 21(23), pp.8213–24.

Liang, J. et al., 2009. RNA-destabilizing factor tristetraprolin negatively regulates NF-kappaB signaling. *The Journal of biological chemistry*, 284(43), pp.29383–90.

Lin, M.-S. & Chen, Y.-W., 1993. B Cell Differentiation. *Cellular Immunology*, 150(2), pp.343–352.

Lin, P. et al., 2005. Lysophosphatidylcholine modulates neutrophil oxidant production through elevation of cyclic AMP. *Journal of immunology (Baltimore, Md.□: 1950)*, 174(5), pp.2981–9.

Lin, Y.C., Brown, K. & Siebenlist, U., 1995. Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B. *Proceedings of the National Academy of Sciences*, 92(2), pp.552–556.

Litvak, V. et al., 2009. Function of C/EBPdelta in a regulatory circuit that discriminates between transient and persistent TLR4-induced signals. *Nature immunology*, 10(4), pp.437–43.

Liu, H. et al., 2002. The scavenger mRNA decapping enzyme DcpS is a member of the HIT family of pyrophosphatases. *The EMBO journal*, 21(17), pp.4699–708.

Lonard, D.M. & O'malley, B.W., 2007. Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. *Molecular cell*, 27(5), pp.691–700.

López de Silanes, I., Quesada, M.P. & Esteller, M., 2007. Aberrant regulation of messenger RNA 3'-untranslated region in human cancer. *Cellular oncology□: the official journal of the International Society for Cellular Oncology*, 29(1), pp.1–17.

Luger, D. et al., 2008. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *The Journal of experimental medicine*, 205(4), pp.799–810.

Luo, M. et al., 2004. Protein kinase A inhibits leukotriene synthesis by phosphorylation of 5-lipoxygenase on serine 523. *The Journal of biological chemistry*, 279(40), pp.41512–20.

Ma, Q. & Herschman, H.R., 1991. A corrected sequence for the predicted protein from the mitogen-inducible TIS11 primary response gene. *Oncogene*, 6(7), pp.1277–8.

Macías-Silva, M. et al., 1996. MADR2 is a substrate of the TGFbeta receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell*, 87(7), pp.1215–24.

MacKenzie, K.F. et al., 2013. MSK1 and MSK2 inhibit lipopolysaccharide-induced prostaglandin production via an interleukin-10 feedback loop. *Molecular and cellular biology*, 33(7), pp.1456–67.

Mahtani, K.R. et al., 2001. Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. *Molecular and cellular biology*, 21(19), pp.6461–6469.

Maier, J. V et al., 2007. Dual specificity phosphatase 1 knockout mice show enhanced susceptibility to anaphylaxis but are sensitive to glucocorticoids. *Molecular endocrinology (Baltimore, Md.)*, 21(11), pp.2663–71.

Makranz, C. et al., 2006. cAMP cascade (PKA, Epac, adenylyl cyclase, Gi, and phosphodiesterases) regulates myelin phagocytosis mediated by complement receptor-3 and scavenger receptor-AI/II in microglia and macrophages. *Glia*, 53(4), pp.441–8.

Mancini, A.D. & Di Battista, J.A., 2011. The cardinal role of the phospholipase A(2)/cyclooxygenase-2/prostaglandin E synthase/prostaglandin E(2) (PCPP) axis in inflammostasis. *Inflammation research: official journal of the European Histamine Research Society ... [et al.]*, 60(12), pp.1083–92.

Manetsch, M. et al., 2012. Corticosteroids and β_2-agonists upregulate mitogen-activated protein kinase phosphatase 1: in vitro mechanisms. *British journal of pharmacology*, 166(7), pp.2049–59.

Manoury-Schwartz, B. et al., 1997. High susceptibility to collagen-induced arthritis in mice lacking IFN-gamma receptors. *Journal of immunology (Baltimore, Md.: 1950)*, 158(11), pp.5501–6.

Mantovani, A. & Marchesi, F., 2014. IL-10 and macrophages orchestrate gut homeostasis. *Immunity*, 40(5), pp.637–9.

Marchese, F.P. et al., 2010. MAPKAP kinase 2 blocks tristetraprolin-directed mRNA decay by inhibiting CAF1 deadenylase recruitment. *The Journal of biological chemistry*, 285(36), pp.27590–600.

Marini, M. et al., 2003. TNF-alpha neutralization ameliorates the severity of murine Crohn's-like ileitis by abrogation of intestinal epithelial cell apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, 100(14), pp.8366–71.

Martinon, F., Mayor, A. & Tschopp, J., 2009. The inflammasomes: guardians of the body. *Annual review of immunology*, 27, pp.229–65.

Massagué, J., 2012. TGFβ signalling in context. *Nature reviews. Molecular cell biology*, 13(10), pp.616–30.

Massie, C.E. & Mills, I.G., 2008. ChIPping away at gene regulation. *EMBO reports*, 9(4), pp.337–43.

Matta, R. et al., 2012. Knockout of Mkp-1 exacerbates colitis in Il-10-deficient mice. *American journal of physiology. Gastrointestinal and liver physiology*, 302(11), pp.G1322–35.

Mauro, C. et al., 2006. ABIN-1 Binds to NEMO/IKK and Co-operates with A20 in Inhibiting NF- B. *Journal of Biological Chemistry*, 281(27), pp.18482–18488.

McCoy, J.M., Wicks, J.R. & Audoly, L.P., 2002. The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *The Journal of clinical investigation*, 110(5), pp.651–8.

McKay, M.M. & Morrison, D.K., 2007. Integrating signals from RTKs to ERK/MAPK. *Oncogene*, 26(22), pp.3113–21.

Medvinsky, A. & Dzierzak, E., 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*, 86(6), pp.897–906.

Medvinsky, A.L. et al., 1996. Development of day-8 colony-forming unit-spleen hematopoietic progenitors during early murine embryogenesis: spatial and temporal mapping. *Blood*, 87(2), pp.557–66.

Medzhitov, R. et al., 2011. Highlights of 10 years of immunology in Nature Reviews Immunology. *Nature reviews. Immunology*, 11(10), pp.693–702.

Medzhitov, R., 2001. Toll-like receptors and innate immunity. *Nature reviews. Immunology*, 1(2), pp.135–45.

Medzhitov, R. & Horng, T., 2009. Transcriptional control of the inflammatory response. *Nature Reviews Immunology*, 9(10), pp.692–703.

Meijsing, S.H. et al., 2009. DNA binding site sequence directs glucocorticoid receptor structure and activity. *Science (New York, N.Y.)*, 324(5925), pp.407–10.

Mele, A. et al., 2014. Single nucleotide polymorphisms at the TNFAIP3/A20 locus and susceptibility/resistance to inflammatory and autoimmune diseases. *Advances in experimental medicine and biology*, 809, pp.163–83.

Mellman, I. & Steinman, R.M., 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell*, 106(3), pp.255–8.

Mikami, F. et al., 2006. The transforming growth factor-beta-Smad3/4 signaling pathway acts as a positive regulator for TLR2 induction by bacteria via a dual mechanism involving functional cooperation with NF-kappaB and MAPK phosphatase 1-dependent negative cross-talk with p38 MA. *The Journal of biological chemistry*, 281(31), pp.22397–408.

Milhollen, M.A. et al., 2010. MLN4924, a NEDD8-activating enzyme inhibitor, is active in diffuse large B-cell lymphoma models: rationale for treatment of NF- B-dependent lymphoma. *Blood*, 116(9), pp.1515–1523.

Miyake, K., 2006. Roles for accessory molecules in microbial recognition by Toll-like receptors. *Journal of endotoxin research*, 12(4), pp.195–204.

Moens, U., Kostenko, S. & Sveinbjørnsson, B., 2013. The Role of Mitogen-Activated Protein Kinase-Activated Protein Kinases (MAPKAPKs) in Inflammation. *Genes*, 4(2), pp.101–33.

Moghaddami, M. et al., 2014. Synovial fluid myeloid dendritic cells display important differences compared to monocyte-derived dendritic cells prepared in vitro. *Clinical & translational immunology*, 3(8), p.e23.

Moretta, A., 2002. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nature reviews. Immunology*, 2(12), pp.957–64.

Moretta, L. et al., 2002. Human NK cells and their receptors. *Microbes and infection / Institut Pasteur*, 4(15), pp.1539–44.

Muhlrad, D. & Parker, R., 2005. The yeast EDC1 mRNA undergoes deadenylation-independent decapping stimulated by Not2p, Not4p, and Not5p. *The EMBO journal*, 24(5), pp.1033–45.

Mukaida, N. et al., 1994. Novel mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappa B is target for glucocorticoid-mediated interleukin 8 gene repression. *The Journal of biological chemistry*, 269(18), pp.13289–95.

Murray, P.J., 2005. The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. *Proceedings of the National Academy of Sciences of the United States of America*, 102(24), pp.8686–91.

Musone, S.L. et al., 2011. Sequencing of TNFAIP3 and association of variants with multiple autoimmune diseases. *Genes and immunity*, 12(3), pp.176–82.

Nagakoa, Suzuki, Toshiyuki, K., *Stability of casein mRNA is ensured by structural interactions between the 3'-untranslated region and poly(A) tail via the HuR and poly(A)-binding protein complex.*

Nakao, M. et al., 1995. HLA A2601-restricted CTLs recognize a peptide antigen expressed on squamous cell carcinoma. *Cancer research*, 55(19), pp.4248–52.

Neu-Yilik, G. & Kulozik, A.E., 2008. NMD: multitasking between mRNA surveillance and modulation of gene expression. *Advances in genetics*, 62, pp.185–243.

Newton, K. & Dixit, V.M., 2012. Signaling in innate immunity and inflammation. *Cold Spring Harbor perspectives in biology*, 4(3).

Newton, R., 2013. Anti-inflammatory glucocorticoids: Changing concepts. *European journal of pharmacology*, pp.1–6.

Newton, R., 2000. Molecular mechanisms of glucocorticoid action: what is important? *Thorax*, 55(7), pp.603–13.

Newton, R. et al., 1998. Repression of cyclooxygenase-2 and prostaglandin E2 release by dexamethasone occurs by transcriptional and post-transcriptional mechanisms involving loss of polyadenylated mRNA. *The Journal of biological chemistry*, 273(48), pp.32312–32321.

Newton, R. & Holden, N.S., 2007. Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? *Molecular pharmacology*, 72(4), pp.799–809.

Nimah, M. et al., 2005. Contribution of MKP-1 regulation of p38 to endotoxin tolerance. *Shock (Augusta, Ga.)*, 23(1), pp.80–7.

Noguchi, T. et al., 1993. Structure, mapping, and expression of erp, a growth factor-inducible gene encoding a nontransmembrane protein tyrosine phosphatase, and effect of ERP on cell growth. *Molecular and cellular biology*, 13(9), pp.5195–205.

O'Mahony, L. et al., 2001. Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. *Alimentary pharmacology & therapeutics*, 15(8), pp.1219–25.

O'Neill, L.A., 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Science's STKE: signal transduction knowledge environment*, 2000(44), p.re1.

O'Shea, J.M. & Perkins, N.D., 2010. Thr435 phosphorylation regulates RelA (p65) NF-kappaB subunit transactivation. *The Biochemical journal*, 426(3), pp.345–54.

Ock, C.Y. et al., 2011. Prevention of Colitis-Associated Colorectal Cancer with 8-Hydroxydeoxyguanosine. *Cancer Prevention Research*, 4(9), pp.1507–1521.

Ogawa, K. et al., 2003. Transcriptional regulation of tristetraprolin by transforming growth factor-beta in human T cells. *The Journal of biological chemistry*, 278(32), pp.30373–81.

Ogle, C.K. et al., 1994. Production of tumor necrosis factor, interleukin-6 and prostaglandin E2 by LPS-stimulated rat bone marrow macrophages after thermal injury: effect of indomethacin. *Inflammation*, 18(2), pp.175–85.

Ollivier, V. et al., 1996. Elevated Cyclic AMP Inhibits NF- B-mediated Transcription in Human Monocytic Cells and Endothelial Cells. *Journal of Biological Chemistry*, 271(34), pp.20828–20835.

Onozaki, K., 2013. [Interluekin-1: from regulation of cell proliferation to chronic inflammatory diseases]. *Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan*, 133(6), pp.645–60.

Opipari, A.W., Boguski, M.S. & Dixit, V.M., 1990. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *The Journal of biological chemistry*, 265(25), pp.14705–8.

Oshimori, N., Oristian, D. & Fuchs, E., 2015. TGF-β Promotes Heterogeneity and Drug Resistance in Squamous Cell Carcinoma. *Cell*, 160(5), pp.963–976.

Ottersbach, K. & Dzierzak, E., 2005. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Developmental cell*, 8(3), pp.377–87.

Owens, D.M. & Keyse, S.M., 2007. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene*, 26(22), pp.3203–13.

Page, T.H. et al., 2010. Nonsteroidal anti-inflammatory drugs increase TNF production in rheumatoid synovial membrane cultures and whole blood. *Journal of immunology (Baltimore, Md.: 1950)*, 185(6), pp.3694–701.

Pang, L. & Hoult, J.R., 1997. Repression of inducible nitric oxide synthase and cyclooxygenase-2 by prostaglandin E2 and other cyclic AMP stimulants in J774 macrophages. *Biochemical pharmacology*, 53(4), pp.493–500.

Panopoulos, A.D. & Watowich, S.S., 2008. Granulocyte colony-stimulating factor: molecular mechanisms of action during steady state and "emergency" hematopoiesis. *Cytokine*, 42(3), pp.277–88.

Park, B.S. et al., 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*, 458(7242), pp.1191–5.

Park, J.H. et al., 2014. The impact of anti-inflammatory agents on the outcome of patients with colorectal cancer. *Cancer treatment reviews*, 40(1), pp.68–77.

Parker, R. & Sheth, U., 2007. P bodies and the control of mRNA translation and degradation. *Molecular cell*, 25(5), pp.635–46.

Parry, G.C. & Mackman, N., 1997. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. *Journal of immunology (Baltimore, Md.: 1950)*, 159(11), pp.5450–6.

Pauli, U., 1994. Control of tumor necrosis factor gene expression. *Critical reviews in eukaryotic gene expression*, 4(2-3), pp.323–44.

Pelaia, G. et al., 2005. Mitogen-activated protein kinases and asthma. *Journal of Cellular Physiology*, 202(3), pp.642–653.

Pelaia, G. et al., 2011. Omalizumab decreases exacerbation frequency, oral intake of corticosteroids and peripheral blood eosinophils in atopic patients with uncontrolled asthma. *International journal of clinical pharmacology and therapeutics*, 49(12), pp.713–21.

Phillips, R.S., Ramos, S.B. & Blackshear, P.J., 2002. Members of the tristetraprolin family of tandem CCCH zinc finger proteins exhibit CRM1-dependent nucleocytoplasmic shuttling. *The Journal of biological chemistry*, 277(13), pp.11606–11613.

Pipkin, M.E., 2006. A reliable method to display authentic DNase I hypersensitive sites at long-ranges in single-copy genes from large genomes. *Nucleic Acids Research*, 34(4), pp.e34–e34.

Pozzi, A. et al., 2004. Colon carcinoma cell growth is associated with prostaglandin E2/EP4 receptor-evoked ERK activation. *The Journal of biological chemistry*, 279(28), pp.29797–804.

Present, D.H. et al., 1999. Infliximab for the treatment of fistulas in patients with Crohn's disease. *The New England journal of medicine*, 340(18), pp.1398–405.

Pritchard, C.C., Cheng, H.H. & Tewari, M., 2012. MicroRNA profiling: approaches and considerations. *Nature reviews. Genetics*, 13(5), pp.358–69.

Qiu, L.Q., Stumpo, D.J. & Blackshear, P.J., 2012. Myeloid-specific tristetraprolin deficiency in mice results in extreme lipopolysaccharide sensitivity in an otherwise minimal phenotype. *Journal of immunology*, 188(10), pp.5150–5159.

Quante, T. et al., 2008. Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. *American journal of respiratory cell and molecular biology*, 39(2), pp.208–17.

Raghavan, A. & Bohjanen, P.R., 2004. Microarray-based analyses of mRNA decay in the regulation of mammalian gene expression. *Briefings in functional genomics & proteomics*, 3(2), pp.112–24.

Raingeaud, J. et al., 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Molecular and cellular biology*, 16(3), pp.1247–55.

Raman, M., Chen, W. & Cobb, M.H., 2007. Differential regulation and properties of MAPKs. *Oncogene*, 26(22), pp.3100–12.

Rao, N.A.S. et al., 2011. Coactivation of GR and NFKB alters the repertoire of their binding sites and target genes. *Genome research*, 21(9), pp.1404–1416.

Rao, P. et al., 2010. IkappaBbeta acts to inhibit and activate gene expression during the inflammatory response. *Nature*, 466(7310), pp.1115–1119.

Rauch, I., Müller, M. & Decker, T., 2013. The regulation of inflammation by interferons and their STATs. *JAK-STAT*, 2(1), p.e23820.

Read, M.A. et al., 2000. Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. *Molecular and cellular biology*, 20(7), pp.2326–33.

Rebuffini, E. et al., 2012. Picibanil (OK-432) in the treatment of head and neck lymphangiomas in children. *Dental research journal*, 9(Suppl 2), pp.S192–6.

Regan, J.W. et al., 1994. Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP2 subtype. *Molecular pharmacology*, 46(2), pp.213–20.

Regard, J.B., Sato, I.T. & Coughlin, S.R., 2008. Anatomical profiling of G protein-coupled receptor expression. *Cell*, 135(3), pp.561–71.

Reily, M.M. et al., 2006. The GRIP1:IRF3 interaction as a target for glucocorticoid receptor-mediated immunosuppression. *The EMBO journal*, 25(1), pp.108–17.

Riddell, J.R. et al., 2012. Peroxiredoxin 1 stimulates endothelial cell expression of VEGF via TLR4 dependent activation of HIF-1α. *PloS one*, 7(11), p.e50394.

Rigby, W.F. et al., 2005. Structure/function analysis of tristetraprolin (TTP): p38 stress-activated protein kinase and lipopolysaccharide stimulation do not alter TTP function. *Journal of immunology*, 174(12), pp.7883–7893.

Rissoan, M., 1999. Reciprocal Control of T Helper Cell and Dendritic Cell Differentiation. *Science*, 283(5405), pp.1183–1186.

Roberts, A.B. et al., 2003. Smad3: a key player in pathogenetic mechanisms dependent on TGF-beta. *Annals of the New York Academy of Sciences*, 995, pp.1–10.

Rocha, S. et al., 2005. Regulation of NF-kappaB and p53 through activation of ATR and Chk1 by the ARF tumour suppressor. *The EMBO journal*, 24(6), pp.1157–69.

Rogler, G. et al., 1998. Isolation and phenotypic characterization of colonic macrophages. *Clinical and experimental immunology*, 112(2), pp.205–15.

Roh, Y.S., Song, J. & Seki, E., 2014. TAK1 regulates hepatic cell survival and carcinogenesis. *Journal of gastroenterology*, 49(2), pp.185–94.

Ronkina, N. et al., 2010. MAPKAP kinases MK2 and MK3 in inflammation: complex regulation of TNF biosynthesis via expression and phosphorylation of tristetraprolin. *Biochemical pharmacology*, 80(12), pp.1915–1920.

Rönnelid, J. et al., 2003. Immune complexes from SLE sera induce IL10 production from normal peripheral blood mononuclear cells by an FcgammaRII dependent mechanism: implications for a possible vicious cycle maintaining B cell hyperactivity in SLE. *Annals of the rheumatic diseases*, 62(1), pp.37–42.

Ross, E.A. et al., 2015. Dominant Suppression of Inflammation via Targeted Mutation of the mRNA Destabilizing Protein Tristetraprolin. *Journal of immunology (Baltimore, Md.: 1950)*, 195(1), pp.265–76.

Ross, S. et al., 2006. Smads orchestrate specific histone modifications and chromatin remodeling to activate transcription. *The EMBO journal*, 25(19), pp.4490–502.

Rossi, J.-F. et al., 2015. Interleukin-6 as a Therapeutic Target. *Clinical cancer research: an official journal of the American Association for Cancer Research*, 21(6), pp.1248–1257.

Rounbehler, R.J. et al., 2012. Tristetraprolin impairs myc-induced lymphoma and abolishes the malignant state. *Cell*, 150(3), pp.563–74.

Ruland, J., 2011. Return to homeostasis: downregulation of NF-κB responses. *Nature immunology*, 12(8), pp.709–14.

Sabroe, I. & Whyte, M.K.B., 2007. Toll-like receptor (TLR)-based networks regulate neutrophilic inflammation in respiratory disease. *Biochemical Society transactions*, 35(Pt 6), pp.1492–5.

Sakaguchi, S. et al., 2009. Regulatory T cells: how do they suppress immune responses? *International immunology*, 21(10), pp.1105–11.

Saklatvala, J., Dean, J. & Clark, A., 2003. Control of the expression of inflammatory response genes. *Biochemical Society symposium*, (70), pp.95–106.

Salojin, K. & Oravecz, T., 2007. Regulation of innate immunity by MAPK dual-specificity phosphatases: knockout models reveal new tricks of old genes. *Journal of leukocyte biology*, 81(4), pp.860–9.

Salojin, K. V et al., 2006. Essential role of MAPK phosphatase-1 in the negative control of innate immune responses. *Journal of immunology (Baltimore, Md.: 1950)*, 176(3), pp.1899–907.

Salvador, J.M., Mittelstadt, P.R., Guszczynski, T., et al., 2005. Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases. *Nature immunology*, 6(4), pp.390–5.

Salvador, J.M., Mittelstadt, P.R., Belova, G.I., et al., 2005. The autoimmune suppressor Gadd45alpha inhibits the T cell alternative p38 activation pathway. *Nature immunology*, 6(4), pp.396–402.

Sambuelli, A. et al., 2000. Serum transforming growth factor-beta1 levels increase in response to successful anti-inflammatory therapy in ulcerative colitis. *Alimentary pharmacology & therapeutics*, 14(11), pp.1443–9.

Sandler, H. et al., 2011. Not1 mediates recruitment of the deadenylase Caf1 to mRNAs targeted for degradation by tristetraprolin. *Nucleic acids research*, 39(10), pp.4373–86.

Sandoval, J. et al., 2004. RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic acids research*, 32(11), p.e88.

Sanduja, S. et al., 2012. The role of tristetraprolin in cancer and inflammation. *Frontiers in bioscience (Landmark edition)*, 17, pp.174–88.

Sanduja, S., Kaza, V. & Dixon, D.A., 2009. The mRNA decay factor tristetraprolin (TTP) induces senescence in human papillomavirus-transformed cervical cancer cells by targeting E6-AP ubiquitin ligase. *Aging*, 1(9), pp.803–17.

Sanjo, H. et al., 2003. TAB2 is essential for prevention of apoptosis in fetal liver but not for interleukin-1 signaling. *Molecular and cellular biology*, 23(4), pp.1231–8.

De Santa, F. et al., 2010. A large fraction of extragenic RNA pol II transcription sites overlap enhancers. *PLoS biology*, 8(5), p.e1000384.

Santana, M.A. & Rosenstein, Y., 2003. What it takes to become an effector T cell: the process, the cells involved, and the mechanisms. *Journal of cellular physiology*, 195(3), pp.392–401.

Sartori, R., Li, F. & Kirkwood, K.L., 2009. MAP kinase phosphatase-1 protects against inflammatory bone loss. *Journal of dental research*, 88(12), pp.1125–30.

Sassone-Corsi, P., 1998. Coupling gene expression to cAMP signalling: role of CREB and CREM. *The international journal of biochemistry & cell biology*, 30(1), pp.27–38.

Sassone-Corsi, P., 2012. The cyclic AMP pathway. *Cold Spring Harbor perspectives in biology*, 4(12), p.a011148–.

Sato, S. et al., 2003. A mammalian homolog of Drosophila melanogaster transcriptional coactivator intersex is a subunit of the mammalian Mediator complex. *The Journal of biological chemistry*, 278(50), pp.49671–4.

Sauer, I. et al., 2006. Interferons limit inflammatory responses by induction of tristetraprolin. *Blood*, 107(12), pp.4790–4797.

Sauer, I. et al., 2006. Interferons limit inflammatory responses by induction of tristetraprolin. *Blood*, 107(12), pp.4790–7.

Saunders, A.M. & Seldin, M.F., 1990. A molecular genetic linkage map of mouse chromosome 7. *Genomics*, 8(3), pp.525–35.

Sawaoka, H. et al., 2003. Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. *The Journal of biological chemistry*, 278(16), pp.13928–35.

Schäcke, H., Rehwinkel, H. & Asadullah, K., 2005. Dissociated glucocorticoid receptor ligands: compounds with an improved therapeutic index. *Current opinion in investigational drugs (London, England: 2000)*, 6(5), pp.503–7.

Schaljo, B. et al., 2009. Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10. *Journal of immunology*, 183(2), pp.1197–206.

Scheinman, R.I. et al., 1995. Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Molecular and cellular biology*, 15(2), pp.943–53.

Scheinman, R.I. et al., 1995. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. *Science*, 270(5234), pp.283–286.

Scheller, J. et al., 2011. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et biophysica acta*, 1813(5), pp.878–88.

Schichl, Y.M. et al., 2009. Tristetraprolin impairs NF-kappaB/p65 nuclear translocation. *The Journal of biological chemistry*, 284(43), pp.29571–81.

Schindler, M. et al., 2013. cAMP-responsive element binding protein: a vital link in embryonic hormonal adaptation. *Endocrinology*, 154(6), pp.2208–21.

Schlitzer, A. & Ginhoux, F., 2014. Organization of the mouse and human DC network. *Current opinion in immunology*, 26, pp.90–9.

Schneider-Brachert, W., Heigl, U. & Ehrenschwender, M., 2013. Membrane trafficking of death receptors: implications on signalling. *International journal of molecular sciences*, 14(7), pp.14475–503.

Schott, J. et al., 2014. Translational regulation of specific mRNAs controls feedback inhibition and survival during macrophage activation. *PLoS genetics*, 10(6), p.e1004368.

Schott, J. & Stoecklin, G., Networks controlling mRNA decay in the immune system. *Wiley interdisciplinary reviews. RNA*, 1(3), pp.432–56.

Schottelius, A.J. et al., 1999. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *The Journal of biological chemistry*, 274(45), pp.31868–74.

Schreiber, S., 2005. The complicated path to true causes of disease: role of nuclear factor kappaB in inflammatory bowel disease. *Gut*, 54(4), pp.444–5.

Schroder, K. et al., 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*, 75(2), pp.163–89.

Schulz, C. et al., 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science (New York, N.Y.)*, 336(6077), pp.86–90.

Schwiebert, L.M. et al., 1996. Glucocorticosteroid inhibition of cytokine production: relevance to antiallergic actions. *The Journal of allergy and clinical immunology*, 97(1 Pt 2), pp.143–52.

Seery, J.P. et al., 1997. Antinuclear autoantibodies and lupus nephritis in transgenic mice expressing interferon gamma in the epidermis. *The Journal of experimental medicine*, 186(9), pp.1451–9.

Seillet, C. et al., 2013. CD8α+ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3. *Blood*, 121(9), pp.1574–83.

Seillet, C. & Belz, G.T., 2013. Terminal differentiation of dendritic cells. *Advances in immunology*, 120, pp.185–210.

Seldon, P.M. et al., 1995. Suppression of lipopolysaccharide-induced tumor necrosis factor-alpha generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase. *Mol. Pharmacol.*, 48(4), pp.747–757.

Serezani, C.H. et al., 2008. Cyclic AMP: master regulator of innate immune cell function. *American journal of respiratory cell and molecular biology*, 39(2), pp.127–32.

Serio, K.J., Reddy, K.V. & Bigby, T.D., 2005. Lipopolysaccharide induces 5-lipoxygenase-activating protein gene expression in THP-1 cells via a NF-kappaB and C/EBP-mediated mechanism. *American journal of physiology. Cell physiology*, 288(5), pp.C1125–33.

Sessler, T. et al., 2013. Structural determinants of DISC function: new insights into death receptor-mediated apoptosis signalling. *Pharmacology & therapeutics*, 140(2), pp.186–99.

Shacter, E., Arzadon, G.K. & Williams, J., 1992. Elevation of interleukin-6 in response to a chronic inflammatory stimulus in mice: inhibition by indomethacin. *Blood*, 80(1), pp.194–202.

Shaw, G. & Kamen, R., 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*, 46(5), pp.659–67.

Shembade, N. et al., 2007. Essential role for TAX1BP1 in the termination of TNF-alpha-, IL-1- and LPS-mediated NF-kappaB and JNK signaling. *The EMBO journal*, 26(17), pp.3910–22.

Shembade, N. & Harhaj, E.W., 2012. Regulation of NF-kappaB signaling by the A20 deubiquitinase. *Cellular & molecular immunology*, 9(2), pp.123–130.

Shembade, N., Ma, A. & Harhaj, E.W., 2010. Inhibition of NF-kappaB signaling by A20 through disruption of ubiquitin enzyme complexes. *Science (New York, N.Y.)*, 327(5969), pp.1135–9.

Sheng, H. et al., 2001. Prostaglandin E2 increases growth and motility of colorectal carcinoma cells. *The Journal of biological chemistry*, 276(21), pp.18075–81.

Shepherd, E.G. et al., 2004. The function of mitogen-activated protein kinase phosphatase-1 in peptidoglycan-stimulated macrophages. *The Journal of biological chemistry*, 279(52), pp.54023–31.

Sheth, U. & Parker, R., 2006. Targeting of aberrant mRNAs to cytoplasmic processing bodies. *Cell*, 125(6), pp.1095–109.

Shi, J. et al., 2010. The prostaglandin E2 E-prostanoid 4 receptor exerts anti-inflammatory effects in brain innate immunity. *Journal of immunology (Baltimore, Md.: 1950)*, 184(12), pp.7207–18.

Shi, Y. & Massagué, J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, 113(6), pp.685–700.

Shim, J.-H. et al., 2005. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes & development*, 19(22), pp.2668–81.

Shipp, L.E. et al., 2010. Transcriptional regulation of human dual specificity protein phosphatase 1 (DUSP1) gene by glucocorticoids. *PloS one*, 5(10), p.e13754.

Shortman, K. & Naik, S.H., 2007. Steady-state and inflammatory dendritic-cell development. *Nature reviews. Immunology*, 7(1), pp.19–30.

Shouval, D.S. et al., 2014. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. *Immunity*, 40(5), pp.706–19.

Shull, M.M. et al., 1992. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature*, 359(6397), pp.693–9.

Sica, A. & Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*, 122(3), pp.787–95.

Silva, A.L. & Romão, L., 2009. The mammalian nonsense-mediated mRNA decay pathway: to decay or not to decay! Which players make the decision? *FEBS letters*, 583(3), pp.499–505.

Singhirunnusorn, P. et al., 2005. Critical roles of threonine 187 phosphorylation in cellular stress-induced rapid and transient activation of transforming growth factor-beta-activated kinase 1 (TAK1) in a signaling complex containing TAK1-binding protein TAB1 and TAB2. *The Journal of biological chemistry*, 280(8), pp.7359–68.

Skoog, T., Hamsten, A. & Eriksson, P., 2006. Allele-specific chromatin remodeling of the tumor necrosis factor-alpha promoter. *Biochemical and biophysical research communications*, 351(3), pp.777–83.

Smale, S.T., 2010. Seq-ing LPS-induced enhancers. *Immunity*, 32(3), pp.296–8.

Smallie, T. et al., 2015. Dual-Specificity Phosphatase 1 and Tristetraprolin Cooperate To Regulate Macrophage Responses to Lipopolysaccharide. *Journal of immunology (Baltimore, Md.: 1950)*, 195(1), pp.277–88.

Smallie, T. et al., 2010. IL-10 inhibits transcription elongation of the human TNF gene in primary macrophages. *The Journal of Experimental Medicine*, 207, pp.2081–2088.

Smith, N.L.D. & Denning, D.W., 2014. Clinical implications of interferon-γ genetic and epigenetic variants. *Immunology*, 143(4), pp.499–511.

Smoak, K. & Cidlowski, J.A., 2006. Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. *Molecular and cellular biology*, 26(23), pp.9126–9135.

So, A.Y. et al., 2008. Conservation analysis predicts in vivo occupancy of glucocorticoid receptor-binding sequences at glucocorticoid-induced genes. *Proceedings of the National Academy of Sciences of the United States of America*, 105(15), pp.5745–5749.

Sohn, B.H. et al., 2010. Functional switching of TGF-beta1 signaling in liver cancer via epigenetic modulation of a single CpG site in TTP promoter. *Gastroenterology*, 138(5), pp.1898–908.

Song, L. et al., 2011. Open chromatin defined by DNaseI and FAIRE identifies regulatory elements that shape cell-type identity. *Genome research*, 21(10), pp.1757–67.

Soucy, T.A. et al., 2010. The NEDD8 Conjugation Pathway and Its Relevance in Cancer Biology and Therapy. *Genes & cancer*, 1(7), pp.708–16.

St-Pierre, J. & Chadee, K., 2014. How the discovery of TNF-α has advanced gastrointestinal diseases and treatment regimes. *Digestive diseases and sciences*, 59(4), pp.712–5.

Stees, J.S. et al., 2012. Recruitment of transcription complexes to enhancers and the role of enhancer transcription. *Biology*, 1(3), pp.778–93.

Steiger, M. et al., 2003. Analysis of recombinant yeast decapping enzyme. *RNA (New York, N.Y.)*, 9(2), pp.231–8.

Steinman, L., 2008. A rush to judgment on Th17. *The Journal of experimental medicine*, 205(7), pp.1517–22.

Stellato, C., 2004. Post-transcriptional and nongenomic effects of glucocorticoids. *Proceedings of the American Thoracic Society*, 1(3), pp.255–63.

Stoecklin, G. et al., 2008. Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. *The Journal of biological chemistry*, 283(17), pp.11689–99.

Stoecklin, G. et al., 2004. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. *The EMBO journal*, 23(6), pp.1313–24.

Van de Stolpe, A. et al., 1994. 12-O-tetradecanoylphorbol-13-acetate- and tumor necrosis factor alpha-mediated induction of intercellular adhesion molecule-1 is inhibited by dexamethasone. Functional analysis of the human intercellular adhesion molecular-1 promoter. *The Journal of biological chemistry*, 269(8), pp.6185–92.

Sugimoto, Y. & Narumiya, S., 2007. Prostaglandin E receptors. *The Journal of biological chemistry*, 282(16), pp.11613–7.

Sun, L. et al., 2007. Tristetraprolin (TTP)-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2a and stabilizes tumor necrosis factor-alpha mRNA. *The Journal of biological chemistry*, 282(6), pp.3766–3777.

Sun, Z. et al., 2012. Serum IL-10 from systemic lupus erythematosus patients suppresses the differentiation and function of monocyte-derived dendritic cells. *Journal of biomedical research*, 26(6), pp.456–66.

Surjit, M. et al., 2011. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell*, 145(2), pp.224–41.

Suzuki, K. et al., 2003. IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-alpha production in mast cells. *The Journal of Experimental Medicine*, 198(11), pp.1717–1727.

Swantek, J.L., Cobb, M.H. & Geppert, T.D., 1997. Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK. *Molecular and cellular biology*, 17(11), pp.6274–82.

Swords, R.T. et al., 2010. Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. *Blood*, 115(18), pp.3796–3800.

Szekanecz, Z. & Koch, A.E., 2007. Macrophages and their products in rheumatoid arthritis. *Current opinion in rheumatology*, 19(3), pp.289–95.

Taga, T. & Kishimoto, T., 1997. Gp130 and the interleukin-6 family of cytokines. *Annual review of immunology*, 15, pp.797–819.

Tahoori, M.T. et al., 2015. Fibroblasts feeder niche and Flt3 Ligand as a novel inducer of plasmacytoid dendritic cells development in vitro. *International immunopharmacology*, 24(2), pp.474–80.

Takeuchi, O. & Akira, S., 2010. Pattern recognition receptors and inflammation. *Cell*, 140(6), pp.805–20.

Tan, F.E. & Elowitz, M.B., 2014. Brf1 posttranscriptionally regulates pluripotency and differentiation responses downstream of Erk MAP kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 111(17), pp.E1740–8.

Tan, N.S. et al., 2004. Essential role of Smad3 in the inhibition of inflammation-induced PPARbeta/delta expression. *The EMBO journal*, 23(21), pp.4211–21.

Tang, E.H.C. et al., 2015. Activation of prostaglandin E2-EP4 signaling reduces chemokine production in adipose tissue. *Journal of lipid research*, 56(2), pp.358–68.

Tang, E.H.C. et al., 2012. Anti-inflammation therapy by activation of prostaglandin EP4 receptor in cardiovascular and other inflammatory diseases. *Journal of cardiovascular pharmacology*, 59(2), pp.116–23.

Taves, M.D., Gomez-Sanchez, C.E. & Soma, K.K., 2011. Extra-adrenal glucocorticoids and mineralocorticoids: evidence for local synthesis, regulation, and function. *American journal of physiology. Endocrinology and metabolism*, 301(1), pp.E11–24.

Taylor, G.A. et al., 1996. A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity*, 4(5), pp.445–454.

Taylor, G.A. et al., 1995. Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase in vitro. *The Journal of biological chemistry*, 270(22), pp.13341–7.

Taylor, G.A. et al., 1991. The human TTP protein: sequence, alignment with related proteins, and chromosomal localization of the mouse and human genes. *Nucleic acids research*, 19(12), p.3454.

Tchen, C.R. et al., 2010. Glucocorticoid regulation of mouse and human dual specificity phosphatase 1 (DUSP1) genes: unusual cis-acting elements and unexpected evolutionary divergence. *The Journal of biological chemistry*, 285(4), pp.2642–2652.

Tchen, C.R. et al., 2004. The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself. *The Journal of biological chemistry*, 279(31), pp.32393–32400.

Tejasvi, T. et al., 2012. TNFAIP3 gene polymorphisms are associated with response to TNF blockade in psoriasis. *The Journal of investigative dermatology*, 132(3 Pt 1), pp.593–600.

Tiedje, C. et al., 2012. The p38/MK2-driven exchange between tristetraprolin and HuR regulates AU-rich element-dependent translation. *PLoS genetics*, 8(9), p.e1002977.

Tobler, A. et al., 1992. Glucocorticoids downregulate gene expression of GM-CSF, NAP-1/IL-8, and IL-6, but not of M-CSF in human fibroblasts. *Blood*, 79(1), pp.45–51.

Toh, M.-L. et al., 2004. Expression of mitogen-activated protein kinase phosphatase 1, a negative regulator of the mitogen-activated protein kinases, in rheumatoid arthritis: up-regulation by interleukin-1beta and glucocorticoids. *Arthritis and rheumatism*, 50(10), pp.3118–28.

Tong, X.-K. & Hamel, E., 2007. Transforming growth factor-beta 1 impairs endothelin-1-mediated contraction of brain vessels by inducing mitogen-activated protein (MAP) kinase phosphatase-1 and inhibiting p38 MAP kinase. *Molecular pharmacology*, 72(6), pp.1476–83.

Treisman, R., 1996. Regulation of transcription by MAP kinase cascades. *Current opinion in cell biology*, 8(2), pp.205–15.

Trynka, G. et al., 2009. Coeliac disease-associated risk variants in TNFAIP3 and REL implicate altered NF-kappaB signalling. *Gut*, 58(8), pp.1078–83.

Tucker, M. et al., 2002. Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae. *The EMBO journal*, 21(6), pp.1427–36.

Tudor, C. et al., 2009. The p38 MAPK pathway inhibits tristetraprolin-directed decay of interleukin-10 and pro-inflammatory mediator mRNAs in murine macrophages. *FEBS letters*, 583(12), pp.1933–1938.

Turer, E.E. et al., 2008. Homeostatic MyD88-dependent signals cause lethal inflamMation in the absence of A20. *The Journal of experimental medicine*, 205(2), pp.451–64.

Uhlenhaut, N.H. et al., 2013. Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. *Molecular cell*, 49(1), pp.158–71.

Vacchelli, E., Vitale, I., et al., 2013. Trial watch: Dendritic cell-based interventions for cancer therapy. *Oncoimmunology*, 2(10), p.e25771.

Vacchelli, E., Eggermont, A., et al., 2013. Trial Watch: Toll-like receptor agonists for cancer therapy. *Oncoimmunology*, 2(8), p.e25238.

De Valck, D. et al., 1999. The zinc finger protein A20 interacts with a novel anti-apoptotic protein which is cleaved by specific caspases. *Oncogene*, 18(29), pp.4182–90.

Valerio, M.S. et al., 2015. Critical role of MKP-1 in lipopolysaccharide-induced osteoclast formation through CXCL1 and CXCL2. *Cytokine*, 71(1), pp.71–80.

Vandevyver, S. et al., 2012. Glucocorticoid receptor dimerization induces MKP1 to protect against TNF-induced inflammation. *The Journal of clinical investigation*, 122(6), pp.2130–40.

Varnum, B.C. et al., 1989. Granulocyte-macrophage colony-stimulating factor and tetradecanoyl phorbol acetate induce a distinct, restricted subset of primary-response TIS genes in both proliferating and terminally differentiated myeloid cells. *Molecular and cellular biology*, 9(8), pp.3580–3.

Varnum, B.C. et al., 1991. The TIS11 primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual Cys-His repeat. *Molecular and cellular biology*, 11(3), pp.1754–8.

Varol, C., Yona, S. & Jung, S., 2009. Origins and tissue-context-dependent fates of blood monocytes. *Immunology and cell biology*, 87(1), pp.30–8.

Vattakuzhi, Y. et al., 2012. Dual-specificity phosphatase 1-null mice exhibit spontaneous osteolytic disease and enhanced inflammatory osteolysis in experimental arthritis. *Arthritis and rheumatism*, 64(7), pp.2201–10.

Vazquez, M.I., Catalan-Dibene, J. & Zlotnik, A., 2015. B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine*.

Vereecke, L. et al., 2014. A20 controls intestinal homeostasis through cell-specific activities. *Nature communications*, 5, p.5103.

Vermeire, K. et al., 1997. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. *Journal of immunology (Baltimore, Md.: 1950)*, 158(11), pp.5507–13.

Verstrepen, L. et al., 2010. Expression, biological activities and mechanisms of action of A20 (TNFAIP3). *Biochemical pharmacology*, 80(12), pp.2009–20.

Verstrepen, L., Carpentier, I. & Beyaert, R., 2014. The biology of A20-binding inhibitors of NF-kappaB activation (ABINs). *Advances in experimental medicine and biology*, 809, pp.13–31.

Vincenzo, B. et al., 2015. Adaptive immunity and inflammation. *International journal of inflammation*, 2015, p.575406.

De Waal Malefyt, R. et al., 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *The Journal of experimental medicine*, 174(5), pp.1209–20.

Wagner, S. et al., 2008. Ubiquitin binding mediates the NF-kappaB inhibitory potential of ABIN proteins. *Oncogene*, 27(26), pp.3739–45.

Walraven, M., Beelen, R.H.J. & Ulrich, M.M.W., 2015. Transforming growth factor-β (TGF-β) signaling in healthy human fetal skin, a descriptive study. *Journal of Dermatological Science*.

Wang, C. et al., 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*, 412(6844), pp.346–51.

Wang, C.-M. et al., 2011. miR-29c targets TNFAIP3, inhibits cell proliferation and induces apoptosis in hepatitis B virus-related hepatocellular carcinoma. *Biochemical and biophysical research communications*, 411(3), pp.586–92.

Wang, D. et al., 2000. Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. *The Journal of biological chemistry*, 275(42), pp.32592–7.

Wang, J., Ford, H.R. & Grishin, A. V, 2010. NF-kappaB-mediated expression of MAPK phosphatase-1 is an early step in desensitization to TLR ligands in enterocytes. *Mucosal immunology*, 3(5), pp.523–34.

Wang, P. et al., 2010. Shear-induced interleukin-6 synthesis in chondrocytes: roles of E prostanoid (EP) 2 and EP3 in cAMP/protein kinase A- and PI3-K/Akt-dependent NF-kappaB activation. *The Journal of biological chemistry*, 285(32), pp.24793–804.

Wang, P., Zhu, F. & Konstantopoulos, K., 2010. Prostaglandin E2 induces interleukin-6 expression in human chondrocytes via cAMP/protein kinase A- and phosphatidylinositol 3-kinase-dependent NF- B activation. *AJP: Cell Physiology*, 298(6), pp.C1445–C1456.

Wang, S. et al., 2013. An enhancer element harboring variants associated with systemic lupus erythematosus engages the TNFAIP3 promoter to influence A20 expression. *PLoS genetics*, 9(9), p.e1003750.

Wang, X. et al., 2014. Abnormal expression of A20 and its regulated genes in peripheral blood from patients with lymphomas. *Cancer cell international*, 14, p.36.

Watford, W.T. et al., 2003. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine & growth factor reviews*, 14(5), pp.361–8.

Watson, I.R., Irwin, M.S. & Ohh, M., 2011. NEDD8 pathways in cancer, Sine Quibus Non. *Cancer cell*, 19(2), pp.168–76.

Weber, A., Wasiliew, P. & Kracht, M., 2010. Interleukin-1 (IL-1) pathway. *Science signaling*, 3(105), p.cm1.

Webster, J.C. et al., 2002. Dexamethasone and tumor necrosis factor-alpha act together to induce the cellular inhibitor of apoptosis-2 gene and prevent apoptosis in a variety of cell types. *Endocrinology*, 143(10), pp.3866–74.

Wei, X. et al., 2014. Activation of the JAK-STAT3 pathway is associated with the growth of colorectal carcinoma cells. *Oncology reports*, 31(1), pp.335–41.

Weinstein, R.S. et al., 2011. Osteoprotegerin prevents glucocorticoid-induced osteocyte apoptosis in mice. *Endocrinology*, 152(9), pp.3323–31.

Weiss, A. & Attisano, L., The TGFbeta superfamily signaling pathway. *Wiley interdisciplinary reviews. Developmental biology*, 2(1), pp.47–63.

Welte, T. & Groneberg, D.A., 2006. Asthma and COPD. *Experimental and toxicologic pathology□: official journal of the Gesellschaft für Toxikologische Pathologie*, 57 Suppl 2, pp.35–40.

Wen, A.Y., Sakamoto, K.M. & Miller, L.S., 2010. The role of the transcription factor CREB in immune function. *Journal of immunology (Baltimore, Md.□: 1950)*, 185(11), pp.6413–9.

Wertz, I.E., O'Rourke, K.M., Zhou, H., et al., 2004. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature*, 430(7000), pp.694–9.

Wertz, I.E., O'Rourke, K.M., Zhang, Z., et al., 2004. Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science (New York, N.Y.)*, 303(5662), pp.1371–4.

Whitehouse, M.W., 2011. Anti-inflammatory glucocorticoid drugs: reflections after 60 years. *Inflammopharmacology*, 19(1), pp.1–19.

Williams, L.M. et al., 1996. Paradoxical effects of a synthetic metalloproteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNF alpha processing in RA synovial membrane cell cultures. *The Journal of clinical investigation*, 97(12), pp.2833–41.

Williams, R.O., Feldmann, M. & Maini, R.N., 1992. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proceedings of the National Academy of Sciences of the United States of America*, 89(20), pp.9784–8.

Wilusz, C.J., Wormington, M. & Peltz, S.W., 2001. The cap-to-tail guide to mRNA turnover. *Nature reviews. Molecular cell biology*, 2(4), pp.237–46.

Winsauer, G. & de Martin, R., 2007. Resolution of inflammation: intracellular feedback loops in the endothelium. *Thrombosis and haemostasis*, 97(3), pp.364–9.

Won, J.-S. et al., 2004. Dual role of cAMP in iNOS expression in glial cells and macrophages is mediated by differential regulation of p38-MAPK/ATF-2 activation and iNOS stability. *Free radical biology & medicine*, 37(11), pp.1834–44.

Won, M. et al., 2010. Novel anti-apoptotic mechanism of A20 through targeting ASK1 to suppress TNF-induced JNK activation. *Cell death and differentiation*, 17(12), pp.1830–41.

Wu, J.J. & Bennett, A.M., 2005. Essential role for mitogen-activated protein (MAP) kinase phosphatase-1 in stress-responsive MAP kinase and cell survival signaling. *The Journal of biological chemistry*, 280(16), pp.16461–6.

Wynn, T.A., Chawla, A. & Pollard, J.W., 2013. Macrophage biology in development, homeostasis and disease. *Nature*, 496(7446), pp.445–55.

Xiao, C.-Y. et al., 2004. Prostaglandin E2 protects the heart from ischemia-reperfusion injury via its receptor subtype EP4. *Circulation*, 109(20), pp.2462–8.

Xu, Y. et al., 2010. Crystal structure of the entire ectodomain of gp130: insights into the molecular assembly of the tall cytokine receptor complexes. *The Journal of biological chemistry*, 285(28), pp.21214–8.

Yamaguchi, K., Carr, B.I. & Nalesnik, M.A., 1995. Concomitant and isolated expression of TGF-alpha and EGF-R in human hepatoma cells supports the hypothesis of autocrine, paracrine, and endocrine growth of human hepatoma. *Journal of surgical oncology*, 58(4), pp.240–5.

Yang, F. & Schoenberg, D.R., 2004. Endonuclease-mediated mRNA decay involves the selective targeting of PMR1 to polyribosome-bound substrate mRNA. *Molecular cell*, 14(4), pp.435–45.

Yang, S., Li, J.-Y. & Xu, W., 2014. Role of BAFF/BAFF-R axis in B-cell non-Hodgkin lymphoma. *Critical reviews in oncology/hematology*, 91(2), pp.113–22.

Yang, X. et al., 1999. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. *The EMBO journal*, 18(5), pp.1280–91.

Yen, D. et al., 2006. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *The Journal of clinical investigation*, 116(5), pp.1310–6.

Yokoyama, U. et al., 2013. The prostanoid EP4 receptor and its signaling pathway. *Pharmacological reviews*, 65(3), pp.1010–52.

Yu, H. et al., 2011. Anti-inflammatory effect of MAPK phosphatase-1 local gene transfer in inflammatory bone loss. *Gene therapy*, 18(4), pp.344–53.

Yu, H. et al., 2011. MKP-1 regulates cytokine mRNA stability through selectively modulation subcellular translocation of AUF1. *Cytokine*, 56(2), pp.245–55.

Yu, J.H. et al., 2005. Ge-1 is a central component of the mammalian cytoplasmic mRNA processing body. *RNA (New York, N.Y.)*, 11(12), pp.1795–802.

Yuan, W. et al., 2011. Systemic lupus erythematosus monocytes are less responsive to interleukin-10 in the presence of immune complexes. *Arthritis and rheumatism*, 63(1), pp.212–8.

Yuan, X.-L. et al., 2011. Gastric cancer cells induce human CD4+Foxp3+ regulatory T cells through the production of TGF-β1. *World journal of gastroenterology : WJG*, 17(15), pp.2019–27.

Zammit, N.W. & Grey, S.T., 2014. Emerging roles for A20 in islet biology and pathology. *Advances in experimental medicine and biology*, 809, pp.141–62.

Zavadil, J. et al., 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proceedings of the National Academy of Sciences of the United States of America*, 98(12), pp.6686–91.

Zelová, H. & Hošek, J., 2013. TNF-α signalling and inflammation: interactions between old acquaintances. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]*, 62(7), pp.641–51.

Zhang, H. et al., 2013. mRNA-binding protein ZFP36 is expressed in atherosclerotic lesions and reduces inflammation in aortic endothelial cells. *Arteriosclerosis, thrombosis, and vascular biology*, 33(6), pp.1212–20.

Zhang, J. et al., 2008. Cyclic AMP inhibits JNK activation by CREB-mediated induction of c-FLIP(L) and MKP-1, thereby antagonizing UV-induced apoptosis. *Cell death and differentiation*, 15(10), pp.1654–62.

Zhang, T. et al., 2012. Mitogen-activated protein kinase phosphatase-1 inhibits myocardial TNF-α expression and improves cardiac function during endotoxemia. *Cardiovascular research*, 93(3), pp.471–9.

Zhang, X. et al., 2005. Genome-wide analysis of cAMP-response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. *Proceedings of the National Academy of Sciences*, 102(12), pp.4459–4464.

Zhang, Y. et al., 2012. Natural resistance to apoptosis correlates with resistance to chemotherapy in colorectal cancer cells. *Clinical and experimental medicine*, 12(2), pp.97–103.

Zhang, Y. et al., 2012. Toll-like receptor 4 ligation confers chemoresistance to docetaxel on PC-3 human prostate cancer cells. *Cell biology and toxicology*, 28(4), pp.269–77.

Zhao, Q. et al., 2006. MAP kinase phosphatase 1 controls innate immune responses and suppresses endotoxic shock. *The Journal of experimental medicine*, 203(1), pp.131–40.

Zhao, Q. et al., 2005. The role of mitogen-activated protein kinase phosphatase-1 in the response of alveolar macrophages to lipopolysaccharide: attenuation of proinflammatory cytokine biosynthesis via feedback control of p38. *The Journal of biological chemistry*, 280(9), pp.8101–8.

Zhong, H., Voll, R.E. & Ghosh, S., 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Molecular cell*, 1(5), pp.661–71.

Zhu, M. et al., 2013. Th17/Treg imbalance induced by increased incidence of atherosclerosis in patients with systemic lupus erythematosus (SLE). *Clinical rheumatology*, 32(7), pp.1045–52.

Zhu, V.F. et al., 2012. Understanding the role of cytokines in Glioblastoma Multiforme pathogenesis. *Cancer letters*, 316(2), pp.139–50.

Zhu, Y. et al., 1998. Smad3 mutant mice develop metastatic colorectal cancer. *Cell*, 94(6), pp.703–14.

Zi, Z., Chapnick, D.A. & Liu, X., 2012. Dynamics of TGF-β/Smad signaling. *FEBS letters*, 586(14), pp.1921–8.

Zigmond, E. et al., 2014. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. *Immunity*, 40(5), pp.720–33.