Investigating the Epigenetic Regulation of Toll-like Receptor 3

By

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Doctor of Philosophy

July, 2018

Declaration

I declare that the work presented in this thesis has not been submitted for any degree or diploma at this, or any other university and that the work described herein is my own.

Signed:…… Date:……..

Acknowledgements

Firstly, I would like to thank my supervisor Declan for the opportunity to undertake this research, it has been a fun four years and I hope you find another puntastic PhD student to replace me. Years from now when you are on your 20th student, remember the days when I was winging it in journal club because I forgot to read the paper, or saying 'Im sure they're around here somewhere', when you ask where Laura and Claire are.

To everyone in CNS who has come and gone over my time here, you have made the experience so much more enjoyable and entertaining than I ever would have thought possible. To Danny, thanks for all the help over the four years, at least I won't be hounding you for reagents or scaring you senseless anymore. Ambrose, thanks for all the help and advice, and I'm sorry that I won't be around to carry things anymore, you're just going to have to ask Danny to do a bit of the heavy lifting.

To my lab mates Laura and Claire. I really couldn't have picked two better people to spend the last four years with. Going through a PhD with people like you makes every bad day a little better, and I know you will both kill it in your last year.

To all my housemates past and present, Mike, Hayley, Susan, Orla, Norma and Ali and Kiah. You made the house a wonderful and fun place to live and I'll miss all the nights out and lazy days in the sitting room.

Norma and Ali, Living with both of you was the most fun times of My PhD, and I am incredibly grateful for having you as friends throughout this whole experience. As a

group of friends we all looked out for each other, and I really can't stress the difference it has made over the last four years. Everyone would be lucky to have such supportive and amazing friends

Des, I'm glad all your other friends left so we could become besties, who needs engineering friends anyway?

To Kiah, we started out on this journey together four years ago, and through all the highs and lows you have always been there to support me. I enjoyed laughing about the stupid things, giving out at length about the most minor annoyances, and struggling through the tough times together. I can never thank you enough for everything you've done for me and I am privileged to call you my friend.

To mum and dad, none of this would have been possible without you. I am so lucky to have parents that love and support me the way you do. You go above and beyond for us, and I know words will never truly express how much I appreciate and love you both, but I can only try. Love you mum and dad and thanks for everything.

And finally, to my girlfriend Orna. Regardless of whether we lived together, or on opposite sides of the world, you have been with me every step of the way. You always make me feel like to most important person in the world, and you know exactly what to say to pick me up when things are bad. I love you more than anything and you are truly the most amazing person I've ever met. Thank you for making me a better person and for making even the smallest thing an adventure.

Abstract

Toll-like receptors are a family of pattern recognition receptors (PRRs), which have evolved to recognise structurally conserved components of microorganisms. They form an important part of the innate immune system, yet in spite of their importance, there is still a paucity of information regarding the expression and regulation of these receptors. Epigenetics is a field of genetics which focuses on non-nucleotide based changes in gene expression, and highlights the implications that changes in chromatin structure and function have for gene expression and regulation. We theorised that TLRs might be susceptible to changes in the epigenetic state, and we theorised that epigenetic modifications may alter the regulation and function of TLRs, most notably TLR3.

Our studies were centred on two cell lines, epithelial HCT 116 cells and monocytic THP-1 cells, two cell lines which play important but distinct roles in the innate immune system. We targeted two families of enzymes involved in remodelling chromatin structure, DNA methyltransferases (DNMTs) and histone deacetylase complexes (HDACs). We initially showed that knockout of DNA methyltransferases in HCT 116 cells produced significant decreases in TLR3 expression, with less dramatic results seen in other TLRs. This finding was replicated with pharmacological inhibition of DNMTs with 5-aza-2-dc and inhibition of HDACs with SAHA. Inhibition of DNMTs and HDACs also inhibited TLR3 function, with no signalling cascade or cytokine release seen with enzyme inhibition. TLR3 function was markedly different in THP-1 and HCT 116 cells, with THP-1 cells not expressing TLR3. This difference in expression led us to search for a potential regulator common to both cell lines. Literature

searches led to IRF8, a potential negative regulator of TLR3, which is known to be highly expressed in THP-1 cells. We determined that high IRF8 expression was correlated with low TLR3 expression, and lack of functional responses. Overexpression of IRF8 in HCT 116 cells, which prevented TLR3 functional responses, suggested that IRF8 may indeed be an epigenetically controlled regulator of TLR3.

Our studies show the immunomodulatory capabilities of these epigenetic drugs, with decreased viral receptor function and inhibition of inflammatory cytokine release seen with inhibition of DNMTs and HDACs. Furthermore, our studies suggest a potential role for epigenetic modifications, which may alter IRF8 expression, in indirectly regulating TLR3 expression and function.

Publications

Peer Reviewed Published Manuscripts

1. **C Hennessy**, DP McKernan (2016). Epigenetics and innate immunity: the "unTolld" story. Immunol. Cell Biol. 94, 631–639. d

Peer Reviewed Published Abstracts

1. **C Hennessy**, DP McKernan. Epigenetic modifications regulate Toll-like receptor 3 expression in intestinal epithelial and monocytic cells. Poster presentation at European congress of immunology, September 2015.

2. **C Hennessy**, DP McKernan. Histone deacetylase complex inhibitors and DNA methylation alter Toll-like receptor expression in an intestinal epithelial cell line. Poster presentation at British Pharmacological Society meeting, December 2014: Proceedings of the British Pharmacological Society.

3. M Clarke, **C Hennessy**, D McKernan. Investigation of the Role of DNA Methylation in Regulating Toll-Like Receptor Activity. Poster presentation at British Pharmacological Society meeting, December 2014: Proceedings of the British Pharmacological Society.

4. DP Mckernan, **C Hennessy**. Epigenetic modifications influence TLR3 expression and activity. The FASEB Journal, vol. 31 no. 1 Supplement1060.5

Other Research Dissemination

International Conferences Attended

1. **C Hennessy**, DP McKernan. The effect of epigenetic modifications on Toll-like receptor 3 expression in HCT-116 cells. Oral presentation at British Pharmacological Society meeting, December 2016.

2. **C Hennessy**, DP McKernan. The effect of epigenetic modifications on Toll-like receptor 3 expression. Poster presentation at Biochemical society conference, November 2016.

3. **C Hennessy**, DP McKernan. Epigenetic modifications regulate Toll-like receptor 3 expression in intestinal epithelial and monocytic cells. Poster presentation at European congress of immunology, September 2015.

4. **C Hennessy**, DP McKernan. Histone deacetylase complex inhibitors and DNA methylation alter Toll-like receptor expression in an intestinal epithelial cell line. Poster presentation at British Pharmacological Society meeting, December 2014.

National Conferences Attended

1. **C Hennessy**, DP McKernan. The effect of epigenetic modifications on Toll-like receptor 3 expression in HCT-116 cells. Poster presentation at Irish Society of Immunology meeting, September 2016.

2. **C Hennessy**, DP McKernan. Epigenetic modifications regulate Toll-like receptor 3 expression in intestinal epithelial and monocytic cells. Poster presentation at Irish Society of Immunology meeting, September 2015.

List of abbreviations

ANOVA	analysis of variance
AGO	argonaute protein
AMP	antimicrobial peptide
APP	acute phase proteins
BIR	Baculovirus Inhibitor of apoptosis protein Repeat
CARD	Caspase activation and recruitment domains
COX2	Cyclooxygenase 2
CD	Crohn's disease
CRP	C-reactive protein
DC	dendritic cell
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
dsRNA	Double stranded RNA
DSS	dextran sodium suflate
EIF2α	Eukaryotic Translation Initiation Factor 2A
ERK	Extracellular signal–regulated kinases
FACS	fluoresence assisted cell sorting
GALT	gut associated lymphoid tissue
GIT	gastrointestinal tract
GTF	general transcription factors
HAT	histone acetyltransferase
HDAC	histone deacetylase complex

HDAi	HDAC inhibitor
HIV	human immunodeficiency virus
IEC	intestinal epithelial cell
IFN	interferon
IFNβ	interferon β
IκB	inhibitor of kB
IKK	IκB kinase
IL6	interleukin 6
IRAK	Interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	Janus kinase
LRR	leucine rich repeat
MAPK	mitogen activated protein kinase
MAL	myelin and lymphocyte protein
MBD	methyl binding domain
MCP1	Monocyte Chemoattractant Protein-1
µg	microgram
µM	micromolar
µl	microliter
MDA5	Melanoma Differentiation-Associated protein 5
MECP2	Methyl CpG binding protein 2
MIP	Macrophage Inflammatory Protein
mg	milligram

mM	millimolar
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTAL	Medullary thick ascending limb
MyD88	myeloid differentiation primary response gene 88
NAD	Nicotinamide adenine dinucleotide
NET	neutrophil extracellular trap
ncRNA	non coding RNA
Ng	nanogram
NK	natural killer
NLR	Nod-like receptor
nm	nanometer
nM	nanomolar
Mx1	myxovirus-resistant gene 1
NOD	Nucleotide-binding oligomerization domain-containing protein
NF-κB	nuclear factor kappa B
NXF1	Nuclear RNA Export Factor 1
NXT1	Nuclear Transport Factor 2 Like Export Factor 1
OA	osteoarthritis
OAS	oligoadenylate synthase
PAMP	pathogen associated molecular pattern
PBS	phosphate buffer saline
pg	picogram

PCR	polymerase chain reaction
PKR	protein kinase R
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
PYD	pyrin domain
RA	rheumatoid arthritis
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
Rb	Rentinoblastoma protein
RIG1	Retinoic Acid Inducible Gene 1
RLR	Rig-like receptor
RNA	ribonucleic acid
RPM	revolutions per minute
RSV	Respiratory syncytial virus
SAHA	suberanilohydroxamic acid
SIGIRR	Single Ig IL-1 Related Receptor
SLE	systemic lupus erythematosus
SOCS	suppressor of cytokine signalling
ssRNA	single stranded RNA
ST2	human IL1 receptor like 1
TAB	TGFβ-activated kinase 1 (TAK1)-binding proteins
TAK	Transforming growth factor beta-activated kinase
TF	Transcription factor

TGFβ	Transforming growth factor beta
Th	T helper cell
TIR	Toll/interleukin-1 receptor homology
TJ	Tight Junctions
TLR	Toll-like receptor
TNFα	tumor necrosis factor α
TNFR	TNF receptor
TRAF	tumor necrosis factor receptor associated factor
TRAM	translocation associated membrane protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
tRNA	transfer RNA
TSA	Trichostatin A
TSS	Transcription start site
UC	Ulcerative colitis
UHRF1	Ubiquitin-like, containing PHD and RING finger domains, 1
Zn	Zinc

Table of Contents

Chapter 1: General introduction

THE IMMUNE SYSTEM

Due to the constant exposure to microorganisms, both pathogenic and commensal, humans require a defence against foreign organisms, which comes in the form of the immune system. The immune system is a sophisticated network of organs, cell types, cytokines and other soluble mediators. It is delineated into two distinct parts, namely the innate and adaptive immune system. The innate immune system acts rapidly in response to pathogens, and it includes physical barriers, such as epithelial cells, as well as cells such as macrophages and dendritic cells, and other factors such as pattern recognition receptors (PRRs), cytokines and the complement system. The adaptive immune response acts less rapidly, but with increased specificity. The adaptive immune response consist of antibody based reactions mediated by B and T lymphocytes. Although the adaptive immune response is not the focus of this thesis, it is worth noting that there is cross talk between the two responses, and they do not operate in isolation (Clark and Kupper, 2005; Getz, 2005).

1.1.1 THE INNATE IMMUNE SYSTEM

The innate immune system is a complex system of cells and structures developed to protect the body from pathogens. Chief among this system is the ability to differentiate pathogenic microbes from the ones which are beneficial to the host, and the ultimate goal of the system is the maintenance of the healthy ‘commensal’ microbes and the detection and elimination of pathogenic microbes. However, the host must provide protection from the outside organisms while also ensuring as little damage as possible

occurs to organs and tissues and as such needs very specific and robust mechanisms of detecting said pathogens. This protection is provided in the form of two different yet complimentary systems, namely the innate and adaptive immune responses. The innate immune system consists of the aspects of the host's immune system that are present at birth and are not learned or developed over time. These are as simple as structural barriers which prevent pathogen entry, such as epithelial cells, which have tight cell junctions which prevent pathogen entry in a rudimentary manner (Anderson and Van Itallie, 2009). Other innate defences include the mucus secretions that line the airways, gastrointestinal tract and the genitourinary tract, which work to trap and, in concert with the epithelial cilia, expunge microbes and pathogens through excretions. The secretions themselves can also contain antiviral or antibacterial elements, neutralising the pathogens before they are afforded the opportunity to cause harm (Elgert, 2009). The innate defences also consist of more sophisticated methods of defence, such as the antimicrobial peptides (AMPs). Defensins and cathelicidins are classified as AMPs, and act as the body's own antibiotics. They act by binding to the bacterial cell wall, and once they are embedded they form pore-like defects, allowing the efflux of essential ions and nutrients, leading to bacterial death. These AMPs are secreted by the cells of the immune system, cathelicidins are stored within the granulocytes of neutrophils and are released following leukocyte activation and the defensins are present in epithelial cells as well as neutrophils (Ganz et al., 1985; Lehrer and Ganz, 2002; Zanetti, 2004). Other bioactive peptides also form an essential part of the host innate immune response, chief among these being the cytokines and chemokines released as a result of immune activation and who serve to propagate the immune response. Membrane bound and cytosolic receptors which serve to recognise structurally conserved molecules on the surface of invading microbes. These receptors are referred to as the pattern recognition

receptors (PRRs) and play a key role in the innate immune system. The Toll-like receptor (TLR) are a family of PRRs, whose function includes sensing bacterial and viral motifs, and they are the main focus of this thesis.

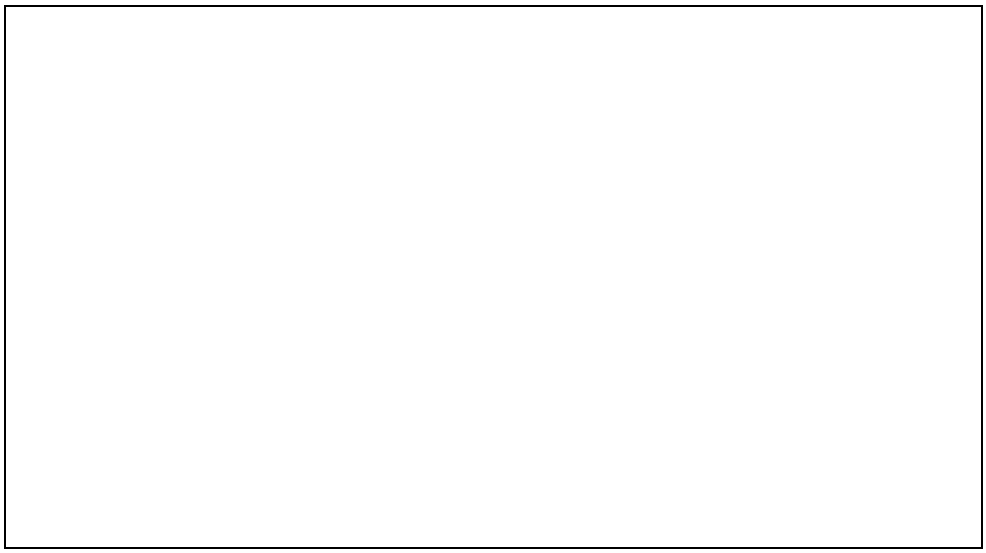


Figure 1.1. Overview of the innate immune responses

The innate immune system provides a rapid but less specific response to pathogens. Innate immunity relies on epithelial barriers, myeloid cells, cytokines, chemokines, cell based receptors and anti-microbial elements. (From (Becknell et al., 2015)

1.1.2 EPITHELIAL BARRIERS IN INNATE IMMUNITY

Commensal and pathogenic organisms interface with the host at a multitude of sites, and with the surface area of mucosae much greater than that of the skin and other sites of exposure, a large proportion of host-pathogen interactions occur in mucosal environments such as the gastrointestinal tract (GIT)(Turner, 2009). The GIT is also host to lymphoid tissues referred to as gut associated lymphoid tissue (GALT), which contain lymphoid follicles (Neutra et al., 2001), and given the constant exposure of these mucosal sites to microorganisms, they serve as important sites of immune regulation.

The epithelium serves as a mediator of the innate immune response by maintaining a barrier between the lymphoid tissues and the contents of the luminal environment. It consists of four cell types which are derived from a common progenitor: AMP secreting Paneth cells, hormone producing enteroendocrine cells, goblet cells and enterocytes, which constitute the majority of the intestinal epithelial cells (IECs) (Yen and Wright, 2006). The anatomical makeup of the epithelium is outlined in figure 1.2. The epithelium of the gut form a selectively permeable barrier through the formation of tight junctions (TJ) between the paracellular spaces, and these junctions protect against pathogens while still allowing the transport of essential nutrients and ions (Shen and Turner, 2006).

The immune function of the epithelia is not confined to their barrier function however, with interactions between dendritic cells (DCs) and the tight junctions in epithelial cells important in bacterial and viral immunity. Dendritic cells have been shown to express tight junction proteins which allow dendrites to pass through tight junctions and sample the luminal environment while preserving barrier integrity (Rescigno et al., 2001). The epithelium is also implicated in the maintenance of colonic

homeostasis and immune tolerance. Expression of Toll-like receptor 9 (TLR9) on the apical and basolateral surfaces of epithelial cells serves to prevent unwarranted immune activation. Where basolateral activation of TLR9 will result in NF-κB activation and a subsequent immune response, apical activation has been shown to result in ubiquitination of IκB, which prevents NF-κB activation, and thus, the immune response is inhibited (Lee et al., 2006). This process is believed to allow necessary immune responses upon activation of basolateral TLR9, while preventing TLR9 activation by the commensal bacteria of the intestinal lumen.

The epithelial cells are defined as being functionally 'polarised', with a distinct apical and basolateral surface, and different protein targets present on each (figure 1.2) (Abreu, 2010). Alternate spatial expression of TLRs has also shown to be present in the IECs, with differences in expression seen between the apical and basolateral surface of cells. For example, TLR5 is expressed exclusively on the basolateral surface of intestinal epithelial cells (Gewirtz et al., 2001), and studies have shown that luminal bacteria flagella can only activate TLR5 following disruption of the epithelium by dextran sodium sulfate (DSS). DSS disruption of epithelium is used to model ulcerative colitis and Crohn's disease in mice by replicating the pathology of human colitis, including the increased gut permeability (Chassaing et al., 2014). This would suggest that TLR5 will only be activated by bacteria which passes the epithelial barrier, whether through injury or another mechanism, thus preventing TLR5 activation in response to bacteria which remain sequestered in the epithelium.

Thus, the epithelial barrier exists as an important component of the innate immune defence, with functions outside of barrier function. There are a number of cell lines which are used for epithelial research, including HCT 116 cells, as well as more complex structures such as organelles. Although not the focus of this thesis, further

detail on the functions of the other cell types, as well as antimicrobial peptides and lectins, can be found in (Abreu, 2010).

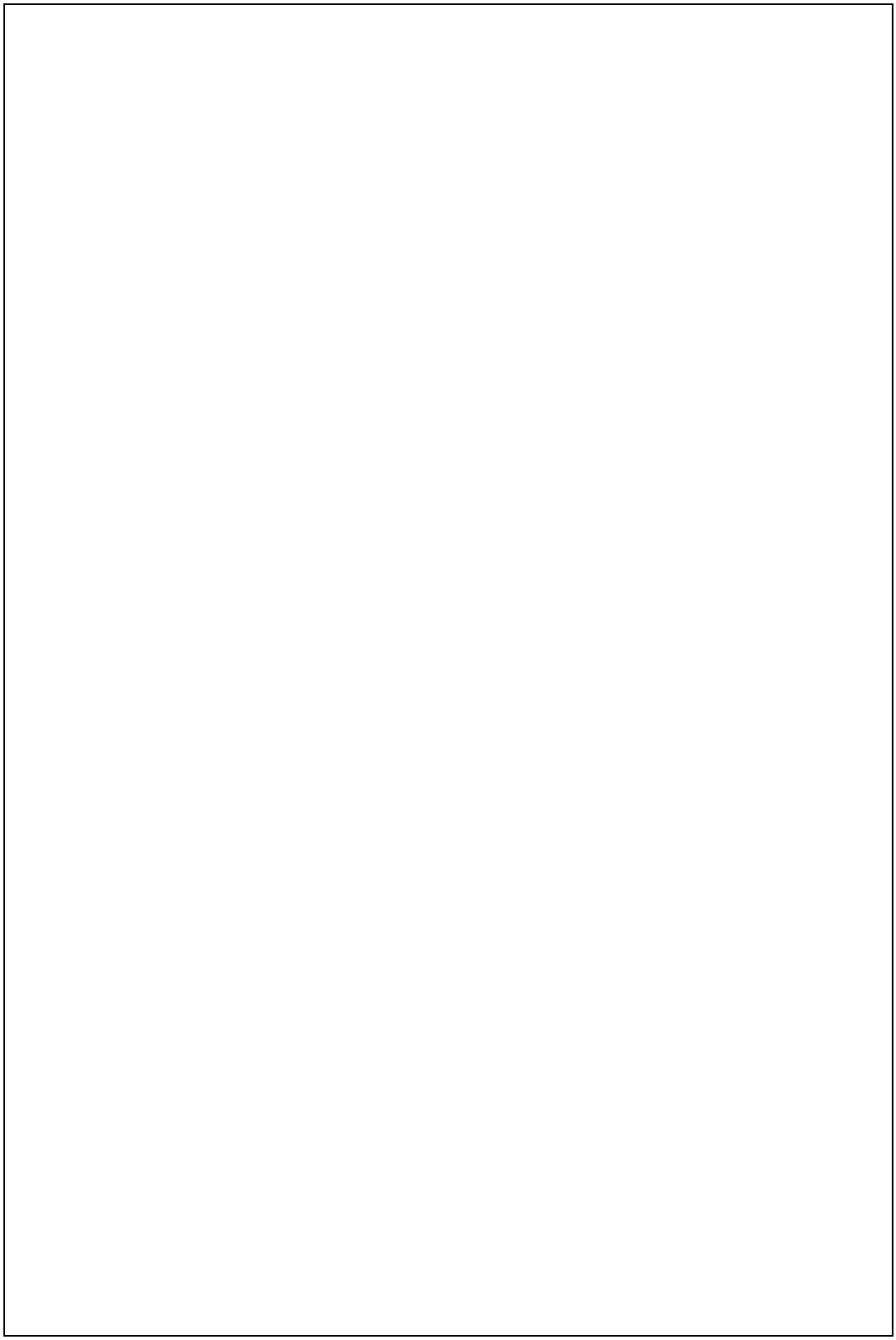


Figure 1.2. Anatomy of the intestinal epithelium. (taken from (Abreu, 2010)

1.2 PATTERN RECOGNITION RECEPTORS.

Pattern recognition receptors play an essential role in the recognition of and response to invading microorganisms. Invading microorganisms possess structurally conserved motifs know as pathogen associated molecular patterns (PAMPs), which are recognised by the PRRs. Through this detection the PRRs allow the innate immune system to respond to neutralise and eliminate these invaders. The PRRs consist of four families of receptors, namely the RIG-like receptors (RLRs), the NOD-like receptors (NLRs), the C-type lectin receptors, and the Toll-like Receptors (TLRs).

The RIG-like receptor (RLR) family consists of RIG1, melanoma differentiation associated gene 5 (MDA5), and LGP2 (Takeuchi and Akira, 2009; Yoneyama and Fujita, 2008). These receptors are located in the cytoplasm and as shown in table 1.2, are activated by genomic RNA from dsRNA viruses as well as dsRNA produced by replication of ssRNA viruses. Recognition by RLRs results in the activation of the innate antiviral responses which ultimately result in the production of type I interferons and inflammatory cytokines, whose goal is to limit viral reproduction (Akira et al., 2006; Kawai and Akira, 2009). The NOD-like receptors (nucleotide-binding oligomerization domain), or NLRs, are intracellular PRRs, whose function is the recognition of PAMPs that exert their effects intracellularly, such as pathogens that are phagocytosed. The NODs are generally described as a scaffold protein on which a signalling complex is assembled following activation of the receptor with a given PAMP (Franchi et al., 2009). The NODs are part of a large family of 23 PRRs in humans, whose actions include the activation of MAPKs and NF-κB signalling,

apoptosis and inhibition of apoptosis. The receptors are divided into subsets based on the homology of their N terminal domains, with CARD, PYD, BIR and acidic domains being the main ones identified to date (Inohara et al., 2005; Inohara and Nuñez, 2003). NOD1 and NOD2 were the first, and the best characterised of the receptor family, with their action being the binding of bacterial associated PAMPs. The receptors recognise components of Gram-positive and Gram-negative bacteria, with NOD1 recognising the muropeptide motif indicative of gram negative bacteria and NOD2 recognising Gram positive bacteria. The recognition of the PAMPs leads to downstream activation of MAPK and NF-κB signalling, with the end product being the production of inflammatory cytokines (Takeuchi and Akira, 2010). The C-Type Lectin Receptors recognise a carbohydrate component of pathogens, and they are present mainly in monocytes, macrophages and dendritic cells. The receptors respond to the glucan, fructose and mannose carbohydrate structures that are present in bacteria, fungi and viruses (Takeuchi and Akira, 2010). Following recognition of a pathogen by a CLR, the pathogen is internalised and subsequently degraded. The CLRs are an important part of both the innate and adaptive immune responses with research suggesting that their activation can lead to modulation of TLR induced genes, as well as activating the NF-κB signalling pathway (Geijtenbeek and Gringhuis, 2009). These three families of PRRs are not the focus of the work of this thesis (for comprehensive reviews see (Franchi et al., 2009; Geijtenbeek and Gringhuis, 2009; Takeuchi and Akira, 2010; Yoneyama and Fujita, 2008). The TLR family of PRRs is the focus of this research.

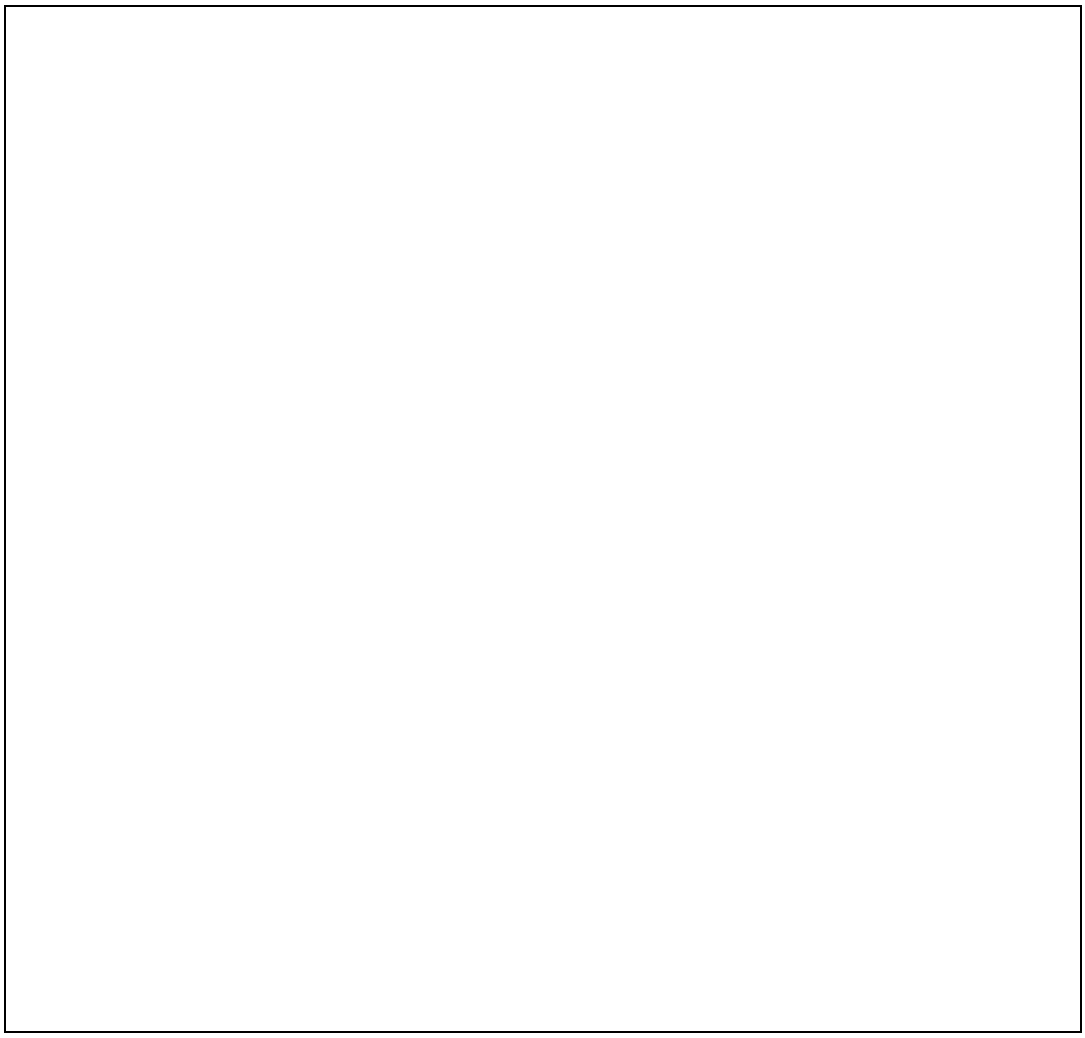


Figure 1.3 The Human Toll-like receptor family and the ligands which activate them

TLR2/1 heterodimers recognise triacyl lipoproteins and the synthetic ligand Pam3CSK4. TLR2/6 heterodimers recognies diacyl lipoproteins (TLR2 recognises HKLM). TLR3 recognises dsRNA and the synthetic mimetic Poly I:C. TLR4 recognises LPS. TLR5 recognises bacteria flagella (flagellin). TLR7 and TLR8 recognise ssRNA. TLR9 recognises dsDNA (CPG ODN-2006)(Liu et al., 2007)

1.3 TOLL LIKE RECEPTORS:

Toll-like receptors are a family of 13 receptors, of which 10 are present in humans, consisting of both intracellular and extracellular receptors (Blasius and Beutler, 2010). The TLRs are known as pattern recognition receptors (PRRs) and they recognise structurally conserved molecules present in bacteria and viruses. These structurally conserved molecules are known as pathogen associated molecular patterns or PAMPs and activate TLRs by interacting and binding with them (Akira et al., 2006). Activation of the TLRs via bacterial, viral or synthetic ligands results in a signal transduction cascade that produces an immune response. This immune response is the result of cytoplasmic activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or interferon regulatory factors (IRF) 3/5/7 transcription factors, which, upon activation translocate to the nucleus of the cell and alter transcription of proinflammatory cytokines such as interferon-α/β, interleukin-6 and tumor necrosis factor α (Akira et al., 2006). Toll-like receptors on the cell surface recognise bacterial and fungal PAMPs and intracellular TLRs recognise viral or microbial PAMPs (Akira et al., 2006). The receptor family is recognised as having a large role in the regulation and control of the immune response to microbial challenge as well as in the development of autoimmunity (Maximiliano Javier Jiménez-Dalmaroni et al., 2015). In figure 1.3, the toll like receptors, and the ligands which activate them, are described.

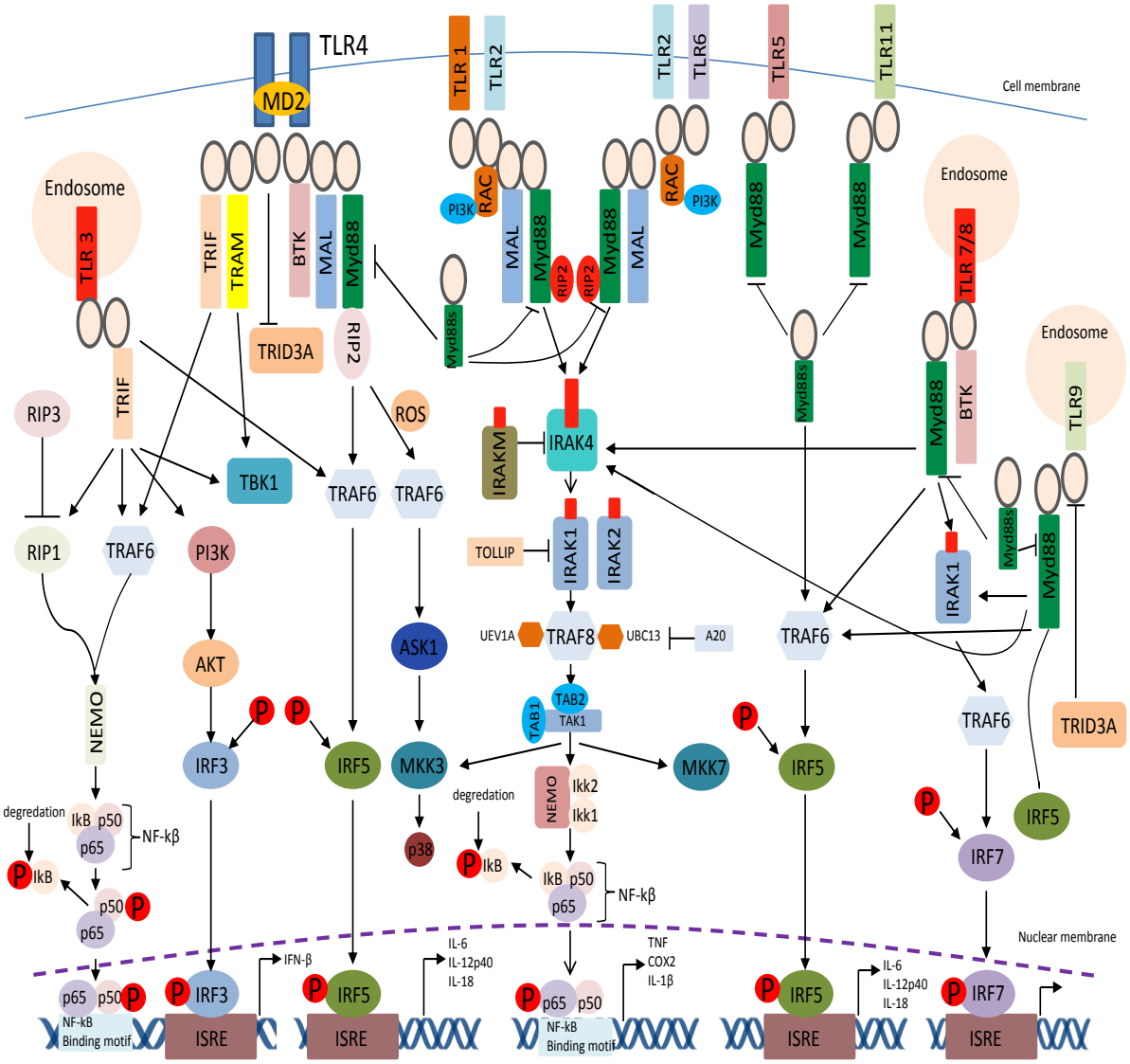


Figure 1.4: Cellular localisation of TLRs and the signalling pathways induced as a result of their activation.

TLR activation results in signalling via MyD88 (except for TLR3, which signals via TRIF) which results in transcription factor activation. Activation of IRF3/5/7 and NF-κB results in transcription of cytokines. (Hennessy and McKernan, 2016).

TLRs possess two key domains, the extracellular domain, which consists of leucine rich repeats (LRRs), whose function is ligand binding, and the intracellular Toll-Interleukin-1 resistance domain (TIR), which is involved in intracellular signalling (Akira and Takeda, 2004). The key signalling output of TLR ligand binding is tightening of the TIR on each TLR, which essentially means there is a conformational change in the structure in the receptor that allows the recruitment of certain signalling molecules (Hennessy et al., 2010). The TIR domain is the intracellular component of the TLRs which is responsible for signalling and it allows the recruitment of adapter molecules such as Myeloid differentiation primary response gene 88 (MyD88) or MyD88 adaptor-like (Mal) which are essential in the TLR signalling (Hoebe et al., 2003). As is evident in figure 1.4, the transduction cascade that results following TLR activation is complex and involves recruitment and activation of many factors via kinases (see (Akira and Takeda, 2004) for an overview of TLR signalling).

TLRs are expressed in a wide variety of cells and tissues. In table 1.1, the cell types that express TLRs, and the TLRs expressed by each is shown. This table is not an exhaustive list however, and does not cover tissue expression of TLRs, which is still poorly understood. Epithelial cells have been shown to express TLRs, however the expression profile has been shown to be different among different tissues. Intestinal epithelial cells have been demonstrated to express TLRs, 2, 3, 4, 5 and 9 (Cario et al., 2000). However, as discussed in the section on epithelial barriers, TLR expression can show 'polarisation' in these intestinal epithelial cells, with TLR5 expressed solely on the basolateral surface of the epithelial cells (Gewirtz et al., 2001). Furthermore TLR9, while expressed both apically and basolaterally, has been demonstrated to trigger immune reactions only when activated basolaterally, thus preventing unwarranted

activation by pathogens which are still sequestered in the lumen of the intestine(Lee et al., 2006). In contrast to the intestinal epithelium, immortalised cell lines derived from vaginal and cervical epithelial tissue have shown that, while they express TLR 1, 2, 3 and 5, they lack the TLR4 expression seen in intestinal cells. However, unlike intestinal epithelium, they do express TLR6 (Fichorova et al., 2002). Thus, definitively classifying a particular cell type as expressing or not expressing TLRs is difficult, as the expression profile seems to depend on more than just the cell type, with differential expression observed across similar cell types in different tissues, and even in different regions of the same tissues. TLRs have been shown to have different basal expression levels in different tissues with certain TLRs having higher levels in certain organs. For example TLR1 expression was shown to be highest in the spleen, while TLR2, 4, 6, 8 and 10 were highest in the lung. TLR3 was highest in the placenta, TLR5 was highest in the trachea, TLR7 was highest in the spinal cord and TLR9 was highest in skeletal muscle (Nishimura and Naito, 2005). This information is valuable when examining alterations in expression of the TLRs in disease states and also serves to highlight the lack of uniformity when it comes to the TLRs in the body, a factor which could be of importance in studies examining changes in tissue TLR expression.

Cell Type	TLRs expressed
Neutrophils	1,2,4,6,8
Mast cells	2,4
Monocytes	1,2,4,5,6,8
Macrophages	1,2,3,4,6,7,9
NK cells	1,7,9
Resting B cells	1,4,6,7,8,9,10
Germinal c B Cells	1,2,6,7,8,9,10
preDC (Monocytes)	1,2,4,5,6,8
iDC ($CD11c^{+}$ iDC)	1,2,3,5,6,8,10
Plasmacytoid DC	1,6,7,9

Table 1.1 TLR expression by cells of the immune system.

This table outlines the TLR expression across a range of cell types important in the immune response. This is not an exhaustive list, and does not take into account expression in tissues. Adapted from (Dembic, 2013)

1.3.1 EXTRACELLULAR TLRS:

As mentioned above the TLRs are divided into two groups, the intracellular and extracellular TLRs which perform different functions as they recognise different structural motifs. The cell surface Toll like receptors consist of TLRs 1, 2, 4, 5, 6 and 11 and the intracellular Toll-like receptors consist of TLRs 3, 7, 8, 9 and 10. The first TLR discovered was TLR4 and is generally regarded as the founding member of the TLR family. It was discovered as being the receptor which responds to lipopolysaccharide, the component of Gram-negative bacteria known for inducing septic shock (Poltorak et al., 1998). The receptor received its name from its similarity to the Toll protein from *Drosophila melanogaster* (Gay and Keith, 1991; Khush et al., 2001; Kopp and Medzhitov, 1999), in which the protein influences the development of dorsoventral polarity (Anderson et al., 1985).

It was determined that in order to signal, TLR4 forms a complex with MD2 on the cell surface and the complex binds to LPS (Shimazu et al., 1999). Formation of this complex with LPS initiates recruitment of intracellular adapters such as MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) which initiate the transduction cascade (Tanimura et al., 2008). While the exact signalling process initiated by TLR4 stimulation has yet to be fully determined, recent research has suggested that upon stimulation the TIR domain facilitates signalling by clustering TLR4 receptors and assembling what is referred to as a signalasome (Guven-Maiorov et al., 2015). TLR4 has been shown to be essential for defence against Gram-negative bacteria, with mutations in *tlr4* resulting in the development of Gram-negative induced sepsis while other immune function remain intact (Poltorak et al., 1998). Furthermore, in studies using C3H/HeJ and C57BL/10ScCr mice, both of which possess mutations in *tlr4* that confer hyporresponsiveness to LPS, it was shown that following challenge with

LPS they produced a much weaker immune response when compared to control mice (Qureshi et al., 1999). This suggests that altered TLR4 function plays a role in the development of the endotoxin resistance seen in these mutant mice.

Toll-like receptor 2 responds to a wide range of structurally conserved motifs from bacteria, fungi and viruses (Akira et al., 2006). Its array of ligands is very broad and ever expanding. Classifying TLR2 ligands proves difficult as it has transpired that there are many different structures recognised by TLR2, however recent research has begun to determine that diacylglycerol may be the ligand moiety for microbial glycolipids and lipoproteins (Maximiliano J. Jiménez-Dalmaroni et al., 2015). For example, TLR2 has been shown to interact with Gram-positive bacteria, yet in spite of this group B streptococci do not signal via the TLR2 receptor (Lien et al., 1999; Takeuchi et al., 1999). Thus, the mechanism underlying the activation of TLR2 is decidedly more complex than some of the other members of the family and it has been speculated that its selectivity for different ligands depends on its association with other receptors. It is understood that it forms heterodimers with other TLRs, namely TLR1 and TLR6, with each combination recognising different structural motifs (Kaisho and Akira, 2002).

When TLR1/2 form a heterodimer, the complex responds to triacylated lipopeptides from Gram-positive bacteria and mycoplasma. However, when a complex is formed with TLR6, it instead recognises diacylated lipopeptides from Gram-negative bacteria and mycoplasma (Jin et al., 2007; Kang et al., 2009). There is also evidence that LPS sensing via the TLR4 receptor requires interaction with TLR2. In a study examining bacterial sepsis and renal function, it was shown that for LPS to induce sepsis via TLR4, TLR 2 was required. The study measured absorption of HCO_3^- by the medullary thick ascending limb (MTAL), a process which is impaired in sepsis and has

been shown to be inhibited by Gram-negative LPS signalling via TLR4 and Gram-positive signalling via TLR2. However, the study showed that in *tlr2*$^{-/-}$ mice LPS challenge had no effect on HCO_3^- absorption, a process which was decreased in wild type mice. The effect seen was linked to the impaired recruitment of MyD88 in the *tlr2*$^{-/-}$ mice when compared to the WT mice. This suggests that interaction between TLR2 and TLR4 is necessary for normal TLR4 function in this context (Good et al., 2012).

TLR5 was initially identified by the presence of the Toll/interleukin (IL-1) receptor homology domain (TIR) (Chaudhary et al., 1998; Rock et al., 1998; Sebastiani et al., 2000) which is a conserved signalling domain considered to be a defining trait of the Toll-like receptor family (Medzhitov et al., 1998). TLR5 recognises the flagellin protein which is the structurally conserved component of bacteria flagella (Akira et al., 2006). Studies have shown that there is high TLR5 expression in the lamina propria dendritic cells (DCs) of the small intestine. Lamina propria DCs are unique in that they promote the differentiation of interleukin 17 producing T helper cells (Th17) and T helper 1 (Th1) cells. Furthermore, they promote the differentiation of naive B cells into immunoglobulin A producing cells in response to challenge by flagellin (Uematsu et al., 2008). It has also been shown to be expressed on monocytes as well as epithelial cells (Cario and Podolsky, 2000; Muzio et al., 2000). Studies have identified its action to be proinflammatory, acting via MyD88 to activate TNFα which in turn stimulates cytokine production (Hayashi et al., 2001).

1.3.2 INTRACELLULAR TLRS:

The intracellular TLRs evolved to detect intracellular foreign nucleic acids, the hallmark of invading viruses. In order to better distinguish foreign DNA from that of the host, the intracellular TLRs act within the endosomal compartment, a compartment which generally excludes host DNA (Blasius and Beutler, 2010). The intracellular TLR, TLR3, was originally identified as recognising the structure of a synthetic analogue of double stranded RNA (dsRNA) known as polyinosinic-polycytidylic acid (poly I:C) (Alexopoulou et al., 2001). This synthetic compound mimics the infection which is induced by the viruses that possess this structurally conserved motif. It does so by inducing the production of type I interferons as well as other inflammatory cytokines. TLR3 interactions with dsRNA were determined by analysis of the domain of TLR3 which was bound to the dsRNA (Bell et al., 2006; Choe et al., 2005). TLR3 is important in the immune response due to its role in recognising ssRNA viruses such as Respiratory Syncytial Virus (RSV), encephalomyocarditis virus, West Nile virus and certain small interfering RNAs (Akira et al., 2006)(Kawai and Akira, 2006). The importance of TLR3 function has been shown in animal studies wherein *tlr3* deficient mice are susceptible to lethal infection by murine cytomegalovirus (MCMV). *Tlr3* knockout mice infected with MCMV possessed a much higher viral titre when compared to controls. Furthermore, although there was no significant increase in death when compared to controls the authors remarked that the $tlr3^{-/-}$ mice appeared to be sicker and trended towards higher mortality (Tabeta et al., 2004).

TLR7 recognises imidazoquinolone derivatives such as imiquimod and resiquimod as well as guanine analogues such as loxoribine. Its purpose in host defence is its recognition of ssRNA from viruses such as Vesicular stomatitis virus (VSV), influenza type A and human immunodeficiency virus (HIV) (Akira et al., 2006)(Kawai

and Akira, 2006). Plasmacytoid dendritic cells (pDCs) show a very high expression of TLR7, and in response to viral challenge produce an immune response by releasing type I interferon as well as other inflammatory cytokines. In the studies above, it was shown that TLR7 is necessary for the immune response of pDCs to viral challenge, and in the absence of TLR7 no release of interferon or cytokines occurs. TLR8 is similar phylogenetically to TLR7, and it also recognises ssRNA. However it was determined that ssRNA sensing in mice is not dependent on TLR8 and that ssRNA sensing was normal even in the absence of TLR8 (Kawai and Akira, 2006)(Akira et al., 2006). A recent study has determined the mechanism behind the activation of TLR8, showing that binding of oligonucleotides at two separate sites is required for activation of the receptor (Tanji et al., 2015). RNA sensing by TLR8 has been implicated in the development of many autoimmune conditions, leading to suggestions that inhibiting it in humans may be beneficial in treating certain autoimmune conditions (Guiducci et al., 2013).

TLR9 recognises the unmethylated CpG motifs that are a hallmark of bacteria and viruses but are rarely seen in mammalian cells. Their activation by these motifs results in activation of dendritic cells, B cells and also initiates a T_H1 response (Bafica et al., 2005). Studies have also shown that in certain cases TLR9 can recognise self CpG-DNA which can lead to the development of autoantibodies and has been implicated in the development of certain autoimmune conditions (Bauer et al., 2001), although recent studies have suggested that activation of TLR9 present on the plasma membrane of B-cells acts as a negative regulator of the endosomal TLR9, preventing the activation and proliferation of the B-cells and could act as a novel approach to controlling TLR9 induced autoimmunity (Guerrier et al., 2014). Current studies have suggested a role for TLR9 in the regulation of the cell cycle, as infection of subjects with the human papilloma virus (HPV) lead to a down regulation of *tlr9* and an increase in cellular

proliferation (Pacini et al., 2015). This increase in cellular proliferation is often seen with HPV infection and often results in cancer development as a result, thus the aforementioned study highlights the potential importance of TLR9 as a regulator of the cell cycle. Until recently the structure and function of TLR10 were unknown, however recent research has illuminated its potential importance in the immune system. The study showed that blocking TLR10 led to increased production of proinflammatory cytokines, including IL1β, following TLR2 stimulation. The study also noted that in humans with polymorphisms in *tlr10*, there was documented increases in IL1β, TNFα, and IL6 upon ligation of TLR2 (Oosting et al., 2014). Thus for the first time a potential role for TLR10 has been suggested, and that is one of a mainly anti-inflammatory nature.

1.4 TOLL LIKE RECEPTORS AND INFLAMMATION

Upon recognition of a particular antigen, the different members of the TLR family recruit specific sets of adaptor proteins, including Myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adaptor protein inducing IFNβ (TRIF, also known as TIR-domain-containing molecule 1, TICAM1), TIR-domain-containing adaptor protein; also known as MyD88-adaptor-like protein, MAL (TIRAP/MAL), or TRIF-related adaptor molecule (TRAM, also known as TICAM 2) (Akira and Takeda, 2004).

1.4.1 MyD88 DEPENDENT SIGNALLING

MyD88 was identified as one of the most important adapter molecules in TLR signaling, with its function being responsible for the action of many of the TLRs (see figure 4 for an overview of TLR signaling). Upon activation of a TLR and the

subsequent formation of the dimer complexes, MyD88 will be recruited to the cytoplasmic TIR domain of the TLR, and will then facilitate the assembly of a signalosome. MyD88 will bind to IRAK4, which mediates IRAK 4 phosphorylation of IRAK1. TRAF6 can then bind to IRAK1 in its phosphorylated state, which will lead to the formation of the TRAF6-IRAK1 complex, which then interacts with a complex consisting of TAK1/TAB1-3. TAK1 then phosphorylates the IKKs, which subsequently phosphorylate the IκBs. This IκB phosphorylation leads to the degradation of IκB and the release of NF-κB. One released, the phosphorylated NF-κB can then translocate to the nucleus to initiate transcription of inflammatory cytokines (Akira and Takeda, 2004). NF-κB activation plays a key role in the development of inflammatory responses, with its phosphorylation leading to the production of pro-inflammatory cytokines such as IL1, IL6, IL8, and TNFα (See (Tak and Firestein, 2001) for a review on the key role of NF-κB in inflammatory diseases).

Activation of TAK1 can also result in the activation of the MAPKs, which initiate further downstream signaling events. The MAPK families of proteins were first identified as having roles in cell differentiation, cell proliferation and apoptosis; however more recent research also shows that they have important roles in inflammation also. The ERK MAPKs are a large family of 20 MAPKs, with ERK 1/2 being the most studied. Although they are not typically involved in directly transcribing inflammatory cytokines, they induce inflammation by other means, such as inducing cell proliferation and migration (Delaney et al., 2008; Grund et al., 2005; Guha et al., 2001). Most research concerning a role for MAPKs in inflammation is centered on two other members of the MAPKs, the P38 and JNK family of proteins. There is strong evidence for the role of P38 in inflammation, with the activation of P38 shown to result in the production of the proinflammatory cytokines IL1β, TNFα and IL6 (Guan et al., 1998).

Its activation is also tied to the production of other inflammatory mediators such as COX2, as well as the induction of differentiation and proliferation of immune cells (Craxton et al., 1998). The JNK family of proteins also have potent pro-inflammatory effects, with phosphorylated JNKs main role being the activation of transcription factors. One activated, these JNK activated transcription factors can then induce the transcription of cytokines, with studies showing that JNK phosphorylation can induce lung epithelial cells to produce TNFα and members of the interleukin family (Novotny et al., 1998, p. 1; Schmeck et al., 2006; Zenz et al., 2008). One of the key transcription factors activated by MAPK signalling is activator protein 1 (AP1), which is involved in cellular proliferation, cell death, and inflammation. AP1 has been shown to be activated in response to both bacterial and viral infections (Seo et al., 2004; Xie et al., 2005), and it has been shown to act by binding to the promoters of inflammatory mediators, with activation of transcription of the proinflammatory mediator IL8 in both hepatitis and HIV shown to be due to an AP1 dependent mechanism (Balasubramanian et al., 2003). The induction of AP1 has been shown to be mediated mostly by P38 and JNK MAPKs (Chang and Karin, 2001), with translocation of the MAPKs to the nucleus increasing transcription of AP1 precursors (Karin, 1995). Thus, it is evident the MAPKs have a very important role in the regulation of inflammatory signalling, especially in the context of a viral infection.

1.4.2 MyD88 Independent signalling

While the MyD88 dependent pathway was originally considered the main mechanism of signal transduction for the TLRs, studies in MyD88 deficient mice showed that there was another pathway by which certain TLRs could induce immune responses. The studies involving mice lacking MyD88 showed there was no induction of TNF or IL6

in these animals when they were exposed to PAMPs associated with TLR 2, 4, 5, 7 or 9 (Adachi et al., 1998). However, further examination of the responses in these deficient animals led to the discovery of a signalling pathway which operated independently of MyD88. Following the revelation that LPS could still elicit an NF-κB response in MyD88 deficient cells, albeit a delayed response, researchers described another adaptor involved in the TLR3/4 transduction cascades, namely the aforementioned TRIF adaptor. The role of TRIF in the MyD88 independent pathway was showcased when TRIF deficient mice were shown to elicit impaired phosphorylation of IRF3 and decreased expression of IFN inducible genes in response to stimulation with TLR3/4 ligands (Yamamoto et al., 2003). Later research uncovered the cascade activated by the TRIF (MyD88 independent) pathway, with TRIF interacting with both TRAF6 and TRAF3. TRAF6 acts to recruit RIP1, which activates the TAK1 complex, leading to phosphorylation of NF-κB and MAPKs, and the induction of inflammatory cytokines (Figure 1.4). TRAF3 however activates the IKKs which results in IRF3 phosphorylation, which ultimately results in the transcription of type I interferons (Kawai and Akira, 2010).

1.4.3 NEGATIVE REGULATORS OF TLRS

While the production of cytokines follows the invasion of a pathogen, if the release of these inflammatory mediators remains unchecked it can lead to severe systemic issues that can ultimately result in death. A prime example of this is endotoxic shock, which can be induced by LPS and has a high mortality rate. Mechanisms self-regulate the TLR responses to prevent excessive and potentially harmful responses. Several molecules which have been demonstrated to have effects that negatively regulate TLR signaling,

namely molecules such as IRAK-M, suppressor of cytokine signaling 1 (SOCS1), MyD88 short (MyD88s), Single Immunoglobulin ILR1 related molecule (SIGIRR), ST2, and IRF8(Akira and Takeda, 2004; Fragale et al., 2011).

IRAK-M was shown to be a negative regulator of TLR signaling when mice lacking IRAK-M were observed to produce markedly more cytokines in response to LPS stimulation. Furthermore, endotoxic tolerance, an important protective mechanism which develops to prevent excessive responses to bacterial invasion, did not develop in the IRAK-M deficient mice (Kobayashi et al., 2002). IRAK-M is believed to exert its effects by inhibiting the dissociation of IRAK and IRAK-4 from MyD88, preventing MyD88 based signaling. SOCS1 is a member of a family of cytokine induced proteins that plays an essential role in the negative regulation of TLRs induced by both LPS and CpG DNA (Dalpke et al., 2001; Stoiber et al., 1999; Yasukawa et al., 2003).

Similarly to IRAK-M, mice deficient in SOCS1 also show increased cytokine production and decreased development of endotoxic tolerance in response to LPS (Kinjyo et al., 2002; Nakagawa et al., 2002). Although there is no consensus on the exact mechanism of SOCS1s negative regulatory effects, several have been proposed. Its though that SOCS1 may bind to the Mal/TIRAP complex, targeting it for degradation, or bind to IRAK and modulate its activity, or lastly it may bind to the p65 subunit of NF-κB, targeting it for degradation also (Galm et al., 2003).

MyD88s, which is an alternatively spliced variant of MyD88, is shown to inhibit TLR signaling because of its inability to bind to IRAK4 and promote IRAK1 phosphorylation (Burns et al., 2003). The membrane bound negative regulators of TLR signaling, SIGIRR and ST2, both possess TIR domains, and through interactions with the TLRs can inhibit their signaling. SIGIRR has been shown to inhibit TLR binding to

IRAk1 and TRAF6, preventing further signaling (Brint et al., 2004). Similarly, ST2 was seen to alter TLR signaling by interfering with NF-κB activation, with its mechanism of action believed to involve the binding and sequestering of MyD88 and MAL (Wald et al., 2003).

One of the more recently characterized negative regulators of TLRs is IRF8. IRF8 was shown to play a critical role in the regulation of TLR3 expression, with a recent study showing that IRF8 interacts with the promoter region of TLR3, preventing binding of IRFs 1/2 to the promoter region of the gene (Fragale et al., 2011). The authors demonstrated that IRF 1 and IRF 2 bound to the promoter region and stimulated TLR3 activity whereas this effect was completely abolished by IRF8. It was posited that IRF8 was binding to the TLR3 promoter, competing with IRF1 and 2 and preventing their activity. There is also a second mechanism by which IRF8 is believed to alter TLR3 expression and function. It has been reported that IRF8 acts a substrate for SHP2 (protein-tyrosine phosphatase 2C), which in turn acts as a negative regulator of TLR signaling in macrophages (An et al., 2006; Gröschel et al., 2008; Huang et al., 2006). Thus IRF8 can serve as a negative regulator of TLR3 by a direct and indirect mechanism, the result of both being the downregulation of the gene and inhibition of responses to stimulation of the receptor.

Thus, an array of molecules are involved in the regulation of TLRs, and the role of negative regulation in the immune response cannot be understated. The aggressive nature of an immune response is only beneficial until it does more harm in its response to a pathogen than good. With outcomes such as endotoxic shock and death from sepsis killing over 250,000 people a year (Epstein, 2016), the importance of an immune response that is kept in check is self-evident.

1.5 CYTOKINES AND THE INFLAMMATORY RESPONSE

Cytokine production as a result of TLR activation is an essential component of the innate immune defence. These inflammatory mediators are key components of the inflammatory response. Recognition of PAMPs by TLRs results in activation of the MAPK and NF-κB pathways, which results in expression of inflammatory cytokines such as TNFα and IL-6. The induction of type I interferons is also induced by activation of TLR3 and TLR4, which aids in host defences against viral infection ((See figure 1.4)(Kawai et al., 2001; Servant et al., 2002). While there are many cytokines and chemokines with important roles in innate immunity, of particular interest in the context of this thesis are the type I interferons, IL6 and TNFα.

The interleukin family of cytokines contains a number of subfamilies of cytokines, with IL1 being a prime example of this. The IL1 family consists of 11 members, which are expressed by different cell types, including macrophages and monocytes (Boraschi et al., 2011). Perhaps the most important cytokine of this family, in the context of inflammation at least, is IL1β. Multiple cell types produce IL1β, however, unlike its constitutively expressed relative IL1α, IL1β is usually produced in response to challenge by microbial pathogens. Stimulation of pattern recognition receptors (PRRs), receptors that form an important part of the innate immune response, can lead to the production of IL1β. Stimulation of Toll-Like Receptors (TLRs) and NOD-Like receptors (NLRs) with specific bacterial and viral components, can lead to the production of IL1β (Brennan and Bowie, 2010; Kawai and Akira, 2011). Once secreted, IL1β serves as a potent pro-inflammatory cytokine, and is involved in triggering vasodilation, and chemotaxis of monocytes and macrophages to the site of the noxious insult. It is also involved in the induction of the systemic hypothalamic fever

response, as well as hyperalgesia (Dinarello, 1996). Another important pro-inflammatory member of the interleukin family is IL6, a pleiotropic cytokine that is also expressed by a number of cell types, such as phagocytes, T cells, B cells, fibroblasts and endothelial cells (Jücker et al., 1991). IL6 can be produced by lymphoid and non-lymphoid cells in response to TLR stimulation, and, along with TNFα, is considered the main product of TLR induction (Scheller et al., 2011). This cytokine is an essential component of both the innate and adaptive defence, demonstrated by its importance in protection against infections such as influenza (Lauder et al., 2013) and *Listeria monocytogenes* (Hoge et al., 2013). IL6 is a key component in the inflammatory response, mostly due to its role as the chief stimulator of acute phase protein (APP) production by hepatocytes (Gauldie et al., 1987). The acute phase proteins are a set of plasma proteins that increase or decrease by at least 25% in the 'acute' phase of inflammation. These APP can activate complement and act as opsonins, targeting pathogens for degradation by phagocytosis (Medzhitov, 2007). Perhaps one of the most well classified acute phase proteins is C-reactive protein (CRP), a liver produced protein that is used as a non specific blood marker for inflammation and inflammatory disorders (Eraly et al., 2014; Horiuchi and Mogi, 2011; Silva and Pais de Lacerda, 2012).. As well as these effects on plasma proteins, IL6 also has roles in the maturation of B cells into plasma cells (Kishimoto, 2010), T cell activation, and regulation of differentiation of different T helper subsets (Smith and Maizels, 2014; Takeda et al., 1998).

The interferons (IFNs) were initially classified as an antiviral substance produced by cells in response to virus infection and it was initially believed to confer resistance on cells to the multiplication of viruses (Isaacs, 1964). Since its initial classification, the interferon family have been shown to have an essential role in the host

defence against viral infection(Belardelli, 1995; Grieder and Vogel, 1999; Muller et al., 1994). Production of type I interferons by TLRs is mediated by the nucleic acid sensing TLRs, namely TLR3, TLR7, TLR8 and TLR9, which recognizes the different conserved viral elements in order to mount an anti-viral response (Blasius and Beutler, 2010). The TLRs signal via MyD88 and IRAK4 to activate IRF3 and IRF7, which leads to type I interferon production. TLR3 is the exception however, as it acts via TRIF in a MyD88 independent fashion to activate IRF3, which initiates type I interferon production (Kono et al., 2013). The interferons are divided into three classes Type I, II and III interferons respectively. The two main classes of interferons are type I and II (Pestka et al., 2004, 1987). While the type III interferons are important, they are often referred to as 'interferon-like molecules', as unlike the type I and II interferons, they bind to different cell surface receptors, exerting their effects through a receptor complex comprised of IFNλR1 (or IL28Ra) and IL10R2 (OR IL10Rb) (Platanias, 2005; Witte et al., 2010). Although type II interferons have established antiviral properties, class I and III interferons are considered the primary antiviral effectors (Schoggins and Rice, 2011) The type I interferons consist most notably of IFNα and IFNβ, and they exert their effects by binding to the cell surface type I interferon receptor. There is only one type II interferon however, IFNγ, and it was originally classified as an interferon due to its ability to 'interfere' with viral infections, a hallmark of the original definition of the interferon family (Isaacs and Lindenmann, 1957; Pestka et al., 1987). IFNγ exerts its effects via the type II interferon receptor, which, much like the type I interferon receptor, is a cell surface receptor. The IFNs have the ability to induce the expression of hundreds of genes (referred to as interferon stimulated genes or ISGs) by signaling via the JAK/STAT pathway, as well as the MAP kinase and NF-κB pathways (Darnell et al., 1994; Platanias, 2003; Stark and Darnell, 2012). While the complement of ISGs are

quite large, with microarray based studies showing up to 1000 ISGs present in certain cell types (de Veer et al., 2001), the 'classical' ISGs have been the most investigated. This classification describes three antiviral effectors, namely myxovirus resistance 1 (Mx1), oxyadenylate synthase/RNAse L (OAS) and Protein kinase R (PKR). Mx1 is purported to exert its effects by forming oligomeric rings around the nucleoproteins of Mx sensitive viruses, inhibiting transcription and replication of viral DNA (Haller and Kochs, 2011). OAS is believed to activate RNAse L, which results in degradation of the viral genome (Kristiansen et al., 2011). PKR exerts its effects by targeting EIF2α, a key component involved in transcription initiation, thus preventing viral replication (Toth et al., 2006). This is a brief overview of some of the effectors interferon stimulated antiviral immunity, more detail on which can be found in (Borden et al., 2007; Ivashkiv and Donlin, 2014; Schneider et al., 2014; Schoggins and Rice, 2011)

The last major cytokine which is important in the context of this thesis is Tumor necrosis factor alpha (TNFα), which was originally described as an endotoxin induced factor that leads to the death of tumor cells (Carswell et al., 1975). Since its initial classification in 1975 however, it has been described as playing major roles in many inflammatory conditions, with its role in the defence against bacterial, parasitic and viral infections of particular interest here (Bradley, 2008). Stimulation of TLRs results in TNF production (Takeuchi and Akira, 2010), and there is evidence that TNFα is important in viral immunity, showing strong anti-influenza and anti-hepatitis actions (Matikainen et al., 2006; Seo and Webster, 2002; Tzeng et al., 2014). The importance of TNFα in the host defense against infection is further highlighted by studies which use TNF deficient mice. Studies showed that, in mice deficient in TNF receptor 1 (TNFR1), they observed a resistance to lethal doses of both LPS and *Staphylococcus aureus*

(Pfeffer et al., 1993; Rothe et al., 1993). TNFα exerts its effects mainly via two receptors, TNF receptor 1 and TNF receptor 2 (TNFR1, TNFR2), with the pro-inflammatory and apoptotic effects of TNFα being mediated by the former (Tracey et al., 2008). One of the major effects of TNFα is the phosphorylation of NF-κB, with the activated TNFR1 though to form a complex with RIP1, which in turn activates MEKK-3 and transforming growth factor-beta (TGFβ)-activated kinase (TAK)1, which subsequently activate the inhibitor of κB (IκB) kinase (IKK) complex, leading to the phosphorylation of IκB, and the degradation of the complex, which releases NF-κB,, allowing it to translocate to the nucleus (Blonska et al., 2005). This activation leads to many of the pro-inflammatory effects of TNFα, as NF-κB has strong proinflammatory effects, namely its action as a transcription factor, with NF-κB important in the synthesis of IL1, IL1β, IL6, and IL8, as well as its activating cytokine, TNFα. Furthermore, NF-κB has also been shown to have a role in the initiation of programmed cell death, with NF-κB induced activation of Fas ligand an important step in initiation of apoptosis (Kasibhatla et al., 1998). Thus TNFα has an undeniably important role in the inflammatory response, with its activation resulting in the downstream propagation of pro-inflammatory mediators.

The cytokines discussed here are products of TLR activation, and these mediators all have important roles to play in the initiation, propagation and resolution of inflammation. While there are many cytokines produced as a result of TLR signalling, IL6, TNFα and IFNβ formed the main focus of experimental cytokine analysis in this thesis.

1.6 LEUKOCYTE RECRUITMENT

One of the main effectors of the innate immune response are the leukocytes recruited and activated as a result of PRR signaling. Activation of PRRs can result in the activation of tissue resident immune cells, namely dendritic cells (DCs) and macrophages. It also results in the recruitment of circulating leukocytes such as monocytes, neutrophils and mast cells, which undergo migration towards the affected tissue.

Neutrophils are key effectors of the innate immune response, recruited rapidly in defence against invading pathogens. Following its recruitment to the site of infection, neutrophils can be activated by a wide range of PAMPs. Neutrophils are known to express the majority of the TLR family, with TLR3 and TLR7 being the exception (Prince et al., 2011). Following activation, neutrophils can respond to pathogens by releasing antimicrobial peptides such as defensins, or proteolytic enzymes such as lysozyme, through the formation of neutrophil extracellular traps (NETs), or by direct phagocytosis of the pathogens. (See (Amulic et al., 2012) for in depth detail on these processes).

Macrophages express a wide array of PRRs, and as table 1.1 shows, this includes a number of TLRs. These receptors allow recognition of pathogenic microorganisms by macrophages following PRR activation (Geissmann et al., 2010; Gordon and Taylor, 2005). Macrophage activation can also be induced by cytokines, with IFNγ being one of the most potent activators of macrophages (Arango Duque and Descoteaux, 2014). Recognition of PAMPs by the PRRs results in macrophages activation, resulting in the phagocytosis of the foreign particle. Macrophages can also act as antigen presenting cells, although they are not as effective as dendritic cells in this regard (Gordon and Taylor, 2005).

Dendritic cells serve as an important bridge between the innate and adaptive immune responses. While antigen uptake and presentation to naïve T cells is the main mechanism of action, they also have other functions. Dendritic cells possess the ability to phagocytose pathogens to a certain extent, although not as effectively as macrophages (Hart, 1997). Dendritic cells have also been shown to express RANTES, MCP-1, IL-8, Mip-1a, MDC and DC-CK1 (An et al., 2006; Godiska et al., 1997), chemokines which act as chemoattractants for innate immune cells including neutrophils and NK cells.

Natural killer (NK) cells act a primary defence against bacterial and virally infected cells. NK cells actions can be induced by activation of PRRs, and they show expression of most TLRs, with TLRs 2,3,5 and TLR6 predominantly expressed (Lauzon et al., 2006). Following activation they exert their cytotoxic effects by two main methods, the release of membrane disrupting perforins and granzymes, or the induction of caspase dependent apoptosis (Mandal and Viswanathan, 2015). NK cells have important roles in viral defences, with activation of TLR3 shown to directly promote NK cell function as well as the release of IFNγ and IL6 from NK cells (Duluc et al., 2009; Schmidt et al., 2004)

Although this provides a brief insight into the cells of the innate immune response, there is evidence that TLRs play an important role in activating these immune cells. The anti-pathogenic effects of these cells require direction, and the activation of TLRs by PAMPs produces cytokines which recruit the cells, and then TLRs present on the recruited leukocytes direct the actions of the cells.

1.7 TOLL-LIKE RECEPTORS AND DISEASE:

The important role of the TLRs in the regulation of innate and adaptive immune responses is reflected in their purported involvement in the pathogenesis of many disease states. Sepsis is a disease that hospitalises over 1.2 million people a year in the United States and one of the main causes is Gram-negative bacteria (Hall et al., 2011). TLR2, the main receptor for bacterial lipoproteins such as those seen in Gram-negative bacteria, has a role in the development of sepsis. Polymorphisms in the *tlr2* gene have been linked to increased susceptibility to certain disease states, with the R753Q polymorphism being implicated in conditions such as *Staphylococcus* infections (Lorenz et al., 2000) and tuberculosis (Ogus et al., 2004). Another polymorphism implicated in disease development is the R677W polymorphism, which has been shown to decrease NF-κB activation following infection with *Mycobacterium leprae* and *Mycobacterium tuberculosis*, increasing susceptibility to both leprosy and tuberculosis (Ben-Ali et al., 2004; Bochud et al., 2003; Kang and Chae, 2001).

Altered regulation of *tlr2* has also been implicated in impaired wound healing in patients with type 2 diabetes mellitus (Singh et al., 2015). Furthermore, methylation differences in the promoter region of *tlr2* have been linked to pulmonary Tuberculosis (TB). Recent studies have also shown increased methylation of CpG sites in the promoter region of the gene and a resulting decrease in *tlr2* expression which has been implicated in the development of the condition (Chen et al., 2014). Hypermethylation of *tlr2* has also been implicated in dysbiosis of the innate immune system as a whole, with studies linking aberrant methylation of the gene to decreased global innate immune responses and susceptibility to disease (Benakanakere et al., 2015)

tlr4 mutations have also been implicated in the development of sepsis, with certain polymorphisms being shown to be present. The D299G *tlr4* mutation has been

shown to increase the risk of infection in human patients at risk of sepsis, with studies linking this polymorphism to the increased susceptibility to gram negative infections (Agnese et al., 2002; Lorenz E et al., 2002). This D299G mutation has shown to cause LPS hypersensitivity which leads to the development of these inflammatory conditions via increase cytokine production due to higher levels of TLR4 (Ohto et al., 2012). Furthermore rare missense mutations in *tlr4* have been shown to increase susceptibility to meningococcal infections in analyses of certain patient populations (Smirnova et al., 2003).

Dysregulation of TLR4 is linked to the development of atherosclerosis and other acute coronary events. The TLR4 hyperresponsiveness seen with the D299G mutation has been shown in several studies to lead to higher incidences of coronary plaque developments and worse cardiac outlook in general in patients who possess this mutation when compared to normal (TLR4) patients (Ameziane et al., 2003; Boekholdt et al., 2003; Kiechl et al., 2002). Polymorphisms in *tlr4* have also been associated with psoriatic arthritis (Akbal et al., 2015) and the development of rheumatoid arthritis (Davis et al., 2015). While there is not evidence at present to directly attribute these effects to TLR4, recent mouse studies have shown that TLR4 antagonists can reduce atherogenesis in mice with type-2 diabetes (Lu et al., 2015). The D299G *tlr4* polymorphism is also linked with ulcerative colitis (UC) and Crohns disease (CD), with a recent study showing a significant correlation between the D299G polymorphism and both UC and CD (Meena et al., 2013).

Furthermore aberrant expression of TLR4 has been associated with the development of acute anterior uveitis (AAU). One study showed a significant difference in the expression of TLR4 in AAU patients when compared to normal controls, and determined that this altered expression was not polymorphism linked (Chang et al.,

2007). Non-polymorphism related upregulation in TLR4 has also been shown to be correlated with ulcerative colitis (UC) and Crohn's disease (CD), with patient samples showing elevated intestinal levels of TL4 (Cario and Podolsky, 2000). This suggests that epigenetic changes affecting TLR4 expression may possibly contribute to the development of IBD.

TLR3 has been linked to the exacerbation of Respiratory syncytial virus (RSV), which is the leading cause of lower respiratory tract infections in young children (Shay et al., 2001). RSV is believed to activate TLR3 which leads to the production of cytokines and chemokines which serve to propagate the immune response and as such the activation of TLR3 is believed to be a major part of the pathogenesis of the disease (Rudd et al., 2005). In mouse models, TLR3 has also been linked to increased morbidity and mortality in response to *vaccinia* infection, in a study which showed that *tlr3*-/- mice had much lower viral replication in the respiratory tract when compared to WT animals. Furthermore there were significantly lower levels of cytokines, specifically IL6, MCP-1, and TNFα, present in the knockout mice when compared to their WT counterparts. All of which suggest a role for TLR3 in the pathogenesis of *vaccinia* infection.

Polymorphisms in *tlr3* have recently been linked to development of type 1 diabetes also, with certain risk alleles being associated with early age diagnosis and worse glycemic control (Assmann et al., 2014). Like TLR4, *tlr3* dysregulation has also been implicated in the development of intestinal inflammatory conditions. However in contrast to TLR4, TLR3 was significantly downregulated in intestinal epithelial cells (IECs) in CD but not in UC (Cario, 2010; Cario and Podolsky, 2000). As with TLR4, this suggests a possible role for epigenetic induced changes in regulation of TLR3 expression.

TLR5 has been shown to play a role in the development of inflammatory conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA). Studies have shown that in the aforementioned conditions that TLR5 expression has been shown to be elevated in synovial tissue, macrophages and endothelial cells. TLR5 was shown to be instrumental in the development of the RA pathogenesis, as there was a direct correlation between TLR5 expression, levels of TNFα in the synovial fluid and the disease activity score. The importance of TLR5 in disease development was determined when TLR5 was blocked and there was a resultant 80% decrease in synovial TNFα as well as a decrease in disease activity (Chamberlain et al., 2012). Thus, it is evident TLR5 plays an important role in the development of inflammatory conditions such as RA. Furthermore, while not strictly an immune condition, increased TLR5 expression has been associated with the development of oral squamous cell carcinoma, with studies showing significant increases in TLR5 expression in the cancer patients, and increased TLR5 expression being correlated with recurrence and poor outlook (Kauppila et al., 2013).

Patients with systemic lupus erythematosus (SLE) have antibodies against endogenous antigens, including nucleic acids (Hahn, 1998). The cause of these autoantibodies is believed to be mediated by signalling via the TLR9 receptor. Although under normal circumstances mammalian DNA is usually inert or inhibitory to signalling via TLR9 (Gursel et al., 2003), it has been shown that signalling via TLR9 can occur when the DNA is present in DNA-immune complexes (Leadbetter et al., 2002; Viglianti et al., 2003). Thus, it is posited that in these patients where the DNA is recognised by the TLR9 receptor, that this can lead to the development of antibodies to one's own DNA, leading to the development of the SLE pathophysiology. Similarly to TLR3/4, TLR9 has also been shown to be dysregulated in IBD, with peripheral B cells taken

from patients with IBD patients shown to have significantly higher expression of TLR9. The study also showed a positive correlation between TLR9 expression and IBD severity (Berkowitz et al., 2013).

The aforementioned examples of the roles of TLRs in the development of a wide array of immune conditions show how important TLRs are in regards to disease. Many of the conditions examined above develop due to mutations or polymorphisms in genes that lead to aberrant regulation and perhaps increased transcription of the gene product, as was the case with TLR4 especially. Given the marked effect traditional genetic changes can have on the expression profile of TLRs, it stands to reason that epigenetic changes could also produce changes in gene regulation that could indeed lead to the development of conditions such as the ones seen above. The dysregulation of several TLRs due to altered expression also lends credence to this theory, especially in the cases where the changes in expression cannot be attributed to a polymorphism.

1.8 REGULATION OF GENE EXPRESSION

The previous section has outlined many disease states which have been attributed to, or correlated with, changes in TLR expression and function. The regulation of gene expression is a complex multistep process, and alterations to any of the steps could produce changes in expression and, subsequently, function of TLRs. The control of gene expression is important in development as well as in response to other exogenous and endogenous stimuli. The tight control of expression is essential in the delineation of stem cells into cells of different lineages. Furthermore, the ability of these regulatory mechanisms to respond to stimuli such as injury or infection is important in mounting an effective response to these stimuli. Outlined in figure 1.5 is a broad overview of the

process of gene expression, from the initial activation of transcription factors to the synthesis of a functional gene product. Transcription factor (TF) binding in DNA regulates the activity of most genes. These transcription factors are divided into two groups, general transcription factors (GTFs) and transcriptional activators (Orphanides et al., 1996). These GTFs bind to regulatory regions such as sites in the vicinity of transcription start sites (TSS), however their distribution is not uniform (Koudritsky and Domany, 2008; Lin et al., 2010). These TF then recruit RNA polymerases (RNA Pol), three of which are present in humans, RNA pol I, II and III, with pol II involved in the transcription of protein coding genes in eukaryotes (Lee and Young, 2000). Once transcription is initiated by pol II, a 'cap' structure is added to the 5' end of the RNA, a process which prevents degradation of the RNA by nucleases and is also believed to have a role in the binding of factors involved in the nuclear export of the mRNA upon completion of transcription (Proudfoot et al., 2002). After the conclusion of the transcription process a poly A tail is added to the 3' end of the RNA, the addition of which is thought to be important in both the cleavage and cessation of translation (Proudfoot et al., 2002). Research also suggests that the poly A tail is important in the nuclear export of the newly transcribed mRNA (Fuke and Ohno, 2008).

Following transcription, the mRNA is exported from the nucleus to the cytoplasm, where translation can occur. Export of mRNA is dependent on mRNA export factors, with the bulk of mRNA exported by a heterodimer complex of NXF1-NXT1 (Herold et al., 2000; Segref et al., 1997). The exported mRNA can then be translated into functional protein by ribosomes (Ramakrishnan, 2002), in a process that is analogous to that seen in transcription. Initiation of translation involves identification of the AUG start codon by a small ribosomal subunit, which then facilitates the binding of a large

ribosomal subunit which generates a translationally active ribosomal unit (Dever, 2002). tRNAs then deliver the amino acids to specific codons, and peptide bonds are formed between the amino acids, facilitating protein assembly (Lodish et al., 2000). This is a basic outline of the processes involved in the regulation of gene expression. Processes which interfere with any of the steps outlined here can result in altered gene expression and protein function, and the effect of chromatin structure and remodeling on gene expression forms part of the main focus of this thesis.

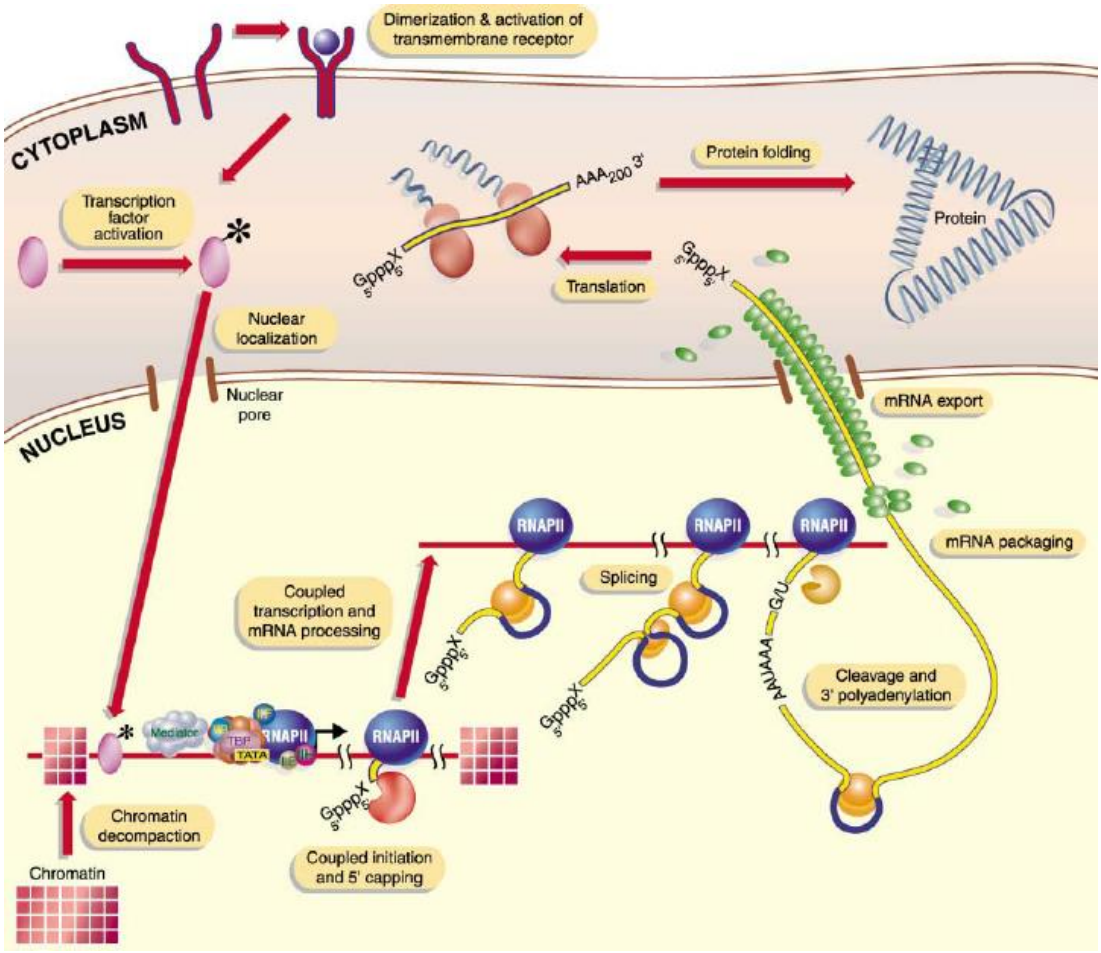


Figure 1.5 The different steps in the pathway from transcription factor activation to protein production.

Transcription factor (TF) activation is followed by translocation to the nucleus of these TFs, which then bind to regulatory regions of DNA. RNApol is recruited and an mRNA transcript is produced, which is exported to the cytoplasm for translation into protein. Protein folding then results in functional products. Taken from (Orphanides and Reinberg, 2002)

1.9 EPIGENETIC MODIFICATIONS:

Human genetics is currently undergoing one of the greatest periods of advancement in the history of the field in what is being dubbed by many as the epigenetic revolution (Carey, 2011; Renaudineau, 2010). For many years we have known that the expression of genes in the human genome is based on a sequence of nucleotides in a specific order, and that changing the position and sequence of these nucleotides can have drastic, even fatal consequences. However, focus is now shifting to so-called epigenetic changes, which are described as changes that result in altered gene expression without changing the nucleotide sequence of these genes. These epigenetic changes are important in normal development and play a huge role in the differentiation of cells into different lineages (Lee et al., 2004; Reik et al., 2001; Wilson et al., 2009). However, when these mechanisms are disrupted it can result in unwanted changes in gene expression and the importance of epigenetic changes in the development of many diseases such as cancer and inflammatory bowel disease is being increasingly appreciated (Esteller, 2008; Jenke and Zilbauer, 2012; Kanwal and Gupta, 2010; Khor et al., 2011; Petronis and Petroniene, 2000).

Epigenetic modifications are described as alterations to the expression of genes without changing the coding sequence by interfering with transcription and translation. While traditional genetics focuses on changes in gene expression that arise due to a change in the nucleotide sequence, such as single nucleotide polymorphisms (SNPs), epigenetics is concerned with changes in gene expression due to modifications to the structure of chromatin which affect gene regulation by repressing or enhancing transcription factor binding through different mechanisms (Berger, 2002; Boyes and Bird, 1991; Sterner and Berger, 2000; Struhl, 1998). While there are many epigenetic

modifications there are two distinct types that will be the main focus of this review, DNA methylation and histone modifications.

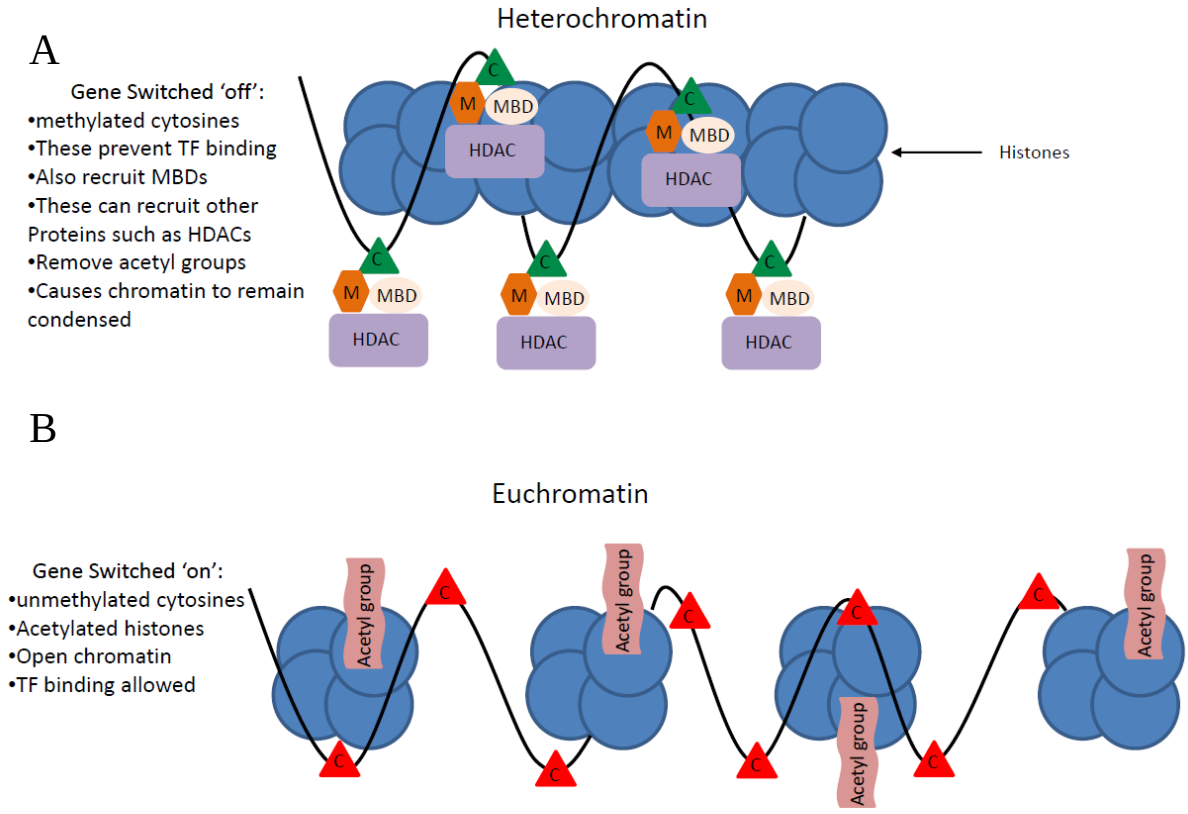


Figure 1.6: Chromatin confirmation and the machinery involved in chromatin remodelling

This figure shows the different states of chromatin and how it affects gene transcription. In (A) the cytosines are methylated, which results in recruitment of methyl binding domains (MBD) which recruit further molecules such as histone deacetylase complexes (HDACs). The removal of acetyl groups causes chromatin to remain in this condensed state known as heterochromatin. In this state TF binding cannot occur and so transcription is impaired. In (B) the cytosines are unmethylated and the acetyl groups are present, maintaining the chromatin in its open confirmation, known as euchromatin. In this state TF binding can occur as the DNA is accessible (Hennessy and McKernan, 2016).

1.9.1 *DNA METHYLATION:*

The methylation of DNA is the most common epigenetic modification in mammals, involving the addition of a methyl group to cytosine residue. The methylation of DNA is associated with development and genomic imprinting (Jurkowska et al., 2011). Methylation of DNA is regulated by the enzyme class known as DNA methyltransferases (DNMTs) (Okano et al., 1998). The DNMTs transfer methyl groups to cytosine residues on a DNA strand specifically at the 5' carbon position of the pyrimidine ring (Chen et al., 1991).

The first DNMT classified on a molecular level and a biochemical level was DNMT1 (Bestor and Ingram, 1983)(Bestor et al., 1988). Generally a cytosine (C) can only be methylated if it precedes a guanine (G) residue (Bird, 1978; Cedar et al., 1979), forming CpG dinucleotides. These CpG dinucleotides occur infrequently in the genome but under normal conditions 80-90% of them are described as being methylated in both a spatially and temporally restrictive fashion (Bird, 2002; Colot and Rossignol, 1999; Ehrlich et al., 1982). However, when these CpG dinucleotides exist as part of CpG islands which are found largely in the promoter regions of genes or in other tissue specific genes, they typically have a high number of non-methylated CpG sites (Bird, 1986). DNA methylation affects chromatin in different ways, generally resulting in epigenetic silencing. It can interfere directly with transcription by blocking transcription factor binding, for example in an unmethylated state the transcription factor erythroblastosis 1 (ETS1) can bind to DNA but when the DNA is methylated, binding does not occur (Maier et al., 2003)(Bell and Felsenfeld, 2000).

The second and most important means by which DNA methylation alters chromatin structure is through the recruitment of chromatin remodelling elements such as HDACs as a result of methylation (figure 1.5). There are currently fifteen proteins

which are known to bind to methylated CpG islands, and they are divided into 3 families; the MBD containing proteins, the methyl-CpG binding zinc fingers, and the SRA domain containing proteins (Hung and Shen, 2003). The MBD group is the largest family, containing 11 of the 15 proteins identified. The MBD group was derived from the methyl CpG-binding protein 2 (MeCP2), and includes members such as methyl CpG-binding domain protein 1, 2, 4 (MBD1) (MBD2) (MBD4)(Hung and Shen, 2003). The methyl-CpG binding zinc finger family are derived from kaisos 3–zinc finger motif found in the C-terminus, which is capable of binding to methylated CpG dinucleotides (Prokhortchouk et al., 2001).

Finally, the SET and RING finger–associated (SRA) domain containing family which only consists of two members UHRF1 and UHRF2 (Parry and Clarke, 2011). These proteins alter chromatin function and transcriptional activity by recruiting chromatin remodelling enzymes. MeCP2 associates with SWItch (SWI)-independent 3A, a transcriptional co-repressor, and also with histone deacetylases to remodel chromatin and leads to repression of transcriptional activity (Jones et al., 1998; Nan et al., 1998). Thus, one of the major actions of the DNA methylation machinery is the recruitment of proteins that aid in the remodelling of chromatin. Once such set of proteins are the enzymes involved in the control of histone acetylation.

1.9.2 HISTONE ACETYLATION/DEACETLYATION:

Transcription in eukaryotes is a highly controlled process with many different factors involved in its regulation. In recent years, it has become evident that acetylation of histones at specific lysine (K) rich sites has a dramatic effect on transcriptional regulation by altering the structure of the histone chromatin complex (Shahbazian and

Grunstein, 2007). The structure of chromatin has been shown to have an effect on the level of transcription of the genetic material contained within the chromatin complex. The nucleosome is the standard organisational unit of DNA in eukaryotic cells, in which 147 base pairs of DNA are coiled around a histone octamer (which is made up of 2 of each of the histone units H2A, H2B, H3, and H4)(Wolffe, 1992). When chromatin is in this nucleosomal state it is generally repressive to transcription (Owen-Hughes and Workman, 1994) and the nucleosomal structure coupled with the DNA histone interactions means the genetic material is inaccessible for transcription and translation and results in epigenetic silencing (figure 1.5). Thus, to enable activation of the genes involved in this DNA-histone complex there must be a means of altering the state of the chromatin structure to enable transcription of genetic material.

Several well-known protein and enzyme complexes can alter chromatin state. For example, the SWI-SNF (SWItch/Sucrose Non-Fermentable) complex alters chromatin packaging in an ATP-dependent fashion (Kingston et al., 1996; Lu and Roberts, 2013). Other methods of modifying chromatin-histone interactions involve processes such as methylation, ubiquitination, ADP-ribosylation, phosphorylation (Bradbury, 1992). The addition of an acetyl group to the nucleosomal complex results in chromatin remodelling due to the interference of the acetyl group with the interaction between the positively charged lysine tail of the histone and the negatively charged DNA (Bannister and Kouzarides, 2011). Neutralising the positive charge on the lysine results in a weakened association between the DNA and the histone protein complex, the result of which is the remodelling of the transcriptionally inactive heterochromatin into the transcriptionally active euchromatin (see figure 2 below). Alternatively, deacetylation of histones results in stronger ionic interactions between the positively charged histones and the negatively charged DNA resulting in the more compact

chromatin structure referred to as heterochromatin. This compact structure results in transcriptionally inactive chromatin due to its condensed state (Johnstone, 2002).

Acetylation and deacetylation of the lysine tail of the histone proteins are controlled by two distinct families of enzymes, the histone acetyltransferases (HATs) and the histone deacetylase complexes (HDACs) (Bannister and Kouzarides, 2011). There are three major families of HATs which have been previously reviewed extensively. General control non-derepressible 5 (Gcn5)-related N-acetyltransferases (GNATs), protein lysine acetyltransferase MYST proteins (Yang and Seto, 2007) and CREB-binding protein/E1A binding protein (CBP/p300) (Goodman and Smolik, 2000) have been well categorised as HATs and their activity in acetylating histones is well documented (Das et al., 2009).

In opposition to the HATs are the enzymes known as histone deacetylase complexes. There are 18 members in this family of enzymes and they are grouped into four distinct classes based on their homology to proteins identified in yeast, namely Rpd3, Hos1 and Hos2 (Class I), HDA1 and Hos3 (Class II) and the sirtuins (Class III)(Verdin et al., 2003)(Blander and Guarente, 2004)(de Ruijter et al., 2003) (Shakespear et al., 2011). Class I, II and IV HDACs are referred to as classical HDACs and their enzymatic activity depends on Zn^{2+} as a cofactor. While class I and II HDACs are evolutionarily similar, class IV HDACs, despite also relying on the Zn^{2+} interaction, are not related to the others. Class III HDACs are known as sirtuins and do not require Zn^{2+} but instead rely on a NAD^{+} cofactor which is utilised by the enzymes to accept acetyl groups (Offermanns, 2008). Many of the HDACs are believed to exist as protein complexes comprising a number of subunits which then target specific genomic regions via interactions with nuclear receptors, transcription factors or other proteins such as methyl binding domains (MBDs) (Glass and Rosenfeld, 2000).

A well characterised mechanism by which HDACs are recruited to DNA is via the interaction between an MBD such as MeCP2 and HDACs. When DNA is methylated the methyl group will recruit additional proteins such as the aforementioned MeCP2 which in turn will recruit a HDAC complex, resulting in the repression of gene transcription (Jones et al., 1998)(Nan et al., 1998). Furthermore, studies in DNA methyltransferase 1 (DNMT1) knockouts show that the methylation state can also affect the ability of HDACs to function. In cancer cells lacking the DNMT1 enzymes, it has been shown that there are elevated levels of histone acetylation at histone H3 and decreased methylation of histone H3. It is speculated that the reason for the hyperacetylation is not due to a direct decrease in HDAC activity, but rather that in the absence of methylation, there is less targeting of the HDACs to DNA sequences due to a decrease in interaction between the HDACs and the heterochromatin protein HP1, which is an important regulator of chromatin remodelling which is involved in transcriptional repression and gene silencing (Espada et al., 2004).

As mentioned previously, HDACs can regulate gene expression independently of DNA methylation, by interacting with nuclear receptors and transcription factors (Glass and Rosenfeld, 2000; Huang et al., 2000; Kato et al., 2004). The E2F family of transcription factors have been shown to play an important role in the control of the DNA replication machinery as well as mitosis during the cell cycle, with some members of the family acting as activators and others as repressors (Ishida et al., 2001). Studies have shown that DNMT1 can form a complex with retinoblastoma protein (Rb), E2F1 and HDAC1 and the resultant complex can repress transcription at promoters which are normally responsive to E2F (Robertson et al., 2000).

1.9.3 REGULATION OF GENE EXPRESSION BY OTHER MECHANISMS

The mechanisms outlined above functionally alter gene expression by preventing the initiation of transcription, either by direct inhibition of transcription factor binding, or by maintaining or establishing an 'unreadable' chromatin complex. However, there are other mechanisms which affect later stages of the gene expression cycle, such as post transcriptional and translational mechanisms, which, although not the focus of this thesis, require mentioning.

1.9.3.1 Post transcriptional modifications

Small noncoding RNAs (ncRNAs), such as miRNA and siRNA, can alter gene expression by cleavage of mRNA or inhibition of translation (Meister and Tuschl, 2004). The action involves the formation of an RNA induced silencing complex (RISC), which then binds to the guide siRNA/miRNA, resulting in mRNA cleavage, or repression of transcription, by a component of the RISC complex known as argonaute (AGO) proteins (Fabian and Sonenberg, 2012; Meister et al., 2005). The transcriptional repressive action of AGO is less well understood than its cleavage of mRNA, however, recent studies have shown that argonaute2 (AGO2) is binding to specific promoter sequences, preventing transcription of target genes (Taliaferro et al., 2013)

Transfer RNAs (tRNAs) are key components in the translation pathway, delivering amino acids to ribosomes in order to translate the mRNA into functional protein (Rodnina and Wintermeyer, 2011). These tRNAs serve as key regulators of gene expression, and the tRNA abundance within a cell can differ depending on the translational needs of the cell at a given time (Fredrick and Ibba, 2010; Ouyang et al., 2000) Modifications and mutations in tRNAs have been associated with disease states,

with mutations leading to altered tRNA function, which impaired protein synthesis (Moraes et al., 1993; Yasukawa et al., 2001).

This is just a brief example of two post transcriptional, pre-translational mechanisms by which gene expression can be regulated. The ncRNAs have important functions in the translation of mRNA into functional protein (tRNA), or the cleavage and degradation of mRNA, preventing transcription (miRNA).

1.10 EPIGENETIC MODIFICATION OF IMMUNE RESPONSES:

It is evident that the Toll-like receptors are important in the immune system's ability to defend against invading pathogens, and thus, any mechanism which interferes with their normal function could be either dangerous or therapeutic. Dangerous from the perspective of defending against foreign bacteria or viruses which are infecting the host, in which case a malfunctioning immune response could prove fatal. However, the ability to modify the immune response could prove useful in situations where the immune response is unwarranted or detrimental to health. In conditions like autoimmune disease, sepsis or indeed any condition where there is unwanted or excess inflammation and immune activation, the ability to dampen or cease this response would be very beneficial. Whether by genetic knock down or knock out, or by epigenetic modification of the genome, interfering with the expression of the proteins that constitute this family of immune receptors could have significant effects on the immune response.

DNA methylation has been shown to have a role in regulating TLR expression. It was determined that decreased methylation in the promoter region of *tlr2* was associated with an upregulation of TLR2 (Furuta et al., 2008). Increased TLR2 expression is believed to be responsible for increased susceptibility to infection that is

associated with cystic fibrosis. An earlier study also linked the upregulation of TLR2 to the dysfunction in the cystic fibrosis transmembrane regulator (CFTR), and that upregulation of TLR2 resulted in an increased inflammatory response to bacterial infection in bronchial epithelial cells (Shuto et al., 2006).

Recent studies by Takahashi and colleagues indicate that hyporresponsiveness to LPS seen in an intestinal epithelial cell line was due to downregulation of TLR4. This downregulation was shown to be due to hypermethylation of the TLR4 promoter (Takahashi et al., 2011, 2009). These papers suggest a direct role for epigenetics in the regulation of TLR4 expression. However, there are few other studies present in the literature examining the role of DNA methylation on the regulation of TLRs, leaving us with the question as to what effect DNA methylation and the DNMTs have on the innate immune system and the TLRs.

While not strictly the subject of this thesis, recent studies have shown that the enzyme Ezh1, which is a histone lysine methyltransferase, promotes TLR induced cytokine release. This would suggest that methylation of histones has a proinflammatory effect, similar to that seen in DNA methylation. The study found that in *ezh1* knockout macrophages, TLR responses were decreased when compared to normal controls, suggesting a role for histone methylation in the maintenance of the immune response (Liu et al., 2015). Dysregulation of *tlr2* has been associated with the development of the inflammatory condition periodontitis, with a recent study showing that hypermethylation of the *tlr2* gene resulted in low transcription of TLR2 (de Faria Amormino et al., 2013). This was implicated in the abnormal response to bacteria that lead to the development of the dental inflammatory condition.

Differential epigenetic regulation of PRRs has also been documented in monocytes and macrophages (Haehnel et al., 2002). Another study has shown that

TLR3 regulation differences seen between adult and new-born derived dendritic cells are due to epigenetic regulation, and suggest a role for epigenetic control of the receptor in the regulation of the innate immune system (Porrás et al., 2008). More recent studies have shown that up-regulation of TLR4 has been linked to increased susceptibility to lupus erythematosus (Elloumi et al., 2017).

1.11 EPIGENETIC MODIFYING DRUGS:

Several drugs that have been indicated for cancer chemotherapy or as antifungals which also may have immunomodulatory activity (table 1.2) based on their potential epigenetic actions. The fungistatic Trichostatin A (Tsuji et al., 1976) is a potent inhibitor of HDACs (Yoshida et al., 1990). The inhibitor of the bromodomain and extra terminal domain (BET) family of proteins mimics acetylated histones and interferes with regulation of genes involved in inflammation (Nicodeme et al., 2010). Thus far, little research has focused on the direct effect of epigenetic modifications and epigenetic modifying compounds on the TLRs.

Epigenetic modification	Treatment	Dose	Time point	Subject	Effect	Reference
HDAC inhibition	Butyrate	Not available	Not available	HeLa cells	Inhibits proliferation	Riggs *et al,*.1977
HDAC inhibition (Pan Inhibitor)	LAQ824	120/40/80nM +/- 1µg/mL LPS	1, 3, 6, 12, or 24 hours	Thp1/dendritic/macrophages/neutrophils	inhibits Th1 function & macrophage chemotaxis	Brogdon *et al.*,2007
HDAC inhibition (Pan Inhibitor)	SAHA	.1-50mg/kg	1hr pre LPS injection	Mice (model of endotoxemia)	decrease in liver damage & cytokine release	Leoni *et al.*, 2002
HDAC inhibition (Pan Inhibitor)	TSA	6.25, 12.5, 25, 50, 100, 200nM	1hr Pre LPS injection. (24hr LPS stimulation)	LPS stimulated macrophages	Decreased levels of cytokine mRNA/protein levels	Han *et al,*. 2009
HDAC inhibition (Pan Inhibitor)	LBH589	2.5, 10, 20nM	24h	Dendritic cells	Decrease in cytokines in response to stim of TLR3/4	Song *et al*., 2011
HDAC inhibition (Pan HDAC)	TSA	100nM	1h before stimulation for 1, 2, 4, and 20 hours with TLR1/TLR2/TLR4 ligands	BMDMs (stim with TLR ligands)	Decrease in cytokines produced and altered expression of TLR1-9	Roger *et al*., 2011
DNMT Inhibition	5-Azacytidine	500 nM of AZA	72 hours	63 cell lines (26 breast cancer, 14 colorectal cancer, 23 ovarian cancer)	Increased immune response	Li et al., 2014)

Table 1.2. The above table summarises the known effects of epigenetic modifying drugs on immune responses showing treatments and effects.

1.12 HDAC INHIBITORS:

The process of acetylation and deacetylation of histones is well documented as having a large role in the regulation of gene expression, and with that, affects cellular proliferation and the cell cycle (Reichert et al., 2012). Given their role in cellular proliferation, it is of little surprise that dysregulation of the HDACs has been implicated in tumorogenisis and they are attractive targets due to their ability to arrest the cell cycle, induce apoptosis and inhibit angiogenesis (Ellis et al., 2009; Kim et al., 2000). Analysis of prostate cancer cells have revealed elevated levels of HDAC1 when compared to healthy patients (Halkidou et al., 2004), which would suggest that decreased acetylation could possibly be playing a role in the development of the disease. Butyrate was one of the first compounds discovered to have HDAC inhibitory effects, with the compound showing reversible inhibition of cellular proliferation and increases in histone acetylation (Riggs et al., 1977). Butyrate however has a myriad of other effects and is not considered a specific HDAC inhibitor. Trichostatin A (TSA) was the first compound discovered to be a specific inhibitor of HDACs. It was originally classified as a fungistatic antibiotic when it was isolated from strains of *Streptomyces hygroscopious* (Tsuji et al., 1976). It was shown to have potent inhibitory effects on mammalian HDACs both *in vitro* and *in vivo* and was also shown to promote cell proliferation and differentiation (Yoshida et al., 1990). Its potential antitumor activity, though not well defined, has been linked to increasing expression of the Rb tumor suppressor gene (Zhao et al., 2005).

Rb normally controls cellular proliferation by modulating the activity of the E2F family of transcription factors, but in many cancers there is a loss of function in the Rb

gene (Sherr and McCormick, 2002). Another HDAC inhibitor, Vorinostat (previously referred to as suberoylanilide hydroxamic acid or SAHA), has been FDA approved as an adjuvant therapy for the treatment of T-cell lymphomas in patients where previous therapies were ineffective (Mann et al., 2007). Like the other HDAC inhibitors its effectiveness is believed to be due to increased expression of pro-apoptotic factors. Given the ability of the HDAC inhibitors to induce remodelling of chromatin and alter gene expression it was posited that they may also have an effect on the regulation of genes involved in the immune response, which would ultimately lead to investigations into their effects on immune regulation.

1.12.1 HDAC INHIBITORS AND THE IMMUNE SYSTEM:

Although these drugs are extremely cytotoxic at the concentrations used in anticancer therapy, HDAC inhibitors could be used to alter immune responses with minimal cytotoxicity when used at lower concentrations. Studies have shown that the HDACs are important in the regulation of certain immune cell functions (Falkenberg and Johnstone, 2014). In one such study, a small molecule inhibitor of HDACs, LAQ824, was found to alter the activation and function of macrophages and dendritic cells in response to TLR4 activation by LPS. Specifically the HDACi prevented activation of T-helper 1 (Th1) cells by dendritic cells but had no effect on T-helper 2 (Th2) cells. Furthermore, the study also showed that HDACi prevented macrophage and monocyte chemotaxis but had no effect on neutrophils (Brogdon et al., 2007). Thus, in this case inhibition of the HDACs was having a highly specific effect on the immune response by altering the Th1 and Th2 balance in response to the LPS challenge.

The *in vivo* applications of SAHA have been examined in a mouse model of endotoxemia. It was found that at non cytotoxic levels, SAHA had a marked effect on the inflammatory response to concanavalin A (Con A) induce liver damage. There was over a 50% decrease in circulating cytokines seen during experimental endotoxemia, including TNF-α, IL-1-β, IL6, and IFN-γ, when compared to non-SAHA treated mice. Furthermore, SAHA treatment suppressed cytokine induced nitric oxide production in mouse macrophages and also prevented hepatocellular damage (Leoni et al., 2002). Recent data has also shown the effectiveness of SAHA in vivo, in a study which examined the immune effects of Vorinostat (SAHA) on patients receiving treatment for graft versus host disease. The study found that in patients receiving treatment with SAHA that there were decreased levels of proinflammatory cytokines when compared to controls, suggesting a clinical anti-inflammatory application for the drug (Choi et al., 2015). TSA has also shown promise as an anti-inflammatory compound, and when compared to five other HDAC inhibitors, its anti-inflammatory effects of LPS stimulated bone marrow derived macrophages (BMDMs) were deemed the most potent. It showed significantly decreased mRNA and protein levels of proinflammatory cytokines TNF-α, IL-1-β, IL6, and increased of IL-10, which is immunosuppressive (Han and Lee, 2009). Finally, a recent study has highlighted a role for HDAC7 in inflammation, with the study exhibiting HDAC7 dependent promotion of the proinflammatory effects of TLR4 activation (Shakespear et al., 2013). Thus, there is sufficient evidence to indicate that there may be a place for HDAC inhibitors as regulators of inflammation.

1.12.2 HDAC INHIBITORS AND TLRS:

Despite the evidence that suggests HDAC inhibitors are altering immune responses and perhaps interfering with the TLR response pathways, there is little evidence at present to suggest whether any of the effects seen are due to a direct action on the expression of TLRs themselves. One study showed the effect of HDAC inhibition by a novel pan-HDAC inhibiting compound LBH589 on TLR3 and TLR4 induced dendritic cell activation. They showed that upon administration of the compound the levels of cytokines released induced by the dendritic cells in response to activation by TLR3/4 was greatly decreased. There were decreases in levels of IL6, IL10, IL12p70 and IL23 released when compared to the untreated controls (Song et al., 2011). The decreases in the levels of these important inflammatory cytokines would suggest that the inhibition of the HDAC complex is having a huge effect on the Toll-like receptor induced immune response. However, the mechanism by which this effect was taking place was indeterminate and despite the implication that TLRs 3/4 are involved, there is no evidence currently to suggest the effect is a direct one.

Another study showed that in a mouse model of atherosclerosis that several different broad spectrum HDAC inhibitors (pan-HDAC inhibitors) successfully inhibited the production of proinflammatory cytokines such as Edn1, Ccl7/MCP3, and IL12p40 in response to TLR4 stimulation with LPS. However the HDAC inhibitors also increased the expression of several pro atherogenic factors COX2 and Pai-1/serpine1 (Halili et al., 2010). A 2006 study showed that activation of TLR4 with LPS resulted in the repression of certain HDACs as well as the increase in expression of others, the role of which the authors speculated was the control of the immune response to prevent excessive inflammation (Aung et al., 2006).

Further studies examining TLR4 activation have also revealed a potential role for HDACs in regulating the immune response. LPS activation of TLR4 was shown to lead to acetylation of the phosphatase mitogen activated protein kinase phosphatase (MKP-1). Activation of MKP1 results in decreased P38 MAPK activation and consequently inhibition of the immune response (Cao et al., 2008). Although it's indeterminate whether TLR4 activation is directly affecting MKP-1, in this case one could speculate that deacetylation of MKP1 could lead to increased p38 activation and an exacerbation of the immune response following TLR4 activation. The act of acetylating histones or inhibiting the deacetylases would usually result in an increase in transcriptional activity given the more open confirmation of the chromatin, however there have been cases identified where HDAC inhibitors have decreased gene expression. In one study it was shown that the inhibitors TSA and SAHA were decreasing TLR mediated increases in inflammatory gene expression. The authors identified the mechanism behind this change was due to the direct impairment of transcription factor recruitment (Bode et al., 2007). Thus the actions of the HDAC inhibitors on gene expression aren't necessarily clear cut or unidirectional.

A study by Roger *et al* examined the effect of the HDAC inhibitor Trichostatin A (TSA) on the innate immune response to stimulation by TLR ligands and infection (Roger et al., 2011). They determined that treatment with the HDAC inhibitor TSA impaired the innate immune response to challenge with the various TLR ligands. The study showed that there were alterations in the levels of cytokines produced in response to the ligands as well as a change in the expression levels of the TLR1-9. These changes were not unidirectional however, with some TLRs expression increasing and others decreasing, suggesting the relationship between histone acetylation, Toll-like receptor expression and the resulting immune response is more complex than originally thought.

This is one of few studies that examine the effects of HDAC inhibitors directly on the expression of the TLRs. Despite this however, it fails to provide any reason for the changes seen, or indeed why the changes in TLR expression were not uniform, with some increasing and others decreasing. A 2015 study has shown that histone acetylation may play a role in the development of tolerance to pathogens in the lungs. In a study which examined the effects of TLR stimulation on chemokine production in alveolar epithelial cells (AECs), it was found that upon repeated stimulation of AECs with TLR ligands it was noted a 'tolerance' response developed, but that addition of HDAC inhibitors circumvented this response (Neagos et al., 2015).

1.12.3 DNMT INHIBITORS:

The role of DNA methylation in the regulation of gene expression is well founded, and given its role in the development of cancers it's no surprise that there has long been interest in inhibitors of methylation as potential antitumor therapeutics. Aberrant methylation of tumor suppressor genes has been implicated in the development of certain cancers due to the excessive methylation preventing the transcription of pro-apoptotic/anti-tumor factors (Herman and Baylin, 2003; Jones and Baylin, 2007). There are a large number of characterised DNMT inhibitors and they are divided into two distinct families: the nucleoside analogs and the non-nucleoside inhibitors. For the purpose of this review we will focus on just the nucleoside inhibitors, specifically the cytidine analogs. 5-aza-cytidine (5-aza) and 5-aza-2-deoxycytidine (5-aza-2dc) are two cytidine analogues that were first synthesized in 1964 (Piskala & Sorm., 1964).

These molecules were initially used as antimetabolites and as chemotherapeutic agents in leukaemia due to their cytotoxicity (Sorm and Veselý, 1968). They were also

shown to be effective in the treatment of solid tumors with the drug inducing remission in patients with breast cancer, melanoma and colon cancer in phase one trials (Weiss et al., 1972). The hypomethylating action of the cytidine analogs was later defined through a series of studies that showed treatment of a non-myoblastic cell line with 5-aza-cytidine or 5-aza-2-deoxycytidine led to the differentiation of these cells into functional myotubules, a process which would have not occurred naturally (Constantinides et al., 1978, 1977). They speculated that the action seen was due to the cytidine analogs interacting with DNA and hypermethlyating, and thus inactivating certain genes involved in repressing the differentiation of the non-functional muscle cells into functional myotubes.

5-azacytidine and 5-aza-2-deoxycytidine are cytidine analogs in which the C5 carbon is replaced by a nitrogen atom and linked to a ribose (5-aza) or a deoxyribose (5-aza-2dc). Ribose analogs (5-azacytidine) are incorporated into both RNA and DNA (Glover and Leyland-Jones, 1987; Li et al., 1970; Stresemann et al., 2008) whereas deoxyribose analogs (5-aza-2-deoxycytidine) are incorporated only into DNA (Stresemann et al., 2008). When these compounds are converted into their tri-phosphorylated active forms however they behave similarly, being incorporated into DNA as substitutes for cytosines. The DNA methylation machinery recognises these azacytidine-guanine dinucleotides as if they were cytosine-guanine dinucleotides. Under normal circumstances the DNMTs would initiate methylation and a covalent bond would form between the enzyme and the carbon-6 atom of the cytosine (Chen et al., 1991; Santi et al., 1984), and this bond would be broken down and the DNMT would continue in this manner indefinitely. However, in the case of the azacytidine this process is blocked which leads to a irreversibly bound complex (Santi et al., 1984). When the

DNMTs are in this trapped state they become targets for proteosomal degradation and thus are eliminated (Ghoshal et al., 2005).

5-aza-2-deoxy cytidine (5-aza-2dc) is the more potent inhibitor of methylation when compared to 5-aza-cytidine (5-aza) (Hollenbach et al., 2010). It is believed that the reason for this is due to 5-aza being a ribose analog, meaning it incorporates into both RNA and DNA, as opposed to just DNA which the deoxyribose analog 5-aza-2dc does. Because of this 5-aza has less incorporation into DNA when compared to 5-aza-2dc as it also effects RNA. Its incorporation into RNA is also speculated to be related to its more significant side effects when compared to 5-aza-2dc. Because of its action 5-aza-2dc can only exert its effects on dividing cells, meaning it is somewhat more selective for rapidly dividing cancer cells, however, the ability of 5-aza to incorporate into both dividing and quiescent cells allows its effects to be more widespread, which is undesirable for a cytotoxic therapeutic (Gravina et al., 2010). The drugs are well established as anticancer drugs in the treatment of leukaemia (Hollenbach et al., 2010) and myelodisplastic syndromes (Raj and Mufti, 2006) due to their efficient anticancer activity, with more recent studies showcasing their efficacy in treatment of myelofibrosis (Daver et al., 2016). Recently, similarly to the previously discussed HDAC inhibitors, they have begun to garner interest as potential immunomodulatory drugs, with studies seeking to examine whether these drugs could be effective at non cytotoxic concentrations (Bracci et al., 2014).

1.12.4 DNMT INHIBITORS AND IMMUNITY:

DNMT inhibitors have proven themselves as effective anticancer therapies, largely due to their highly potent cytotoxic action in rapidly dividing cells as mentioned above.

Given the importance of DNA methylation in regulating gene expression one would assume that inhibition of methylation would exert some effect on the immune system. However, the effects of DNA methylation and the inhibition of methylation on the immune response have received little attention to date. One of the primary outcomes of TLR activation is the production of type one interferons, this includes the inflammatory cytokines such as IL6, TNF-α and IFN-β (Uematsu and Akira, 2007). As such the effect any given treatment on TLR activation is often measured in terms of the resulting change in cytokine production.

One study has examined the effects of methylation on the suppressor of cytokine signalling (SOCS) family of proteins, namely SOCS1. These proteins serve as negative regulators of cytokine signalling. In this study the authors found that hypermethylation of the *SOCS1* gene, which resulted in a loss of function of this gene, lead to increased signal transduction of IL6, a proinflammatory cytokine which is also a driving factor in the development of multiple myeloma (MM) (Galm et al., 2003). While this case highlighted the effect methylation was having on MM development, the main factor under consideration was increased signal transduction of IL6, a cytokine with a well-established role in the development of chronic inflammation, autoimmune disorders and other immune related conditions (Barnes et al., 2011; Scheller et al., 2011). It stands to reason that inhibition of the DNMTs could reverse the increase in IL6 due to the hypermethylation of *SOCS1*, thus providing a potential avenue to alter immune response that may be due to excess IL6.

There is very little research on the effect of DNA methylation on the expression of TLRs, and in this area of the field there is currently a large knowledge gap. There is however a precedent for methylation having a role, with one study showing the effect of decreased methylation on TLR2 expression in cystic fibrosis (CF) patients. The study

showed that decreased methylation of the TLR2 promoter was resulting in an up regulation of TLR2, which the author believed was possible for the immune effects seen in the disease such as the enhanced proinflammatory response to bacterial challenges (Furuta et al., 2008). Other papers have shown that methylation may have an important role in the control of the immune system as a whole, with one study showcasing that DNA demethylating drugs had a broad immunostimulatory role in the immune system (Li et al., 2014).

Within the field of immunology the epigenetic control of the immune system provides a novel and important target for future research. Drugs targeting the enzymes involved in maintaining the epigenome could provide future anti-inflammatory treatment options for a wide range of disorders. However the level of current knowledge regarding how epigenetic modifications are affecting the immune system is still underdeveloped. This exposes a knowledge gap for research into the potential immunomodulatory effects of inhibitors of methylation and examining whether they alter the expression of the cytokines released following TLR activation or alter the expression of the TLRs themselves.

1.13 PERSPECTIVES:

TLRs are important innate immune receptors, with their role in recognising PAMPs essential in the host defence against microorganisms. Their role as the moderators of many immune related conditions such as sepsis, autoimmune disease and rheumatoid arthritis, as well as their role in other conditions such as atherosclerosis and acute coronary syndromes, indicate the importance of researching their potential as drug targets. However, despite the importance of the TLRs we currently know little about

how they are regulated. The system of complex signal transduction cascades initiated following stimulation of even a single member of the family makes determining a cause of change in expression of either the TLRs themselves or their products a difficult task. To help better understand these processes, researchers have sought to examine situations where diseases have resulted from what have been determined to be changes in the nucleotide sequence of TLR genes.

The effect of traditional genetic changes such as single nucleotide polymorphisms on the expression or activity of certain TLRs have been shown to affect the development of certain medical conditions such as increased susceptibility to lupus and tuberculosis, and worsening of conditions of inflammation. With the emergence of evidence that non nucleotide changes in genes were resulting in altered gene expression, it was reasonable for researchers to assume that epigenetic changes may indeed affect TLRs in the same way traditional nucleotide changes have. Thus, researchers sought to examine the importance of methylation and acetylation and their related enzymes in gene regulation, unearthing in the process a new generation of cytotoxic anticancer drugs in the form of DNMT inhibitors and HDAC inhibitors. Rising to prominence due to their effective antitumor activity, research into the mechanism of action of these drugs would reveal their potential for use as modulators of the immune system. The epigenetic modifying drugs, especially the HDAC inhibitors, have been shown to have effective anti-inflammatory action in a handful of studies both *in vivo and in vitro*, mainly due to decreased transcription of inflammatory cytokines and chemokines. However these studies are few and how the drugs are exerting the effects seen are not fully understood. One study has measured how HDAC inhibitors alter TLR mRNA expression following stimulation with ligands, however the changes seen were neither unidirectional nor shown to be direct.

Thus, we are left with a large family of receptors with a major role in the immune system, whose potential to be manipulated by epigenetic modifying drugs remains relatively untapped. This thesis introduction highlights the importance of these receptors in a medical context, and how using them as drug targets could open the door to many new and effective anti-inflammatory medications. To achieve this however more needs to be understood about how epigenetic drugs affect TLRs, and how exactly this effect is happening. Determining whether the effects are direct or whether one of the many TLR response genes is instead responsible is a difficult undertaking, but the potential impact of the rewards justify the potential difficulty of the task.

1.14 HYPOTHESES

We hypothesize that epigenetic modifications alter the expression of TLRs. And we believe that inhibition of DNA methylation and histone acetylation will alter the expression of TLRs as well as the effect of TLR stimulation. Thus, with these specific hypotheses in mind, in the following section we outline our aims with regard to the studies in this thesis.

1.15 AIMS

The first aim of the studies in this thesis was to classify the response of the TLR family members to changes in methylation status. In order to accomplish this we analysed changes in mRNA expression of the TLRs in the WT and DKO HCT 116 intestinal epithelial cell lines, as well as the THP-1 monocytic cell line. These cell lines were chosen due to their importance in the immune function, with epithelial HCT 116 cells being the first barrier against infection, forming a physical wall that prevents the entry of foreign pathogens. THP-1 cells were chosen for their importance as members of the

white blood cell lineage, and as one of the first responders in many forms of noxious insult. Our initial examinations of the expression of the different TLRs led us to our particular receptor of interest, TLR3. Given the dramatic changes seen with this receptor we then wished to examine the effects of inhibition of DNA methylation and histone acetylation on the expression of this receptor. Furthermore, we wished to examine the effect of the epigenetic modifications on the inflammatory signalling and subsequent release of cytokines as a result of stimulation of the receptor. And finally we wished to identify a possible mechanism by which the changes in epigenetic state were leading to the observed changes in TLR3 expression and signalling

Chapter 2: Materials & Methods

2.1 LIST OF MATERIALS USED

Materials		
Cell Culture	**Product number**	**Supplier**
McCoys 5a Medium	M8403	Sigma Aldrich LTD
RPMI-1640	G7513	Sigma Aldrich LTD
L-glutamine	G7513	Sigma Aldrich LTD
Penicillin/Streptomycin	P0781	Sigma Aldrich LTD
Fetal bovine serum	10270-106	Gibco (life technologies)
Hanks Balance Salt solution	H9394	Sigma Aldrich LTD
Dubelcos Phosphate Buffered Saline	D8537	Sigma Aldrich LTD
Trypan Blue	T8154	Sigma Aldrich LTD
Trypsin EDTA	T4174	Sigma Aldrich LTD
Cytokines		
Recombinant Human IL6	rcyec-hil6	Invivogen, san diego, USA
Recombinant Human TNFα	rcyc-htnfa	Invivogen, san diego, USA
Recombinant Human IFNγ	rcyec-hifng	Invivogen, san diego, USA
Drug treatments		
SAHA	inh-SAHA	Invivogen, san diego, USA

TSA	met-tsa-1	Invivogen, san diego, USA
5-aza-2-deoxycytidine	A3656	Sigma Aldrich LTD
5-azacytidine	A2385	Sigma Aldrich LTD
TLR Ligands		
poly I:C HMW	tlrl-pic	Invivogen, san diego, USA
LPS	tlrl-eklps	Invivogen, san diego, USA
HKLM	tlrl-hklm	Invivogen, san diego, USA
ODN	tlrl-2006-1	Invivogen, san diego, USA
CpG DNA		Invivogen, san diego, USA
Pam3CSK4	tlrl-pm	Invivogen, san diego, USA
Western Blotting		
Cell lysis & Bradford assay		
Na2EDTA	E5134	Sigma Aldrich LTD, Wicklow, Ireland.
EGTA	E4378	Sigma Aldrich LTD, Wicklow, Ireland.
Triton	T8787	Sigma Aldrich LTD, Wicklow, Ireland.
sodium pyrophosphate	P8315	Sigma Aldrich LTD, Wicklow, Ireland.
beta-glycerophosphate	G9422	Sigma Aldrich LTD, Wicklow, Ireland.

Na3VO4	450243	Sigma Aldrich LTD, Wicklow, Ireland.
Protease inhibitor cocktail	P8340	Sigma Aldrich LTD, Wicklow, Ireland.
B-Mercaptoethanol	M6250	Sigma Aldrich LTD, Wicklow, Ireland.
NP-40	I7771	Sigma Aldrich LTD, Wicklow, Ireland.
NaF	S7920	Sigma Aldrich LTD, Wicklow, Ireland.
SDS	71736	Sigma Aldrich LTD, Wicklow, Ireland.
KH2PO4	P8416	Sigma Aldrich LTD, Wicklow, Ireland.
Bovine Serum Albumin	A9647	Sigma Aldrich LTD, Wicklow, Ireland.
Bradford reagent	71729	Sigma Aldrich LTD, Wicklow, Ireland.
Western Blotting		
30% Acrylamide	A3699	Sigma Aldrich LTD, Wicklow, Ireland.
Ammonium Persulfate	A3678	Sigma Aldrich LTD, Wicklow, Ireland.
TEMED	T9281	Sigma Aldrich LTD, Wicklow, Ireland.
Glycine	G8898	Sigma Aldrich LTD, Wicklow, Ireland.
Primary Monoclonal anti β-Actin antibody	A5441	Sigma Aldrich LTD, Wicklow, Ireland.

Tween 20	P1379	Sigma Aldrich LTD, Wicklow, Ireland.
Trizma Base	T1503	Sigma Aldrich LTD, Wicklow, Ireland.
1° Antibodies		
MAPK Family Antibody Sampler	#9926	Cell Signalling Technology, Danvers, Massachusetts, USA
Phospho-MAPK Family Antibody Sampler Kit	#9910	Cell Signalling Technology, Danvers, Massachusetts, USA
NF-κB Pathway sampler kit	#9936	Cell Signalling Technology, Danvers, Massachusetts, USA
IRF-3 (D83B9) Rabbit mAb	#4302	Cell Signalling Technology, Danvers, Massachusetts, USA
2° Antibodies		
IRDye® 800CW Goat anti-Rabbit	P/N 926-32211	LI-COR Biosciences, Nebraska, USA.
IRDye® 680LT Goat anti-Mouse IgG	P/N 926-68020	LI-COR Biosciences, Nebraska, USA.
Nitrocellulose membrane	162-0115	Bio-Rad, Germany.
qPCR		
TLR2 Primer	101225	Roche, Germany
TLR3 Primer	111008	Roche, Germany
TLR4 Primer	135752	Roche, Germany

TLR5 Primer	103674	Roche, Germany
TLR7 Primer	111012	Roche, Germany
TLR8 primer	103816	Roche, Germany
TLR9 primer	143252	Roche, Germany
IRF8 Primer	116597	Roche, Germany
IL6 Primer	144013	Roche, Germany
TNFα Primer	163295	Roche, Germany
IFNβ Primer	145386	Roche, Germany
CDKN2A Primer		Roche, Germany
DNMT1 Primer		Roche, Germany
DNMT3b primer	110289	Roche, Germany
β-Actin Primer	143636	Roche, Germany
RNA Isolation Kits	11828665001	Roche, Germany
cDNA Synthesis Kit	4368813	Applied Biosystems, Dublin, Ireland
PCR Mastermix	04707494001	Roche, Germany.
Flow Cytometry		
CD283 (TLR3)-PE, human (clone: TLR3.7**)**	130-096-887	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-IRF-8-APC, human and mouse (clone: REA516)	130-108-196	Miltenyi Biotec, Bergisch Gladbach, Germany
ELISA		
Human IL6 ELISA	#88-7066-22	Affymetrix Bioscience, Ca, USA
Human TNFα ELISA	#88-7346-22	Affymetrix Bioscience, Ca, USA
Human IFNβ ELISA	luex-hifnb	Invivogen, san diego,

		USA
Immunocytochemistry		
2° Antibodies		
Alexa Flour 546 goat anti rabbit	A11035	Life technologies, Ca, USA
Alexa Flour 488 Goat anti mouse	A11001	Life Technologies, Ca, USA
Normal Goat serum	G9023	Sigma Aldrich LTD, Wicklow, Ireland.
Plasmid transfection reagents		
pUNO *hIRF8* Plasmid		Invivogen, san diego, USA
pUNO *hTLR3* Plasmid		Invivogen, san diego, USA
Lipofectamine™ 3000 Transfection Reagent	L3000008	Thermo Fisher Scientific, MA, USA
Midi Xtra plasmid purification kit	740410.50	MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany
Gene Sequencing		
TLR3 Primers		Eurofins scientific, Luxembourg.
Taq Polymerase kit	18038042	Thermo Fisher Scientific, MA, USA
EZ DNA methylation kit	D5001	Zymo research, Ca, USA
Roche DNA isolation Kit	11814770001	Roche, Germany

2.2 CELL CULTURE

2.2.1 HCT 116 CELL CULTURE

The First cell line used throughout this project was the HCT-116 human intestinal epithelial cell line (figure 2.1). These cells were originally isolated from a primary culture of cells isolated from a human colonic carcinoma (Brattain et al., 1981). The particular cell line used in our lab was obtained from the lab of professor Burt Vogelstein (Rhee et al., 2002). According to (Rhee et al., 2002), the deletion of both DNMT1/3b results in almost complete ablation of methyltransferase activity as well as greater than 95% reductions in genomic DNA methylation (figure 2.3). According to the article, the knockout of DNMT3b involved the deletion of exons 2 to 21, however they did not clarify exactly as to what exons are removed in DNMT1. We utilised both the wild type variant of the cells as well as the DNMT1/3B double knockout variant generated by homologous recombination in the Vogelstein lab. The HCT 116 cell line was chosen due to their role in the immune system. Their role as epithelial cells results in them being the first point of contact for many pathogens and they have been recognised as important regulators of homeostasis and barrier function in the gut (Peterson and Artis, 2014).

The cells in question are an adherent cell line which grows in a monolayer in cell culture. They are cultured in McCoy's 5a media (Sigma Aldrich Ltd, Wicklow, Ireland), which was supplemented with 10% Fetal Bovine serum, 5% penicillin/streptomycin and 1% L-Glutamine (All Sigma Aldrich Ltd, Wicklow, Ireland). The cells were stored in an incubator which was maintained at 37°C with 95% air and 5% CO_2. Cells were

maintained in T75 Cell culture flasks (Sarstedt LTD, Wexford, Ireland) and were subcultured once they reached 80-90% confluency (every 2-3 days) as required. When required the cells were subcultured as follows; the existing cell media was removed and discarded into 1% virkon solution. The cells were then washed with PBS (Sigma Aldrich Ltd, Wicklow, Ireland), which was removed and discarded into the virkon. To release the cells from the surface of the flask a solution of 1% trypsin-EDTA (1 ml of 10% trypsin diluted in 9 ml of hanks balanced salt solution (Sigma Aldrich Ltd, Wicklow, Ireland) was used. The cells were then placed in the incubator for 5-8 minutes to allow cells to detach. The flasks were examined under a microscope to ensure complete cell detachment, then the trypsin was neutralised by adding an equal volume of culture media. This solution was added to sterile 15ml tubes, (Sarstedt LTD, Wexford, Ireland) which were then inserted into a tabletop centrifuge and spun at 300g for 5 minutes to pellet the cells. The supernatant was removed and the cells were resuspended in fresh cell culture media. The cells were then returned to the flasks or harvested for use in experiments. Subcultivation ratios of 1:2 and higher were used depending on the confluency of cells.

2.2.2 THP-1 CELL CULTURE

The second cell line used was the THP-1 cell line which is a human monocytic leukemia cell line isolated from a one year old human male (Tsuchiya et al., 1980)(ATCC, Va, USA)(figure 2.1). The role of monocytes, and their descendents macrophages, in the innate immune defences against invading pathogens makes them an interesting experimental platform for investigating the regulation of Toll-like receptors. THP-1 cells

exist as a suspension in cell culture media and were maintained using RPMI-1640 cell culture media (Sigma Aldrich Ltd, Wicklow, Ireland) which was supplemented with 10% Fetal Bovine serum, 5% penicillin/streptomycin and 1% L-Glutamine (All Sigma Aldrich Ltd, Wicklow, Ireland). The cells were stored in an incubator which was maintained at 37°C with 95% air and 5% CO_2.Cells were maintained in T175 Cell culture flasks (Sarstedt LTD, Wexford, Ireland) and were subcultured once they reached 80-90% confluency every 2-3 days as required. When required the cells were subcultured as follows; the cells were pipette into sterile 15ml tubes (Sarstedt LTD, Wexford, Ireland). The tubes were then inserted into a tabletop centrifuge and spun at 300 x g for 5 minutes to pellet the healthy cells. The supernatant was removed and fresh cell culture media was used to resuspend the pellet. The cells were then subcultured in ratios of 1:2 or higher depending on their concentration, or they were removed for use in experiments as needed.

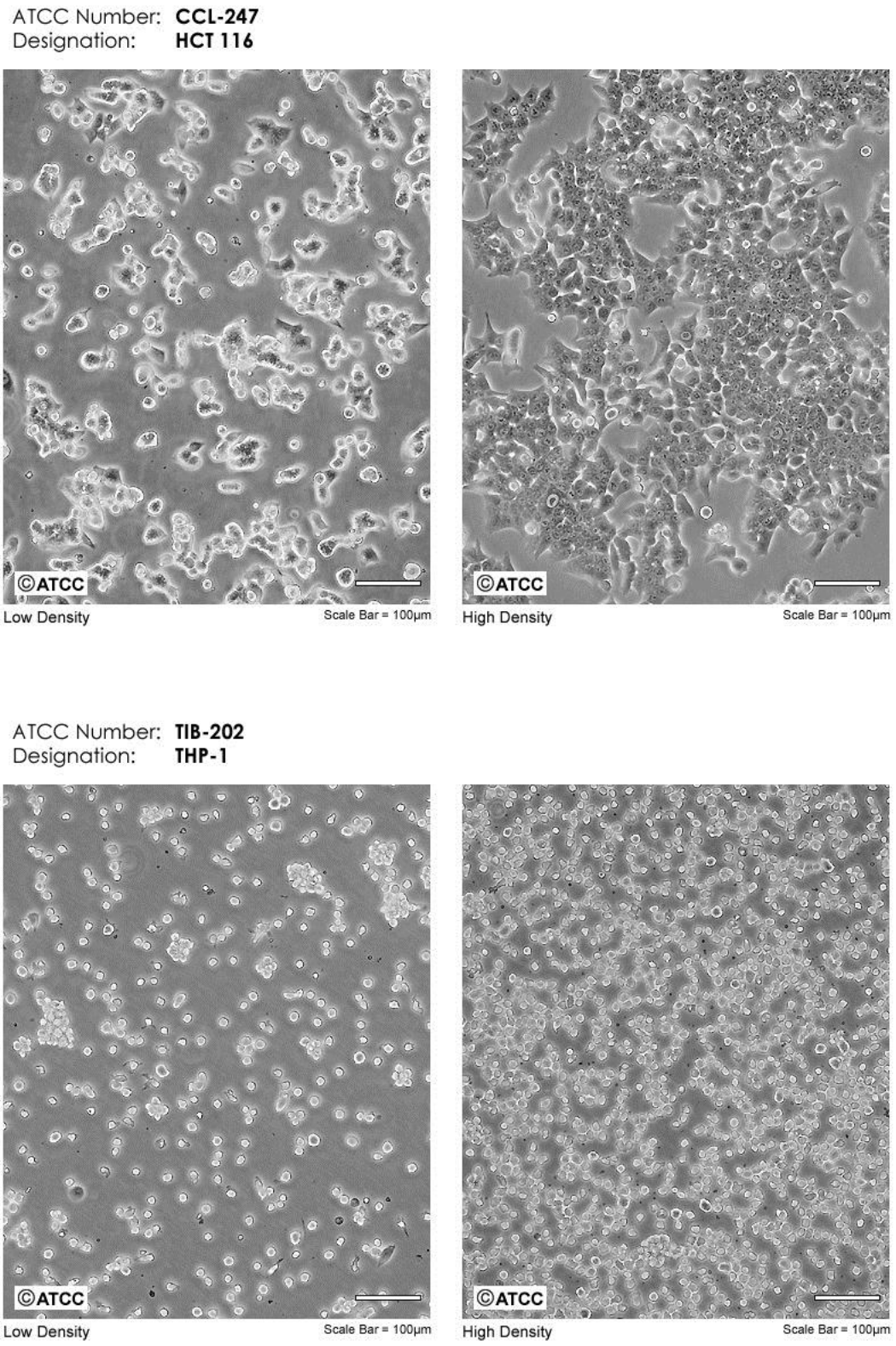


Figure 2.1. Microscopic image of HCT 116 and THP-1 cells at low and high density taken from the ATCC website.

2.2.3 DIFFERENTIATION OF THP-1 CELLS INTO MACROPHAGES

THP-1 cells are pre-monocytes which are committed to the monocytic cell lineage (Takashiba et al., 1999). In order to differentiate THP-1 cells into macrophages, we treated with phorbol 12-myristate 13-acetate (PMA) at a concentration of 10 ng/ml for 24hrs. Following 24hr treatment with PMA, differentiation can be confirmed with western blotting for RSK1, as well as identification of cell adherence to the surface of well plates (Park et al., 2007). We observed that following 24 hrs of 10 ng/ml PMA treatment we observed cell adherence (figure 2.2 B), as well as increased expression of RSK1 (figure 2.2C), as measured by Western blot. Once cells are differentiated they can be used for cell treatments as described previously.

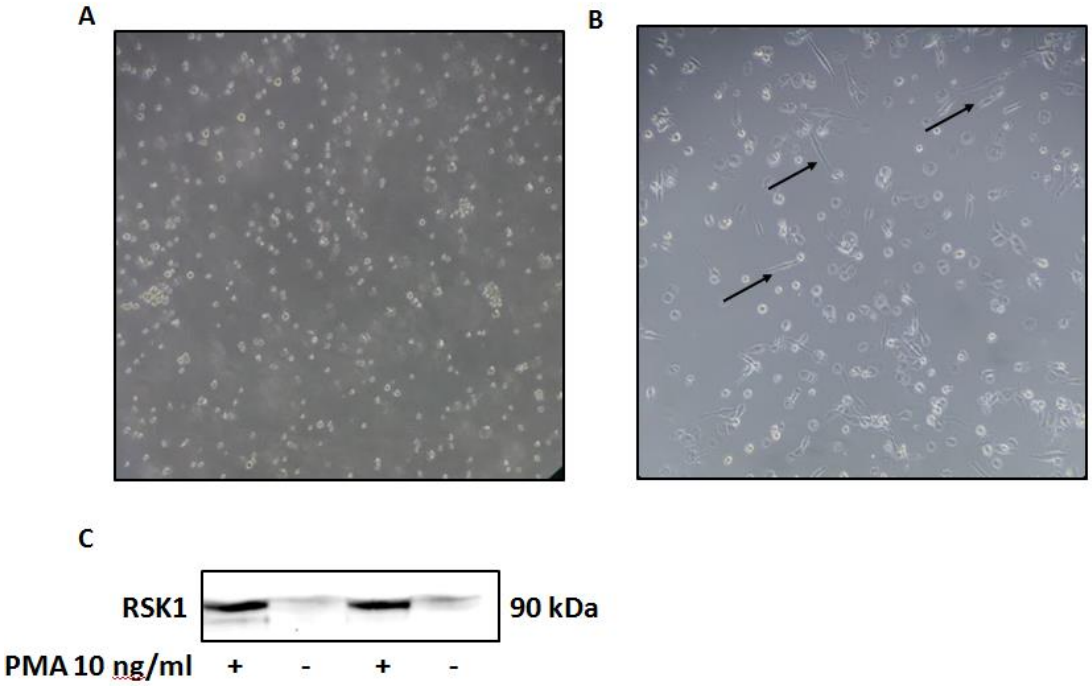


Figure 2.2 Differentiation of THP-1 cells into macrophages.

THP-1 cells were differentiated into macrophages using 10 ng/ml PMA for 24hrs. Following stimulation cells were visualised under a microscope, with THP-1 cells (**A**),

being compared to macrophages (**B**). The arrows indicated examples of cells adhering to the surface of the 6 well plate. **C)** Shows the increase in RSK1 expression, which is a marker for macrophages, and is used as a confirmation of differentiation.

2.2.4 CELL COUNTING

In order to seed cells at the required density for treatments, cells were required to be counted to ensure that in every case there was equal number of cells at the outset of each experiment. A small sample of cells was removed from a well suspended culture flask to ensure an accurate cell count. Once removed, a 20 µl sample of the cells was mixed with 20 µl of Trypan blue solution (Sigma Aldrich Ltd, Wicklow, Ireland). Trypan blue dye is a cell stain which can be used to quantify cells and determine their viability, as healthy cells will exclude the trypan blue dye from their nucleus, whereas non-viable cells will allow the dye to enter the nuclear envelope. Once the cells were mixed with the trypan blue dye, a 10 µl sample of the solution was pipette onto a haemocytometer (figure 2.4) which was then placed under a microscope (Olympus CKX31). The haemocytometer is designed such that the number of cells in one of the 4x4 quadrants multiplied by 10^4 is equal to the amount of cells per ml. To obtain an accurate count the total number of cells in 4 of the 4x4 quadrants is divided by 4 to get an average for one quadrant. This number is then multiplied by 2 to account for the trypan blue dilution factor, and then by 10^4 to give a value of cells per ml of cell culture suspension.

Figure 2.3 From (Rhee et al., 2002) showing the generation of human cells deficient in DNMT1/3b.

a, b, Targeted deletion of *DNMT3b*. Numbered boxes represent exons. Neomycin- or hygromycin-resistance genes (N/H) replace conserved DNA cytosine methyltransferase motifs (Roman numerals), including the critical prolylcysteinyl dipeptide (PC). A probe distinguishing *Nco*I fragments from wild-type (WT) and targeted (KO) alleles allowed Southern blot confirmation (b) of the indicated genotypes. c, Western blot of wild-type (WT), *DNMT3b*$^{-/-}$ (3bKO) and *DNMT3b*$^{-/-}$;*DNMT1*$^{-/-}$ (DKO) cells using antibodies described in Methods. d, DNA methyltransferase activity as a percentage of wild-type activity (± s.d.) of triplicate measurements. MT1KO$^{N/H}$ designates *DNMT1*$^{-/-}$(MT1KO) clones retaining two drug cassettes

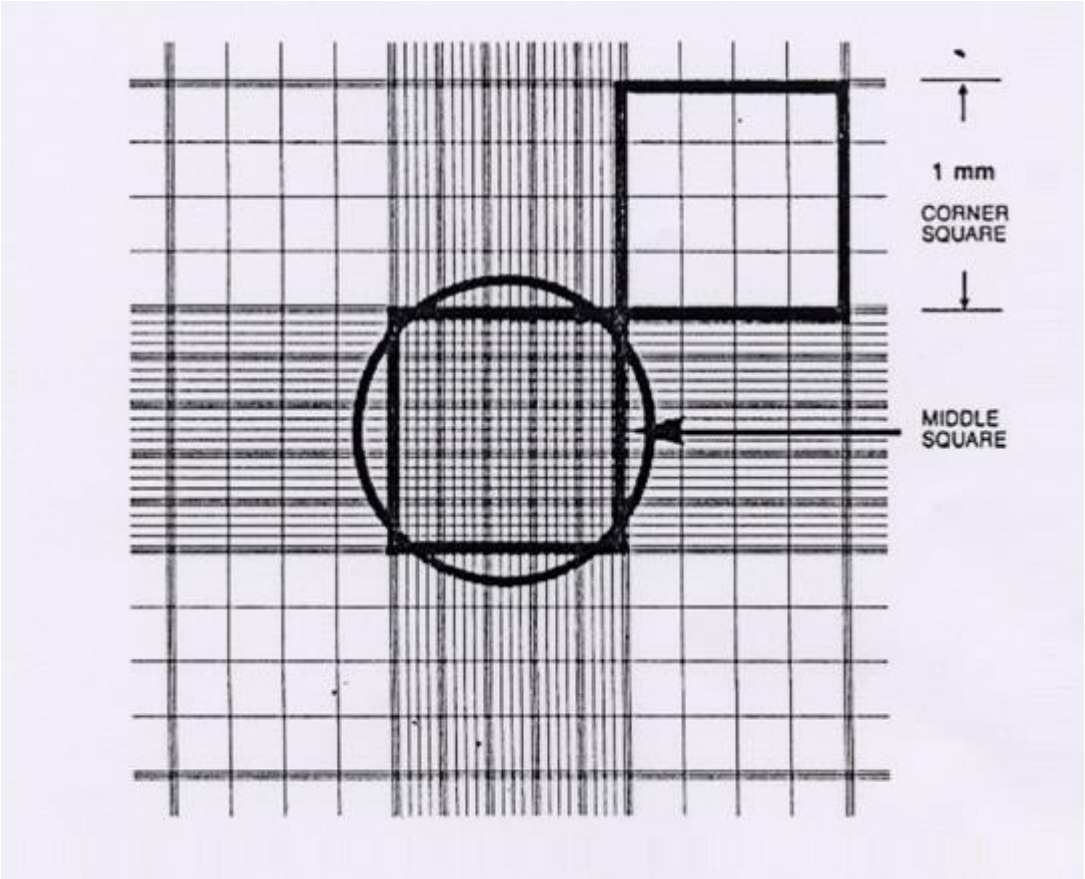


Figure 2.4: The haemocytometer as seen under a microscope.

In order to count cells accurately, a haemocytometer such as the one above was used. Cells are counted in four of the 4x4 squares (Black outline in top right corner). This count was then divided by 4 to give a count for one 4x4 square or 1 square mm, which amounts to 0.1 µl of the cell suspension. We then multiply this number x 10^4 to give us the concentration of cells in 1 ml.

2.3 CELL STIMULATIONS

For the purpose of cell stimulations both cell lines were seeded in the appropriate plates (either 6 well plates or 5 ml dishes ((Sarstedt LTD, Wexford, Ireland)) at concentrations of 250,000 cells per ml, with 2 mls being seeded per well in the 6 well plates and 5 mls in the 5 ml dishes. For the purpose of cell death and cell proliferation assays, cells were seeded in 96 well plates, with 200 µl of cells at 200,000 cells per ml being seeded into each well. Cell counts were obtained by the method described above and the cells were then harvested from their flasks and diluted to the required concentrations using the appropriate cell culture media. The cells were then seeded in the plates at the required concentrations. The HCT 116 cells were required to be left overnight to ensure they had fully adhered to the treatment dishes whereas the THP-1 cells could be treated immediately following seeding if required. Stimulations were performed using the ligands and concentrations in table 2.1. Upon completion of a study, treatments were stopped in two distinct ways, depending on the cell line used. For HCT 116 and THP-1 derived macrophages, cells were required to be lifted from the plates, given their adherent nature. The medium was removed and discarded, and 1 ml of PBS was placed in each well. The cells were then scraped with cell scrapers, following which they were transferred into 1.5 ml Eppendorf tubes. They were then removed from the cell culture hood and spun at 1500 RPM for 5 minutes to pellet the cells. The supernatant was then discarded and the cells were resuspended in 1 ml of PBS to wash them. They were again spun at 1500 RPM for 5 minutes, following which they were resuspend in RNA lysis buffer for PCR, protein lysis buffer for western blotting, or FACS buffer for flow cytometry. THP-1 cells were processed in the same manner except they did not require the initial cell scraping step due to them existing in suspension.

TLR	LIGAND	Stock concentration	Final Concentration
2	HKLM	10^{10} Cells	10^{8} Cells
3	poly I:C	1 mg/ml	10 μg/ml
4	LPS	100 μg/ml	1 μg/ml
5	Flagellin	100 μg/ml	1 μg/ml
7	CpG DNA		
9	ODN	500 μM	5 μM

Table 2.1: TLR stimulation ligands. Both cell lines were treated with the TLR ligands at the above concentrations for 6 & 24 hours for mRNA and protein analysis respectively.

2.3.1 DRUG TREATMENTS

Drug treatments were performed in 6 well plates as well as 5 ml dishes, and samples were stored for RNA and protein work. The drugs used were the HDAC inhibitors SAHA and TSA, and the methyltransferase inhibitors 5-azacytidine and 5-aza-2-deoxycytidine (All drugs from Sigma Aldrich LTD, Wicklow, Ireland). Drugs were made up according to the manufacturer's instructions and used at the following concentrations;

Drug	Stock Concentration	Final concentration	Time Point
SAHA	1 mM	10 μM	48hr
TSA	1 mM	10 μM	48hr
5-azacytidine	500 μM	5 μM	72hr
5-aza-2-deoxy-cytidine	50 μM	500 nM	72hr

Table 2.2: Drug treatments and concentrations. Both cell lines were treated with the above concentrations of drug for the timepoints shown. These treatments were then followed by subsequent ligand treatments.

2.3.2 CYTOKINE TREATMENTS

For the cytokine treatments, cells were seeded as previously described. The cells were treated with each of the cytokines individually, as well as all three together. Cytokines were used at a final concentration of 50 ng/ml. The cytokines used were human IL6, human TNFα and human IFNβ (all Invivogen, San Diego, USA).

2.4 CELL VIABILITY ASSAYS

In order to determine the extent to which each treatment undertaken in this thesis affected cell viability, we performed cell viability assays. The concentrations of each compound used, and the timepoints which they were used at, are outline in table 2.5.

MTT Solution	
MTT	5 mg
PBS	Up to 1 mL

Table 2.3. MTT solution (5 mg/ml). Solution should be filter sterilized after adding MTT.

MTT Solvent	
NP-40	50 µl
1 M HCl	200 µl
Isopropyl Alcohol	Up to 50 ml

Table 2.4 MTT solvent (4 mM HCl, 0.1% Nondet P-40 in isopropanol).

Treatments	Concentrations	Time points measured
Ligands		
poly I:C	1, 10, 100 µg/ml	24h, 48h, 72h
LPS	0.1, 1, 10 µg/ml	24h, 48h, 72h
ODN	100nm, 1, 10 µM	24h, 48h, 72h
HKLM	10^7, 10^8, 10^9 cells	24h, 48h, 72h
Pam3csk4	1, 10, 100 µg/ml	24h, 48h, 72h
Flagellin	100 ng/ml, 1, 10 µg/ml	24h, 48h, 72h
ssRNA	100 ng/ml, 1, 10 µg/ml	24h, 48h, 72h
Drugs		
5-azacytidine	1, 5, 10 ,100 µM	24h, 48h, 72h
5-aza-2-deoxycytidine	100, 500 nM, 1, 10 µM	24h, 48h, 72h
SAHA	1, 10, 100 µM	24h, 48h, 72h
TSA	1, 10, 100 µM	24h, 48h, 72h
Cytokines		
IL6	10, 50, 100 ng/ml	24h, 48h, 72h
TNFα	10, 50, 100 ng/ml	24h, 48h, 72h
IFNβ	10, 50, 100 ng/ml	24h, 48h, 72h

Table 2.5 This table outlines the concentrations and time points used for the MTT and Alamar blue assays.

2.4.1 MTT ASSAY

Cells were seeded in 96 well plates at 200,000 cells/ml. Each of the drug treatments was performed in triplicate for the concentrations and time points shown in table 2.5. Following the incubations, MTT solution (table 2.3) was added so so MTT is diluted 1:10 (22 µl MTT/ 220 µl total volume in well). The plates were then incubated for 3 hours at 37^{o}C in a cell culture incubator. After incubation for 3hrs, the medium/MTT solution mix was removed and 150 µl of the MTT solvent (table 2.4) was added to each well. The plate was then covered and placed on a shaker for 10 minutes. Absorbance was then read at 590 nm with a reference filter of 620 nm.

2.4.2 ALAMAR BLUE ASSAY

Cells were seeded as described for the MTT assay. Drug treatments were performed for the concentrations and timepoints outlined in table 2.5. At the end of each timepoint, Resazurin reagent (Sigma Aldrich Ltd, Wicklow, Ireland) was diluted 1:10 in PBS. Medium was aspirated from the 96 well plates and 100 µl of PBS diluted Resazurin was added to each well. Plates were then incubated for 3 hours at 37^{o}C in a cell culture incubator. Fluorescence was detected using a fluorescence excitation wavelength of 570 nm.

2.5 PCR

2.5.1 RNA ISOLATION

Following sample collections as outlined above, RNA isolations were performed using the Roche Total Pure RNA isolation kit (Roche, 11828665001). The cell pellets were resuspended with 200 µl of phosphate buffer saline, and following this, 400 µl of the provided Roche lysis buffer was added to the cell pellet. This was then vortexed for 15 seconds, and following the insertion of a filter column into a waste collection tube, the sample was transferred to the filter column. Once all the samples had been added to the filter columns they were inserted into a table top centrifuge and spin at 8000 x g for 15 seconds. Tubes were removed from the centrifuge and 100 µl of the DNAse mix (10 µl DNAse (10,000 U/mg) and 90 µl DNAse incubation buffer per sample) was added to each filter column. The columns were incubated at room temperature for 15 minutes according to the manufacturer's instructions. Wash buffers 1 and 2 were prepared by adding 20 ml and 40 mls of absolute molecular ethanol respectively. 500 µl of wash buffer 1 was added to each column and they were again spun for 8000 x g for 15 seconds. The tubes were then removed from the centrifuge, the waste was discarded and 500 µl of wash buffer 2 was added to the filter columns. The columns were again spun at 8000 x g for 15 seconds. Once again the tubes were removed from the centrifuge and the waste was discarded, and a final wash of 200 µl of wash buffer 2 was added. This wash was spun at 13,000 x g for 2 minutes to ensure removal of any residual buffer. Following this step, the tubes were removed from the centrifuge and the collection tubes were discarded. The Filter columns were then inserted into new 2 ml RNase/DNase free safe seal tubes (Sigma Aldrich Ltd, Wicklow, Ireland). Then, 75 µl of elution buffer was

added to the filter columns and the filter colums-2 ml tube combinations were inserted into the centrifuge and spun at 8000g for 1 minute to elute the RNA from the columns. RNA concentrations were determined using a Masetrogen nanodrop spectrophotometer (MSC, Dublin, Ireland). RNA concentrations were measured in ng/µl and Δ260/280 and Δ260/230 values were used to determine RNA purity. RNA was then stored at -80°C to be used for cDNA synthesis.

2.5.2 cDNA Synthesis

cDNA synthesis was carried out using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Dublin, Ireland). Following determination of RNA concentration as outlined previously, RNA was normalised to 250 ng per reaction. The reverse transcriptase was used at a concentration of 50 U/µl as part of a reverse transcriptase mix. Reactions were carries out in 0.5 ml RNase/DNase free tubes with the standard reaction volume being 20µl. For this volume, a reverse transcription mastermix was made as follows; 2 µl reverse transcriptase (50 U/µL), 4 µl 10x RT buffer, 4 µl of 10x random primers, 1.6 µl dNTPs (100 mM) and 8.4 µl of nuclease free water. This provided a 2x mastermix to which RNA/nuclease free water was added in quantities that resulted in a standard concentration of 250 ng per reaction. The tubes were then inserted into a thermalcycler (Fisher Scientific, Dublin, Ireland) and run at 95°C for 8 minutes, 37°C for 2 hours, and then held at 4°C for up to 20 hours. Following synthesis of cDNA it was stored at -80°C for later use.

2.5.3 QTPCR

To quantify mRNA levels the Roche LightCycler 480 was used (Roche, Manneheim, Germany). Probes were designed to anneal to specific target regions and the specific primer sequences for each probe can been seen in table 2.7. For the purpose of these studies β-actin and GAPDH were used as endogenous controls, but β-Actin was the most stable and thus was used most frequently. Ten microliter reactions were carried out in triplicate in Roche 96 well plates (Roche, Manneheim, Germany), with each plate containing endogenous and negative controls. The ten microliter PCR reaction mastermix contained 0.5 µl of probe, 3.5 µl of nuclease free water and 5 µl of 2x Roche mastermix. The 96 well plate was placed in the LightCycler 480 and the programme was set up as seen in table 2.6. Upon completion of the experimental run, curves of fluorescence vs cycle were generated and a cycle threshold (Ct) was generated to exclude background fluorescence (figure 2.5). Ct values were then generated for each sample, which were then transformed using the $2^{-\Delta ct}$ method and following this fold change was calculated (for an overview of the theory behind the $2^{-\Delta ct}$ method, see (Livak and Schmittgen, 2001)). The Ct values are normalised to an endogenous housekeeping gene β -Actin.

Process	Temperature	Time	Cycle number
Pre-Incubation	95°C	10 Minutes	1
Amplification	95°C/60°C	10 Secs/30 Secs	45
Cooling	40°C	10 Secs	1

Table 2.6. qtPCR programme setup.

Target Gene	**Assay I.D.**	**Forward Primer**	**Reverse Primer**
ACTB	143636	TCCTCCCTGGAGAAGAGCTA	CGTGGATGCCACAGGACT
IRF8	116597	GAGGTGGTCCAGGTCTTCG	CGGCCCTGGCTGTTATAG
TLR1	111000	AGGGGACAATCCATTCCAA	TTGGTCTATATTTTTGACAAATTCTCC
TLR2	101225	TGTCATTCTTTCTTCCTGCTAAGA	CTAGGTAGGACAGAGAATGCCTTT
TLR3	111008	GCTGGAAAATCTCCAAGAGC	GTGAAAACACCCTGGAGAAAA
TLR4	135752	CAAGATGCCCCTTCCATTT	TCCTTAGGAATTAGCCACTAGACTTT
TLR5	103674	GACACAATCTCGGCTGACTG	TGTCAGGAACATGAACATCAATC
TLR7	111012	CCAGTGTCTAAAGAACCTGGAAA	GGGACAGTGGTCAGTTGGTT
TLR9	143252	CGCTACTGGTGCTATCCAGA	AGCCCAGGGAGGAGCTAAG
IL6	144013	ACCGGGAACGAAAGAGAAG	GAAGGCAACTGGACCGAAG
TNF	103295	TCCTCACCCACACCATCAG	GATGGCAGAGAGGAGGTTGA
IFNB1	145386	CGACACTGTTCGTGTTGTCA	GAAGCACAACAGGAGAGCAA

Table 2.7. Roche PCR Primer Sequences and Assay I.D.

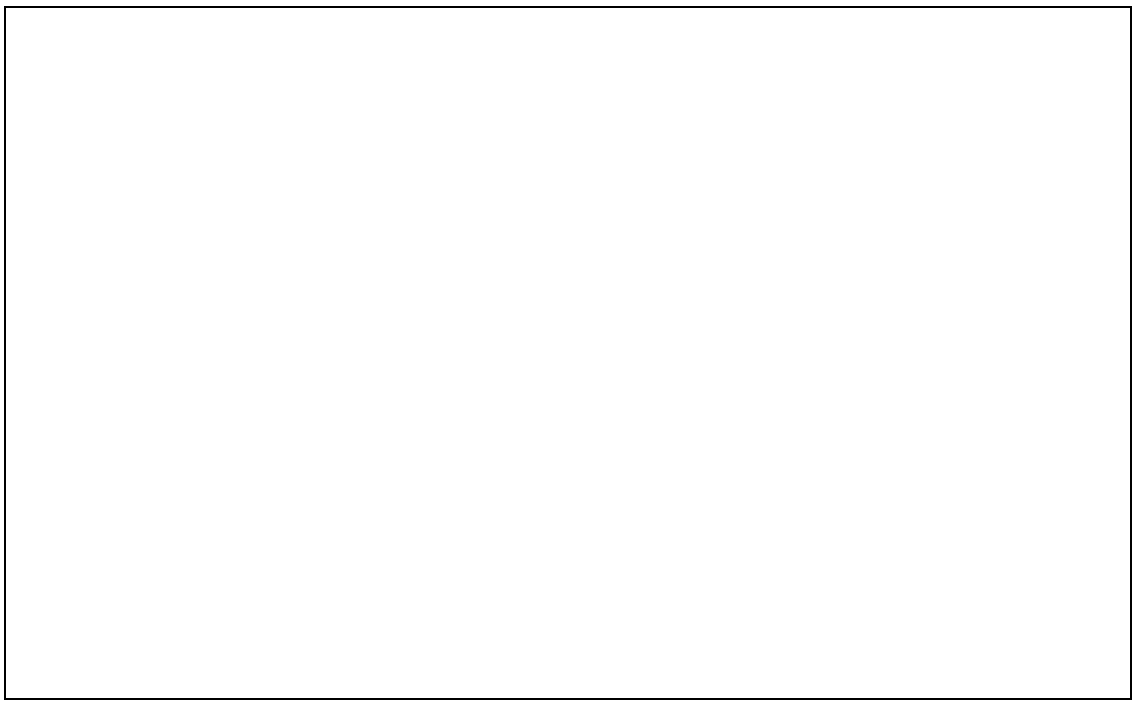


Figure 2.5 Threshold subtracted fluorescence versus number of PCR Cycles.

PCR amplification occurs in three stages. The first stage is defined as the initiation phase, and it occurs during the first PCR cycles where the emitted fluorescence cannot be distinguished from the baseline. During the exponential or log phase there is an exponential increase in fluorescence, before the plateau phase is reached. In this last phase, the reagents are exhausted, and no increase in fluorescence is observed. Only in the exponential phase, quantification is possible (Talkington, 2013).

2.6 WESTERN BLOTTING

The technique of western blotting was utilised in order to determine changes in the expression of certain protein following various experimental conditions. The process involved cell lysis, determination of protein concentration, SDS-PAGE gel electrophoresis, electrotransfer and antibody specific protein detection. The process involved in each step will be outline below.

2.6.1 CELL LYSIS

Cells were removed from 5 ml dishes or 6 well plates and placed in 1.5 ml Eppendorfs, which were then spun in a tabletop centrifuge at 300 x g for 5 minutes. The supernatant was discarded and 25-100 µl of lysis buffer was added to each pellet. The lysis buffer consisted of the following components 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4 (All Sigma Aldrich Ltd, Wicklow, Ireland). This provided a 10x stock of lysis buffer that was then diluted with PBS to give a 1x stock solution, which was aliquoted in volumes of 990 µl. 10µl of protease inhibitor cocktail (Sigma Aldrich Ltd, Wexford, Ireland) was added to each aliquot immediately prior to use. The Pellets were then vortexed for 15 seconds before being placed on ice. They were then vortexed every 10 minutes for 30 minutes total. Following this step the samples were sonicated with a Branson sonifier 150, which was set to 1 on the output rating. The samples were sonicated for 5 seconds 3 times each, and placed on ice in between each round of sonication to ensure the samples did not overheat. Upon completion of the sonication step the samples were spun at 13000 x g for 15 minutes to

pellet the cell debris. The supernatant was removed and added to fresh 1.5 ml Eppendorfs and stored for quantification and later use.

2.6.2 BRADFORD ASSAY

To determine the concentration of protein in each sample generated for use in western blotting we used the Bradford assay. Standard curves were generated using an 8 point serial dilutions curve using a stock of 2 mg/ml bovine serum albumin (BSA, Sigma Aldrich, LTD. Wexford, Ireland). The standards were added in triplicate and samples were diluted 1:5 in Eppendorf tubes before being added to the 96 well plate. 250 µl of Bradford reagent was added to each well and the assay was left at room temperature for 45 minutes. Following the incubation period the plate was read at 595 nm using a spectrophotometer and a standard curve was generated using graph pad prism. The unknown sample concentrations were then interpolated from the standard curve using linear regression.

2.6.3 SDS PAGE

Following determination of the protein concentration of each sample by the Bradford assay, the samples were standardised to concentrations of 25 ng/µl by diluting as necessary in distilled water. 4x sample buffer (5% Tris-HCL, pH 6.8, 8% glycerol. 10% SDS, 5% betamercaptoethanol, dH20, Bromophenol Blue) was added to each sample, following which the samples were boiled in a heating block at 95°C for 8 minutes. Samples were then ready to be loaded onto polyacrylamide gels which consisted of a 5% stacking gel (30% acrylamide, 1.5 M Tris-HCL pH 6.8, 10% SDS, 10% APS,

Tetraethylenemethylenediamene) and 10% resolving gels (30% acrylamide, 1M Tris-HCL pH 8.8, 10% SDS, 10% APS, Tetraethylenemethylenediamene). When preparing gels the resolving gel was poured into the gel casts first and a thin layer of 2-propanol (fisher scientific, Dublin, Ireland) was added to ensure the gel set evenly. Once the resolving gel had set, the stacking gel was poured on top of the resolving gel and the gel comb was inserted to form the structure of the wells. The gels were then allowed to set for a minimum of 30 minutes or more, after which time they were inserted into an electrophoresis tank (BIO-RAD, Dublin, Ireland). 1x running buffer (1:5 dilution of 5x running buffer (0.12M Tris (15 g) 1.44M Glycine (108 g) 0.5% of 20% SDS (25 mls), dissolve to make a final volume of 1 litre in dH_2O)) was then added to the tank and the gel comb was removed to allow the samples to be loaded into the gel wells. 20 µl of sample was added to each well and 5µl of the Li-cor chameleon duo molecular weight marker was added to each gel prior to running. The gels were then run at 80 V until the protein had passed the stacking gel and then at 120 V for the remainder of the run time.

2.6.4 ELECTROPHORETIC TRANSFER

Upon completion of the gel electrophoresis the gels were removed from the tank. The transfer apparatus was then prepared for the electrophoretic transfer. 1x transfer buffer was prepared by diluting the 10x transfer buffer (Tris (30 g) Glycine (144 g). Dissolve to make a final volume of 1 litre in dH2O) as follows; 150 mls of 10x transfer buffer, 300 mls of methanol and 1050 mls of dH20. This 1x transfer buffer was then placed on ice to cool it. Following preparation of the transfer buffer, filter paper and nitrocellulose membrane (Both Thermo fisher, Dublin, Ireland) were submerged in the transfer buffer

and left for 10 minutes. This was done to ensure the membrane was thoroughly soaked in transfer buffer before it came in contact with the gel, which resulted in more consistent transfers with very few blemishes/air bubbles. The filter paper was then removed from the transfer buffer and placed on the cassettes. The gels were then separated from their plates by using a blade to pry apart the plates and transfer buffer was pipette under the gel to loosen it from the plate. The gel was then placed on the filter paper, following which the membrane was placed on top of the gel, and a small roller was used to remove any air bubbles, which ensured a complete transfer. The gel/cassette assembly was then placed inside the transfer tank which was then placed inside a Styrofoam box, surrounded by ice packs. The ice packs help to keep the temperature low during transfer, which was determined to produce a more optimal transfer and eliminated hotspots in the transfer buffer, which can lead to incomplete or dysfunctional transfer in certain cases. The transfer was run at 70 V for 105 minutes with the current set to the maximum value the powerpack allowed (400 mA). Upon completion of the transfer, the cassettes were removed from the transfer tanks and the membrane was covered in Ponceau stain to ensure successful transfer. The Ponceau stain was then washed off with 3x TBS washes and prepared for the blocking step.

2.6.5 ANTIBODY DETECTION

To prevent non-specific antibody binding the membrane was blocked with 5% BSA in TBST (10x TBS 1:10 (50 mM Tris (6.05 g), 150 mM NaCl (8.75 g), dissolved in dH_2O, pH adjusted to 7.6. Make a final volume of 1 litre in dH_2O) for 1 hour at room temperature with 0.1% Tween 20 (Sigma Aldrich Ltd, Wexford, Ireland) added).

Following the hour long blocking, the solution was discarded and 5 ml of the antibody of choice, made up in 5% BSA in 0.1% TBST, was added to the membrane and left overnight at 4°C. Removal of the primary antibody was achieved by washing the membranes with 0.1% TBST for 3 x 5 minute washes, following which the secondary antibodies were added for 1hr at room temperature. The secondary antibodies were diluted in 1% BSA in 0.1% TBST and were required to be kept in the dark due to the fluorescent nature of the antibodies. Secondary antibodies were removed by washing with 0.1% TBST for 3 x 5 minutes as outlined above, after which the membranes were ready to be imaged.

The following primary antibodies were used, p44/42 MAPK (Erk1/2), SAPK/JNK Antibody 9252, p38 MAPK (D13E1) XP® Rabbit mAb 8690 (All from MAPK Family Antibody Sampler Kit #9926), Phospho-p38 MAPK (Thr180/Tyr182) (D3F9) XP® Rabbit mAb 4511, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb 4370, Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb 4668, NF-κB p65 (D14E12) XP® Rabbit mAb 8242, Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb 3033, IκBα (L35A5) Mouse mAb (Amino-terminal Antigen) 4814, Phospho-IκBα (Ser32) (14D4) Rabbit mAb 2859. Primary antibody binding was detected using IRDye® 800CW Goat anti-Rabbit IgG (H + L), 0.5 mg [P/N 926-32211] and IRDye® 680LT Goat anti-Mouse IgG (H + L), 0.5 mg [P/N 926-68020] where appropriate. All primary antibodies used were obtained from Cell Signalling (Danvers, Massachusetts, United States) and used at 1:1000 unless otherwise stated, and all secondary's were obtained from Licor (Lincoln, Nebraska, United States) and used at the recommended 1:10000. The imaging instrument used was the Licor odyssey (Lincoln, Nebraska, USA). Blots were placed on the scanner and the fluorescent

secondaries were excited at different wavelengths, with protein bands appearing in either the red (800 nm) or Green (680 nm) wavelength channels. Once the images were generated, these could then be analysed using the Image Studio (Licor, NA, USA) software, which allowed quantification of protein bands using densitometry.

2.7 INTRACELLULAR FLOW CYTOMETRY

Flow Cytometry was performed using the BD FACSCanto II Multicolour flow cytometer (BD Biosciences, Wisconsin, USA). Cells were removed from plates and counted to determine cell concentration. Cells were then added to V-bottom plates (Sarstedt LTD, Wexford, Ireland), with 100,000 cells being added to each well of the plate. The plate was then spun in a tabletop centrifuge at 400 x g for 5 minutes to pellet the cells in the V-bottom. The supernatant was then discarded and cells were fixed in 100 µl 2% PFA at 4°C for 10 minutes. Cells were then washed with FACs buffer (PBS, 0.5% BSA or 5% FBS, 0.1% NaN3 sodium azide) and spun again at 400 x g. The supernatant was then discarded and 50 µl of an antibody-saponin solution was added to each sample. For HCT 116 cells, 10 µl of Anti-IRF-8-APC (130-108-196), human and mouse (clone: REA516) and CD283 (TLR3)-PE, human (clone: TLR3.7, 130-096-887) (both Miltenyi Biotec, Bergisch Gladbach, Germany) was diluted in 40 µl of saponin (Sigma Aldrich, Wicklow, Ireland). For THP-1 cells, 5 µl of each antibody was diluted in 45 µl of saponin. Cells were incubated in the saponin-antibody solution for 10 minutes at 4°C.

Following staining, cells were washed with FACS buffer and spun at 400g 3 times before being resuspended in 150µl of FACs buffer and being transferred to flow

cytometry tubes (Sarstedt LTD, Wexford, Ireland). Samples were then run on the BD FAC FACSCanto II Multicolour flow cytometer and the outputs were analysed using FlowJo Flow cytometry software (FlowJo LLC, Ashland, Oregon, USA). Cells were shown on graphs of forward scatter area (FSC-A) vs side scatter area (SSC-A) (fig 2.6A, 2.7A, 2.8A). Cells were the gated to ensure only a homogenous cell population was analysed. Following this cells were graphed as forward scatter height (FSC-H) vs forward scatter area (FSC-A), which allowed cells to be gated for single cell populations. Then cells were shown as histograms of either PE/APC dye fluorescence intensity which was a measure of protein expression.

2.7.1 GATING OF CELLS FOR FLOW CYTOMETRY.

In order to interpret flow cytometry accurately, proper gating strategies are required to be used by the analyser of the data. To this end, in figures 2.6, 2.7 and 2.8, I have outlined the gating strategy which was used for each of the cell populations, giving a clear representation of how each cell line was analysed. In fig (2.6, 2.7, 2.8) A we can see the initial plot obtained from the cytometer, cells are presented based on their values for Forward scatter (FSC) and Sidescatter (SSC). With the correct voltage settings our cells of interest should appear as a defined population, with enough space to identify doublets and dead cells/debris. Cells were then gated as is shown in (fig (2.6, 2.7, 2.8) A) to ensure that only single cells are being considered for analysis, excluding any cells that may be stuck together or cells which are non viable, both of which can adversely affect fluorescence values. Following this in fig (2.6, 2.7, 2.8) B we have our second gate, where cells are represented in a plot of FSC height vs FSC area. This representation allows us to gate out any doublets which escaped our primary gating procedure, leaving us with a population of true single cells. This then allows us to generate histograms such as those in fig (2.6, 2.7, 2.8) C, which show the fluorescence detected in the channel for our dye of interest.

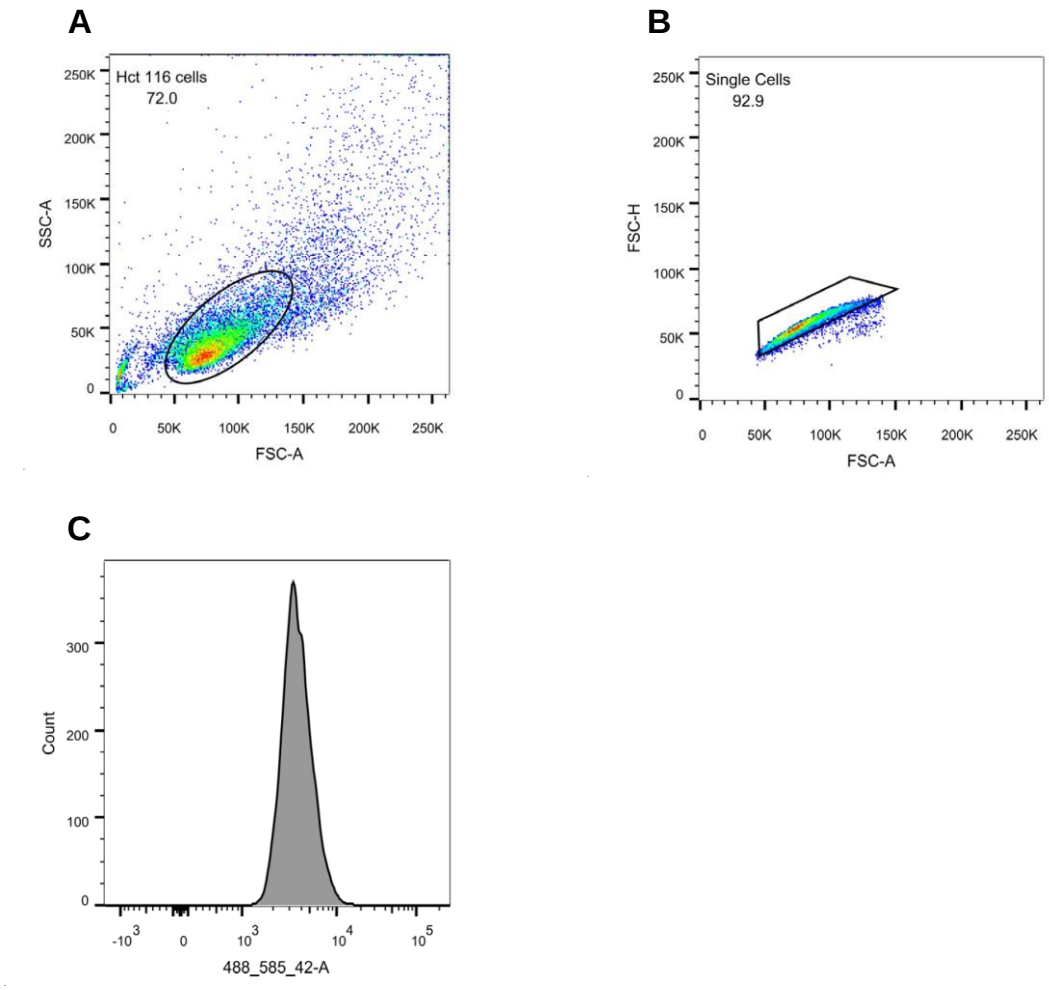


Figure 2.6 Flow cytometry gating strategies for HCT 116 cells.

A) HCT 116 cells are gated to exclude the cell debris as well as doublets **B)** Cells are then further gated to ensure only single cells are represented. **C)** Fluorescence intensity of the stain used is represented as a histogram. Data was collected using the BD FACS Canto II flow cytometer, and was analysed in FlowJo software.

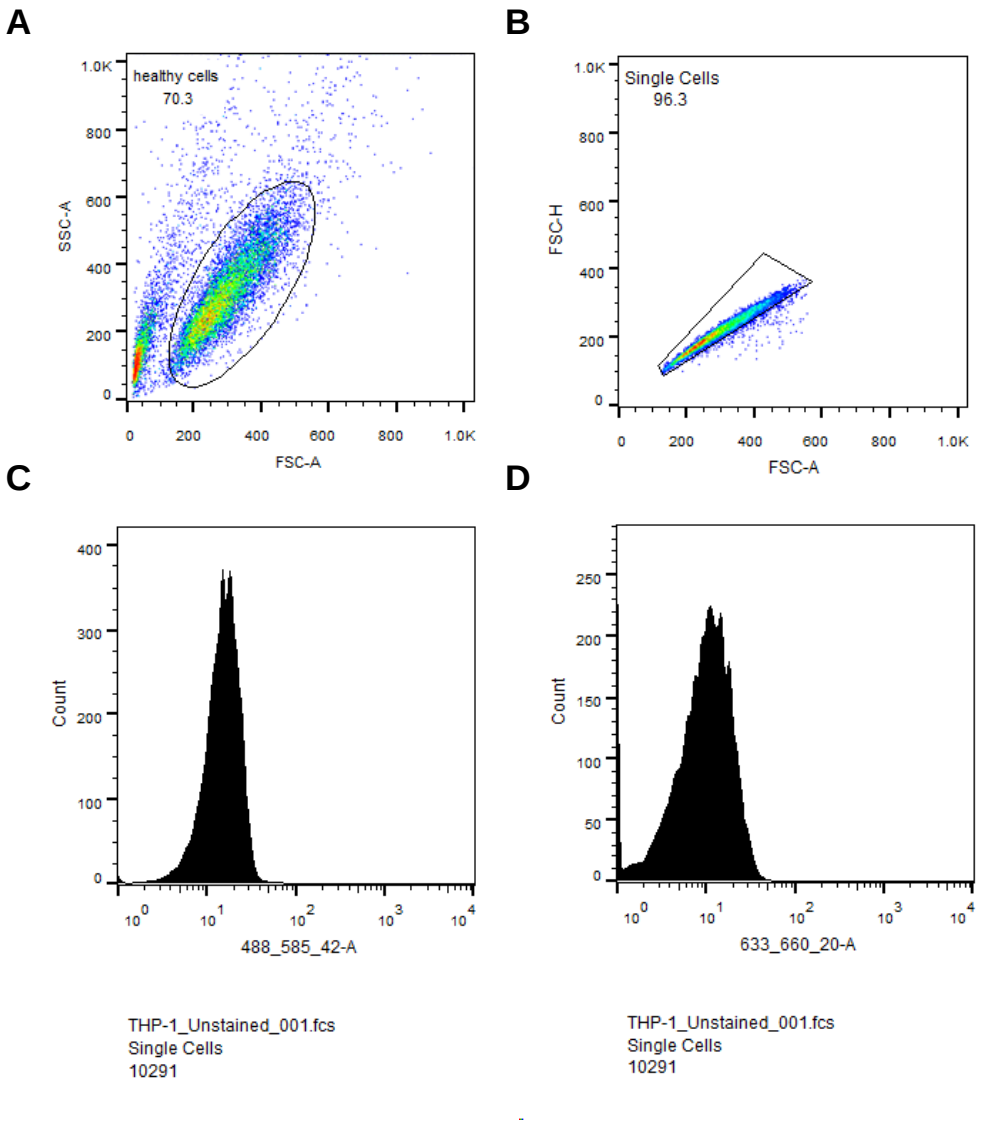


Figure 2.7. Flow cytometry gating strategies for THP-1 cells.

A) THP-1 cells are gated to exclude the cell debris as well as doublets **B)** Cells are then further gated to ensure only single cells are represented. **C)** Fluorescence intensity of the stain used is represented as a histogram. Data was collected using the BD FACS Canto II flow cytometer, and was analysed in FlowJo software.

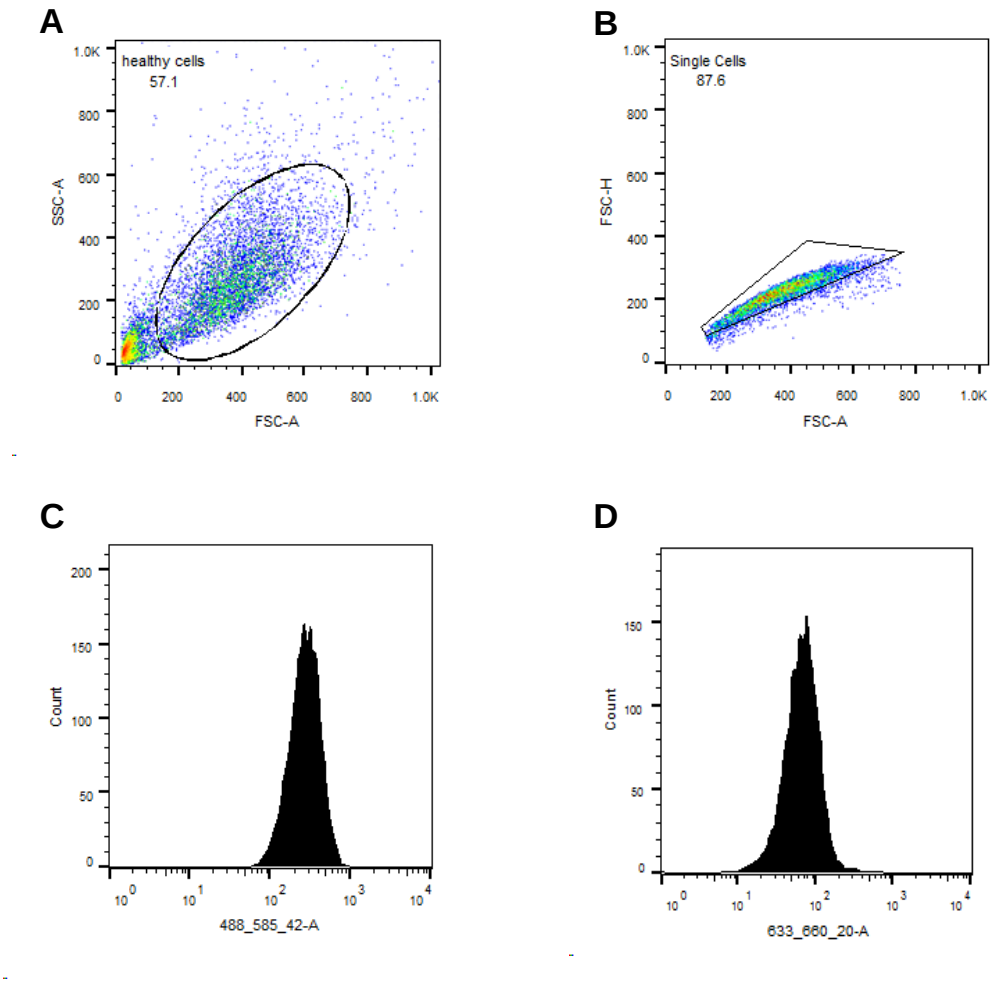


Figure 2.8. Flow cytometry gating strategies for macrophages

A) THP-1 cells are gated to exclude the cell debris as well as doublets **B)** Cells are then further gated to ensure only single cells are represented. **C)** Fluorescence intensity of the stain used is represented as a histogram. Data was collected using the BD FACS Canto II flow cytometer, and was analysed in FlowJo software.

2.7.2 TLR3 FLOW CYTOMETRY ANTIBODY TITRATION

A titration of the TLR3 flow cytometry antibody was performed to ensure an optimal concentration of antibody was utilised. The antibody is provided at a concentration of 30 µg/ml (30 ng/ µl). In figure 2.9A we observe the different volumes of antibody added to each sample. We used 1.25 µl, 2.5 µl, 5 µl and 10 µl as out test concentrations. Adequate positive staining was not achieved with any volume other than 10 µl, which resulted in 96% positive staining for TLR3. Rat IgGa isotype control was used. In each case to total volume of antibody + saponin added was 50 µl. We selected the 10 µl per sample volume of antibody (final concentration of 6 ng/µl). Figure 2.9B shows histograms of antibody staining at 3 ng/µl (5 µl volume) and 6 ng/µl (10 µl volume). As was seen in 2.9A, the 10 µl volume gives adequate separation, with the 5 µl volume proving insufficient.

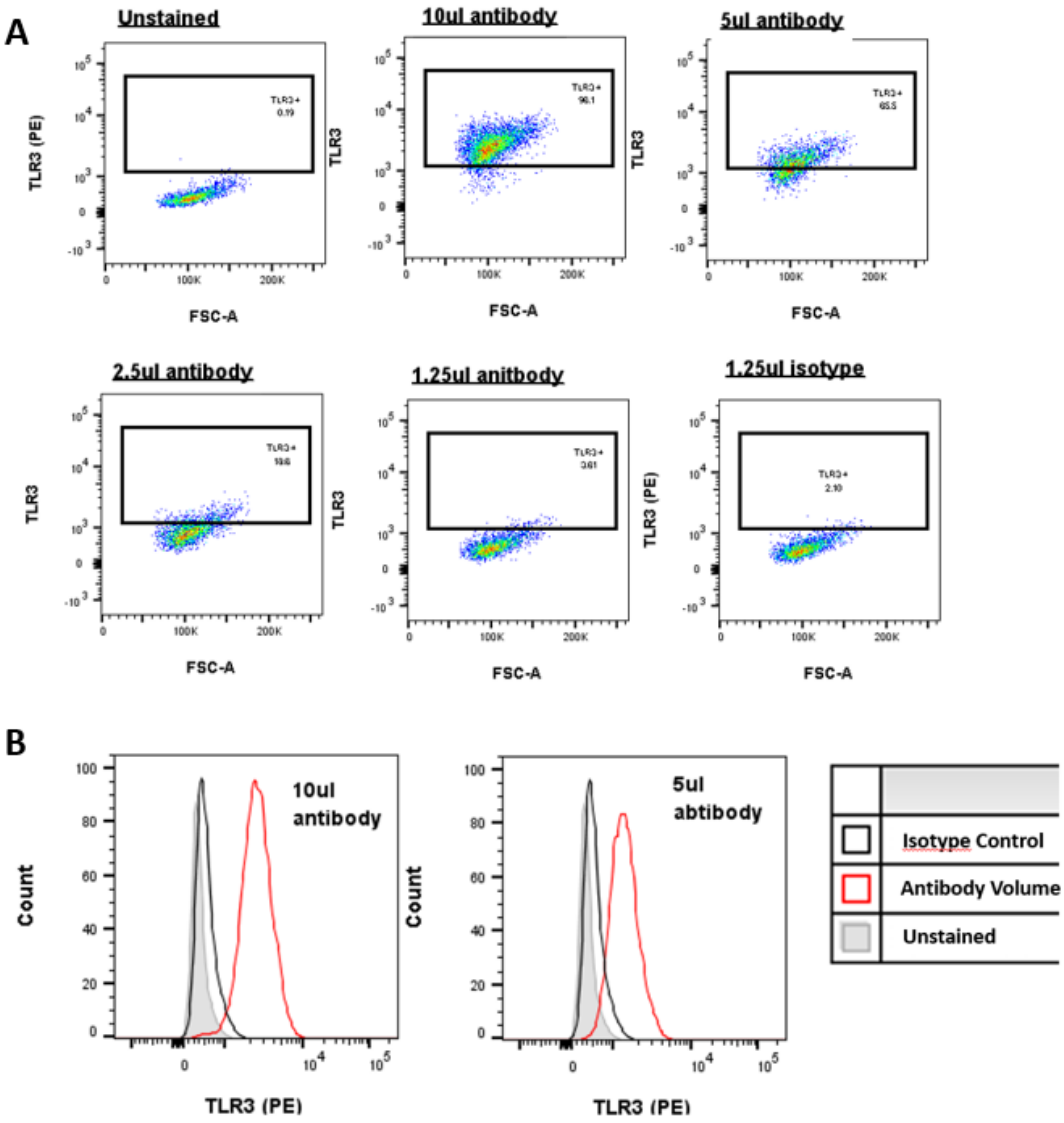


Figure 2.9 TLR3 Antibody titration.

A) Cells were stained with different volumes of antibody ranging from 1.25 µl to 10 µl. Antibody concentration was 30 ng/ µl. The isotype was rat IgGa. **B)** Histogram of the antibody at 5 µl and 10 µl per sample shown against the unstained control and the isotype control.

2.8 ELISA

2.8.1 EBIOSCIENCE READY SET GO ELISAS

To measure the release of extracellular cytokines form cells ELISA Ready Set Go! Human Elisa kits were used (eBioscience INC, San Diego, California). To prepare for the ELISA A wash buffer (1x PBS, 0.05% Tween 20) was prepared as well as a stop solution (1M H_3PO_4). NUNC Maxisorp plates (Nunc, Denmark) were coated overnight with 100µl/well of capture antibody (48 µl of antibody added to 12 ml of the provided 1x coating buffer). The plate was then covered with a sealing foil and left overnight at 4°C. The next day the wells were aspirated and washed 3x with 250 µl per well of washing buffer. 10 ml of ELISA/ELISAPOT diluent was added to 40 ml of deionised water. Wells were then blocked for 1 hour at room temperature with 250 µl/well of this blocking solution.

The lyophilised standards were then prepared by adding 100 µl of the provided standard to a volume of ELISA/ELISAPOT diluents to dilute the standard from the 15 ng/ml provided to the necessary top standard (200 pg/ml for TNF & IL6). A serial dilution of the standards was then performed and 100 µl of each standard was added per well. 100 µl of sample was added to each well as required and the plate was then sealed and incubated at room temperature for 2 hours. Following this the plate was washed 3 times and 100 µl of detection antibody was added to each well. The plate was resealed and incubated at room temperature for 1 hour. The plate again underwent 3 washes, following which 100 µl of the avidin/HRP solution was added and incubated for 30 minutes. Plates were again aspirated and washed, but this time for 5-7 washes with the wash buffer being left to soak for 2 minutes for each wash. 100 µl/well of TMB solution

was then added and the plate was incubated for 30 minutes at room temperature. 50 µl of the stop solution was then added to halt the reaction and the plate was read at 450 nm.

2.8.2 LUMIKINE™ XPRESS HUMAN IFNB ELISA KIT

The IFNβ ELISA kit used was different to that used for the other cytokines. The detection antibody is fused transcriptionally to Lucia, a secreted luciferase. Levels of IFNβ are determined by measuring the luminescence instantaneously produced by Lucia luciferase after hydrolysis of its substrate. The procedure for this ELISA is as follows; 100 ml of Capture Antibody (diluted to 1 mg/ml in Coating Buffer) was added to each well of a white flat-bottom MaxiSorp® 96-well plate. The plate was covered with an adhesive seal and incubated overnight at room temperature. We removed excess capture antibody by flicking the plate over a sink and patting it against clean paper towels to remove any remaining drops. We then added 200 ml of blocking buffer (PBS containing 2% BSA and 0.05% Tween 20, 0.2 µM filtered) to each well and incubate for 2 hours at 37 °C. We removed blocking buffer by flicking the plate over a sink and patting it against clean paper towels. The plate is now ready for sample addition. We then added 100 ml of sample or standard diluted in reagent diluent (PBS containing 1% BSA and 0.05% Tween 20, 0.2 µM filtered), per well. The plate was then covered with an adhesive seal and incubated for 2 hours at 37 °C. We then removed the liquid by flicking the plate over a sink, and each well was washed with 200 ml of wash buffer (PBS containing 0.05% Tween 20) x 3. Repeat the washing process twice for a total of three washes. After the last wash, we removed any remaining wash buffer by patting the plate against clean paper towels. We then added 100 ml of streptavidin-lucia conjugated

antibody (diluted to 10 ng/ml in reagent diluent). The plate was then covered and incubated for 2 hours at 37 °C. We then prepare the QUANTI-Luc™ assay solution by pouring the powdered QUANTI-Luc into a 50 ml tube with 25 mls of sterile water. We then repeated the washing process. The luminometer (Biocompare, Ca, USA) reading time was set to the minimum value (0.1-0.5 second), and we added 50 ml of QUANTI-Luc™ to each well and proceeded immediately with the measurement.

2.9 IMMUNOCYTOCHEMISTRY

For the purpose of performing Immunocytochemistry cells were either seeded in 8 well chamber slides (Ibidi, Germany), with treatments being performed as usual in the chamber slides, or by removing suspension cells from treatment plates and spinning onto slides with a cytospin (Thermofisher Scientific, MA, USA). Once cells were on slides/once medium was removed from chamber slides they followed the same protocol. Cells were first fixed by submerging in ice cold methanol for 10 minutes. Following this step the cells were washed for 5 mins with TBS (10x TBS 1:10 (50mM Tris (6.05 g), 150 mM NaCl (8.75 g), dissolved in dH_2O, pH adjusted to 7.6. Make a final volume of 1 litre in dH_2O). Following this cells were incubated in blocking solution (1 ml Normal goat serum, 60 µl Triton X-100, 20 µl of 10% Sodium Azide and 18.92 ml TBS (with 1% BSA)) for 1hr at room temperature. After completion of the blocking step, the blocking solution was removed and the cells were incubated with primary antibody (Antibody of choice at recommended dilution for IHC, 0.01% sodium azide, 1% BSA TBS) overnight at room temperature. The following day the primary antibody is removed and the cells are washed 3 x 10 minutes with TBS and the cells are then

incubated with a fluorescent secondary antibody (Goat anti-rabbit A.F. 546 (Red) at 1:1000 or Goat anti-mouse A.F. 488 (Green) at 1:1000, 1% Normal goat serum, TBS) for 3 hours at room temperature. Upon completion of the 3 hour incubation the secondary antibody was removed and the cells were washed for 3 x 5 minutes with TBS. They were then incubated with 1 µg/ml DAPI in TBS for ~5 min. Following one more set of 3 x 10 minute washes with TBS they were ready for imaging. Slides were imaged using the Optigrid structured illumination system.

2.10 PLASMID TRANSFORMATION AND TRANSFECTION

In order to perform transfection studies P-UNO plasmids for both human TLR3 and human IRF8 were purchased from Invivogen. Plasmids are provided at 20 µg of lyophilized plasmid. In order to ensure adequate plasmid for future studies the purchased plasmids were transformed using *E. coli* based transformation. The Invivogen pUNO kits also provided *E. coli* Fast-Media®, which are ready to use media for the preparation of agar plates and broth with a selection antibiotic included.

To perform the transformation we removed dH5 alpha cells from storage in -80°C and they were thawed on ice. Agar plates were removed from the fridge and let warm to room temperature. DNA (2.5 µg) was mixed with 100 µl of cells in a 1.5 ml Eppendorf. The mixture was then incubated on ice for 30 minutes. The transformation was then heat shocked by placing the tube in a heating block at 42°C for 45 seconds, following which cells were placed back on ice for 2 minutes. The mixture was then diluted with 1000 µl of SOC medium (20 g/L Tryptone, 5 g/L Yeast Extract, 4.8 g/L MgSO4, 3.603 g/L dextrose, 0.5g/L NaCl, 0.186 g/L KCl). This mixture was then

incubated at 37°C for 1hr in a shaking incubator. This step is important to allow the cells to develop the antibiotic resistance (in our case *blasticidin* resistance) encoded on the plasmid backbone. These cells were then plated on the agar plates at 37°C overnight. The following day once colonies had developed, colonies were selected for further expansion, with colonies being placed in broth for 12hr at 37°C in a shaker incubator to allow further bacterial expansion. Following the expansion, the bacterial cells were spun in a centrifuge at 15000 RPM for 15 minutes to pellet the cells, which were then ready to be used for plasmid purification

2.10.1 PLASMID PURIFICATION

Plasmids were purified from bacterial cultures using Macherey-Nagel plasmid purification kits (MACHEREY-NAGEL GmbH & Co. KG, Duren, Germany). Cell pellets from step 2.10 were resuspended with 8mls of resuspension buffer + RNAse A. Following this, 8mls of lysis buffer was added to the cell suspension, which was inverted 5 times to mix. Do not vortex at this step as it will shear and release contaminating chromosomal DNA from cellular debris into the suspension. We incubated the mixture at room temperature for 5 minutes. During the incubation step, we added 12 mls of equalisation buffer to the NucleoBond® Xtra Column together with the inserted column filter. Then we allowed the column to empty by gravity flow. Following the 5-minute incubation with lysis buffer, we added 8 mls of neutralisation buffer to the cell suspension and mix by inverting. Then we loaded the lysate onto the column and allowed it to empty by gravity flow. Following this, we washed the NucleoBond® Xtra column filter and Nu-cleoBond® Xtra column with equilibration buffer. Next, we removed the column filter and discard it, then washed the column with 8 mls of wash

buffer. The plasmid DNA was then eluted with 5 mls of elution buffer. To precipitate the DNA, we added 3.5 mls of room temperature isopropanol. Then, we centrifuged the tube containing the DNA/isopropanol at 15,000 x g for 30 min at 4 °C. We then carefully discarded the supernatant and added 10 mls of room temperature 70% ethanol to the pellet. Then, we centrifuged again at 15,000 x g for 30 min at 4 °C. Finally, we discarded the supernatant and allowed the pellet to dry at room temperature. Following evaporation of the remaining ethanol, we resuspended the DNA in nuclease free water. DNA was then be quantified using a spectrophotometer.

2.10.2 TRANSFECTION

Cells were transfected using Lipofectamine™ 3000 Reagent (Invivogen, san diego, USA). To transfect cells, cells were seeded to be 70-90% confluent at transfection. Transfections were performed in 24 well plates with ~100,000 cells per ml. One hour before transfection, medium was removed from cells and replaced with low serum medium, which contains 5% FBS as opposed to the regular 10% FBS. The Lipofectamine™ 3000 Reagent was diluted in 25 µl of medium per well in one tube, while in another tube the plasmid DNA (1 µg per well) was combined with the p3000 reagent (2 µl per µg of DNA) and 25 µl of reduced serum medium. The two tubes were then combined and incubated at room temperature for 15 minutes. The DNA lipid complex were then added drop-wise to the cells and the plates were shaken in a figure 8 to ensure even distribution of the DNA lipid mix. We then incubated the cells for 48 hours before performing treatments or analysing cells.

2.11 METHYLATION SPECIFIC SEQUENCING

In order to determine whether there were changes in the methylation status of the TLR3 promoter region, we wished to sequence DNA which had been bisulfite converted. The process of treating DNA with bisulfite converts cytosine residues to uracil, but leaves methylated cytosine residues unconverted. Thus, bisulfite treatment of DNA introduces specific changes in the DNA sequence that depend on the methylation status of individual cytosine residues, yielding single-nucleotide resolution information about the methylation status of a segment of DNA.

2.11.1 DNA ISOLATION

In order to isolate DNA for the purpose of bisulfite conversion, we used the Roche DNA isolation kit for cells and tissues. Cells were removed from plates as previously described. Cells were then spun at 1500 RPM for 5 minutes. The supernatant was removed and 400 µl of cell lysis buffer from the kit was added to the pellets. The pellets were then vortexed for 15 seconds. 5 µl of RNAse A (50 U/mg) was then added and the Eppendorfs were incubated at 37°C for 1 hour. Following this the cells were incubated with 10 µl of proteinase k (2.5 U/mg) for 2 hours at 65°C. The proteinase was then denatured by heating to 95°C for 10 minutes. Following the proteinase inactivation the samples were loaded onto nucleic acid binding columns. Columns were spun at 8000g for 15 seconds to pass sample through column, allowing nucleic acids to bind to the column. Following this step, 500 µl of wash buffer I was added to the column, and the column + collection tube was spun at 8000g for 15 seconds. This wash step was repeated with wash buffer 2. A final was step was then performed with wash buffer 2,

and the column and collection tubes was spun at 130000 x g for 2 minutes to remove any residual wash buffer. The column was then inserted into a new nuclease free tube, and 20-100 µl of elution buffer was added to the column, which was then spun at 8000 x g for 1 minutes. Following elution the DNA was quantified using a nanodrop spectrophotometer.

2.11.2 BISULFITE CONVERSION

In order to bisulfite convert the DNA we used the Zymo research EZ DNA methylation lightning kit (Zymo research, Ca, USA). Following isolation of DNA and its subsequent quantification, DNA was bisulfite converted in order to measure changes in methylation status of cytosines. DNA was diluted in 0.2 ml PCR tubes such that each tube contained 500 ng of DNA. One hundred and thirty µl of lightning conversion reagent was then added to each tube, with each tube being centrifuged to ensure no drops were on the sides or cap of the tube. The tubes were then placed in a thermal cycler and the following steps were performed.

Process	Temperature	Time
Pre-Incubation	98°C	8 Minutes
Amplification	54°C	60 minutes
storage	4°C	Up to 40 hours

Table 2.3. Bisulfite conversion PCR programme setup.

Following the PCR step the tubes were removed from the thermal cycler. Six hundred µl of M-binding buffer was added to a Zymo Spin column, which was then placed in a collection tube. The samples in the PCR tubes were then loaded into the column containing the binding buffer. The cap was closed and the tube was inverted several times to mix. The tubes were then centrifuged at full speed (> 10,000 g) for 30 seconds. The flow through was discarded. One hundred µl of M wash buffer was added to the column which was spun at full speed for 30 seconds. 200 µl of L-desulphonation buffer was added to the column, which was then let stand at room temperature for 20 minutes. 200 µl of M wash buffer was added and the tubes were again spun at 10,000g for 30 seconds. This wash step was then repeated. The column was then placed in a 1.5 ml eppendorf tube and 10 µl of M elution buffer was added. The columns were then spun at full speed for 30 seconds to elute the DNA.

2.11.3 PRIMER DESIGN FOR BISULFITE SEQUENCING

Designing primers for bisulfite converted DNA is a difficult process due to the nature of the conversion. Following bisulfite treatment the DNA will be significantly fragmented, as the strands are no longer complementary, and almost completely devoid of cytosine. There are a few criteria suggested for primers for bisulfite PCR; bisulfite PCR primers need to be long (usually between 26-30 bases), the amplicon size should be relatively short (between 150-300 bp), the primers should not contain CpG sites and finally its important to note hot start polymerases are strongly recommended as non-specific amplification is relatively common with bisulfite-converted DNA due to it being AT-rich. In light of these recommendations we designed the following PCR primers.

Primer set	Length (BPs)	Sequence
Set 1 Forward	30	TTAGTTGTTTTATGATTTGTGTGATTGAAG
Set 1 reverse	34	ACRTAAAATAAACACCTTCACTTACTTAAATAAC
Set 2 forward	25	ATAAGGGAGTTAGATTAGGTGGTTT
Set 2 reverse	24	CTTTCCCTTCTTTCGTCTAACTTT
Set 3 forward	25	AAATAAGGGAGTTAGATTAGGTGGT
Set 3 reverse	25	CTTTCCCTTCTTTCGTCTAACTTTT

Table 2.5 Primer sequences for bisulfite PCR.

2.11.4 PCR AMPLIFICATION OF REGION OF INTEREST

In order to amplify the regions of interest we performed PCR using a *Taq* PCR kit (New England BioLabs INC, Ma, USA). The following reaction mixture was used;

Component	25 µl reaction	Final concentration
10X Reaction Buffer	2.5 µl	1x
10 mM dNTPs	0.5 µl	200 µM
10 µM Forward Primer	0.5 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	0.2 µM
Template DNA	Variable	< 1000 ng
Taq DNA Polymerase	0.125 µl	1.25 U/50 µl PCR
Nuclease-free water	To 25 µl	

Table 2.6 Reaction mixtures for 25 µl PCR reaction

And the following cycling conditions were used;

Cycle step	Temperature	Time	Cycles
Initial denaturation	95°C	30 seconds	1
Denaturation Annealing Extension	95°C 45-68°C 68°C	15-30 seconds 15-60 seconds 1 minute per kb	30
Final extension	72°C	5 minutes	1
Hold	4°C		

Table 2.7 PCR cycling conditions for bisulfite converted DNA amplification

Following the PCR amplification, DNA samples were run on a 1% agarose gel with ethidium bromide, in order to visualize PCR products.

2.12 INTESTINAL BIOPSY SAMPLE COLLECTION AND PROCESSING

In order to further understand TLR3 expression, we wished to examine its expression in primary human cells. In collaboration with Prof Larry Egan (Pharmacology & Therapeutics) we obtained ethical approval (Ref: C.A. 491, UCHG Clinical research ethics committee.) to collect intestinal biopsies from patients attending UCHG. Samples were harvested from both healthy patients as well as patients diagnosed with IBD/Crohn's disease, by the attending medical registrar/consultant during the course of endoscopic investigations. Samples were collected from the ileum, caecum, transverse colon and rectum, with samples being transferred into 1.5 ml Eppendorfs which contained 400 µl of RNAlater (Sigma Aldrich, Wicklow, Ireland), which prevented RNA degradation. Samples were stored in the fridge in the endoscopic suite and collected each evening. RNAlater was removed from the samples and 400 µl of cellular lysis buffer from the Roche RNA isolation kit was added to each sample. Samples were homogenised for 10 seconds using the IKA T10 basic ultra turrax homogenizer (IKA, Staufen, Germany). Following homogenisation, samples were then isolated using the Roche RNA isolation kits as previously described.

2.13 STATISTICAL ANALYSIS AND DATA PROCESSING

Data was analysed in IBM SPSS statistics (version 22) engine (IBM, Dublin, Ohio, USA) and Graph Pad prism 5 (Ca, USA). Shapiro Wilke tests of normality and levene's test for homogeneity of variance were carried out and upon determination of normality & homogeneity of variance, 2 way ANOVAs and students t-tests were used where appropriate. The appropriate post-hoc test was used to determine where differences

between groups existed. Data is expressed as mean ± SEM. $P < 0.05$ was considered significant. Significance was represented as follows * = $p < 0.05$. ** = $p < 0.01$, *** = $p < 0.001$. Data was plotted using Graph Pad prism 5 software (Ca, USA).

Chapter 3: Initial Analyses of TLR expression

3.1 INTRO

Toll-like Receptors (TLRs) are a family of innate immune receptors which are part of a larger family of pattern recognition receptors (PRRs). This family of receptors recognise structurally conserved molecules known as pathogen associated molecular patterns (PAMPs) present on the surface of bacteria and viruses (Akira et al., 2006). Activation of these receptors results in the phosphorylation of cytoplasmic nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or interferon regulatory factors (IRF) 3/5/7 transcription factors, which, following activation translocate to the nucleus of the cell and induce transcription of inflammatory cytokines such as interferon-α/β, interleukin-6 and tumor necrosis factor α. (Akira et al., 2006) The receptor family is accepted as having a large role in the innate immune response to microbial challenges as well as in the pathogenesis of autoimmunity (Maximiliano Javier Jiménez-Dalmaroni et al., 2015).

The role of DNA methylation and histone acetylation in the regulation of gene expression is well established (Razin and Cedar, 1991; Verdone et al., 2005), however whether the epigenetic machinery can be manipulated to alter the expression and regulation of the TLRs is unknown. In this chapter we sought to characterise the basal expressions of the TLR family in our WT and DNMT1/3b DKO cell lines, and determine which, if any, experienced altered expression as a result of the removal of the DNA methylation enzymes. Much of this chapter will involve investigations of the drugs and compounds used throughout our studies and developing profiles of cell death

responses to treatments. For the purposes of measuring cell viability we initially set out to compare the MTT and the Alamar blue cell viability assays. Both assays have been shown to be effective at measuring cell viability (Mosmann, 1983; Rampersad, 2012), with the effective reduction of a dye, and a subsequent colour change used as a measure of mitochondrial respiration. These cell viability studies were followed by examination of the basal expression of the TLR family, which ultimately results in our selection of TLR3 as our receptor of interest based on the responses seen here.

3.2 METHODS

The methods used in this chapter did not differ in any way from those outlined in chapter 2.

3.3 EXPERIMENTAL DESIGN

The first set of results shown in this chapter consists of the Alamar Blue™ and MTT cell viability assays. These studies were performed across 3 timepoints of 24, 48 and 72 hours post treatment, with each treatment being performed in triplicate. The drug concentrations used are outline in table 3.1. The examination of basal TLR expression was performed on cell culture isolates, where WT and DKO cells were lysed, and RNA was isolated. cDNA was synthesized. Samples were then used for qPCR. For the cytokine studies in the THP-1 and HCT 116 cells, cells were plated at 250,000 cells/ml and left overnight. The following day cells were treated with the cytokines individually

or in the combinations shown, for 24hr, after which cells were isolated for qPCR and flow cytometry, as outlined in chapter 2.

Treatments	Concentrations	Timepoints measured
Ligands		
poly I:C	1, 10, 100 µg/ml	24h, 48h, 72h
LPS	0.1, 1, 10 µg/ml	24h, 48h, 72h
ODN	100 nM, 1, 10 µM	24h, 48h, 72h
HKLM	10^7, 10^8, 10^9 cells	24h, 48h, 72h
Pam3csk4	1, 10, 100 µg/ml	24h, 48h, 72h
Flagellin	100 ng/ml, 1, 10 µg/ml	24h, 48h, 72h
ssRNA	100 ng/ml, 1, 10 µg/ml	24h, 48h, 72h
Drugs		
5-azacytidine	1, 5, 10 ,100 µM	24h, 48h, 72h
5-aza-2-deoxycytidine	100, 500 nM, 1, 10 µM	24h, 48h, 72h
SAHA	1, 10, 100 µM	24h, 48h, 72h
TSA	1, 10, 100 µM	24h, 48h, 72h
Cytokines		
IL6	10, 50, 100 ng/ml	24h, 48h, 72h
TNFα	10, 50, 100 ng/ml	24h, 48h, 72h
IFNβ	10, 50, 100 ng/ml	24h, 48h, 72h

Table 3.1. Concentrations and timepoints of each treatment assessed in the cell viability assays.

3.4 RESULTS

3.4.1 CELL VIABILITY ASSAYS

3.4.1.1 Cell Viability in HCT 116 cells was measured following administration of TLR ligands.

In order to determine the optimal concentration and timepoint with which to use each TLR ligand, it was necessary to do a series of dose and time response studies to determine the cytotoxicity of each ligand. In order to determine cell viability, we used two cell viability assays, the Alamar Blue™ assay, and the MTT assay. Each assay involved three doses of each ligand at 24, 48 and 72 hours, with data being calculated as percentage cell viability relative to the untreated control. Data shown is mean % cell viability ± SEM. In Figure 3.1A and Figure 3.2A we see the effect of poly I:C on HCT 116 cells measured by both Alamar Blue™ and MTT assays. The highest dose of poly I:C (100 µg/ml) shows significant reduction of cell viability at all three time-points in both assays (Alamar Blue™ (UT 100% vs poly I:C (24hr (72% ± 5.288 $P < 0.001$, 48hr (74% ± 3.32 $P < 0.01$), 72hr (67% ± 3.84 $P < 0.001$)) (MTT (UT 100% vs poly I:C (24hr (69% ± 4.9 $P < 0.001$), 48hr (72% ± 2.5 $P < 0.001$), 72hr (79% ± 1.98 $P < 0.001$)), however the lower doses (1 µg/ml & 10 µg/ml) only show significant reductions in cell viability at 72h, again seen in both assays (Alamar Blue™ (UT 100% vs (1 µg/ml 72h (80% ± 2.2 $P < 0.05$) (10 µg/ml 72h (81% ± 7.1, $P < 0.05$)) (MTT (UT 100% vs (1 µg/ml 72h (80% ± 2.2 $P < 0.001$) (10 µg/ml 72h (81% ± 7.1, $P < 0.001$)). As shown in Figure 3.1B/Figure 3.2B, in both assays, treatment with LPS only seems to

significantly affect cell viability after 72h and with the highest does used. 10 μg/ml LPS showed a significant decrease in cell viability after 72h (Alamar Blue™ (UT (100% ± 1.7) vs LPS 10 μg/ml (82% ± 2.8, $P < 0.01$), MTT (UT (100% ± 0.5) vs LPS 10 μg/ml (80% ± 2.9), $P < 0.01$)). Figure 3.1C/Figure 3.2C shows the effect of ODN on cell viability, with significant changes seen in 5 μM group at 48h (Alamar Blue™ (79% ± 3, $P < 0.001$), MTT (82% ± 3.1, $P < 0.01$) and 72h (Alamar Blue™ (81% ± 6.5, $P < 0.01$), MTT (76% ± 3.6, $P < 0.001$). In the MTT assay significant changes were also seen in the 1 μM group at 72h (82% ± 3.1, $P < 0.01$), however this was not seen in the Alamar Blue™ assay for this dose. In Figure 3.1D and Figure 3.2D, the effect of HKLM treatment on HCT 116 cell viability is shown. The 10^9 cells dose of HKLM was seen to cause significant cell death across all timepoints in both assays (Alamar Blue™ (24h (84% ± 4.4, $P < 0.01$), 48h (87% ± 1.6 $P < 0.05$), 72h (58% ± 5.5, $P < 0.001$), MTT (24hr (80% ± 4.6, $P < 0.001$), 48h (87% ± 1.6 $P < 0.05$), 72h (58% ± 5.5, $P < 0.001$)). In the Alamar Blue™ assay, a significant reduction in cell viability was also seen with the 10^8 dose of HLKM at 48h (85% ± 2, $P < 0.05$) and 72h (65% ± 5, $P < 0.001$), however in the MTT assay the 10^8 cells dose only showed significant cell viability reduction at 72h (61% ± 5.6, $P < 0.05$). In Figure 3.1E/3.2E we have the Alamar Blue™ and MTT results for Pam3CSK4. As is shown in the graph, there appears to be no significant reduction in cell viability in either assay at the 24hr and 48hr timepoints. However, there are significant reductions seen in the Alamar Blue™ assay with all doses of the ligand at 72hrs (1 μg/ml (84 ± 1.3, $P < 0.05$), 10 μg/ml (74% ± 5.8, $P < 0.001$), 100 μg/ml (71% ± 5.3, $P < 0.001$)). However in the MTT assay, significant reduction of cell viability was only seen in the 10 μg/ml (71% ± 4.9, $P < 0.01$) and 100 μg/ml (69% ± 5.4, $P < 0.01$) doses. Flagellin induces a significant reduction in cell viability at the 10 μg/ml dose in

both assays at 72hr (Alamar Blue™ (81% ± 5.4, $P < 0.01$), MTT (78% ± 5.8, $P < 0.001$). There are also significant reductions in cell viability seen with flagellin in the 1 μg/ml treatment group at 72h in the MTT assay (89% ± 1.4, $P < 0.05$); however a similar significant effect is not observed in the Alamar Blue™ assay. The final TLR ligand examined in the HCT 116 cells was ssRNA, and from both the MTT and Alamar Blue™ assay it was determined that there was no significant effect seen with any of the doses and timepoints used. The results obtained from the MTT assays and Alamar blue assays were very similar. Given that they produced similar results and they both measure mitochondrial respiration, we then elected to use just one assay, the MTT assay.

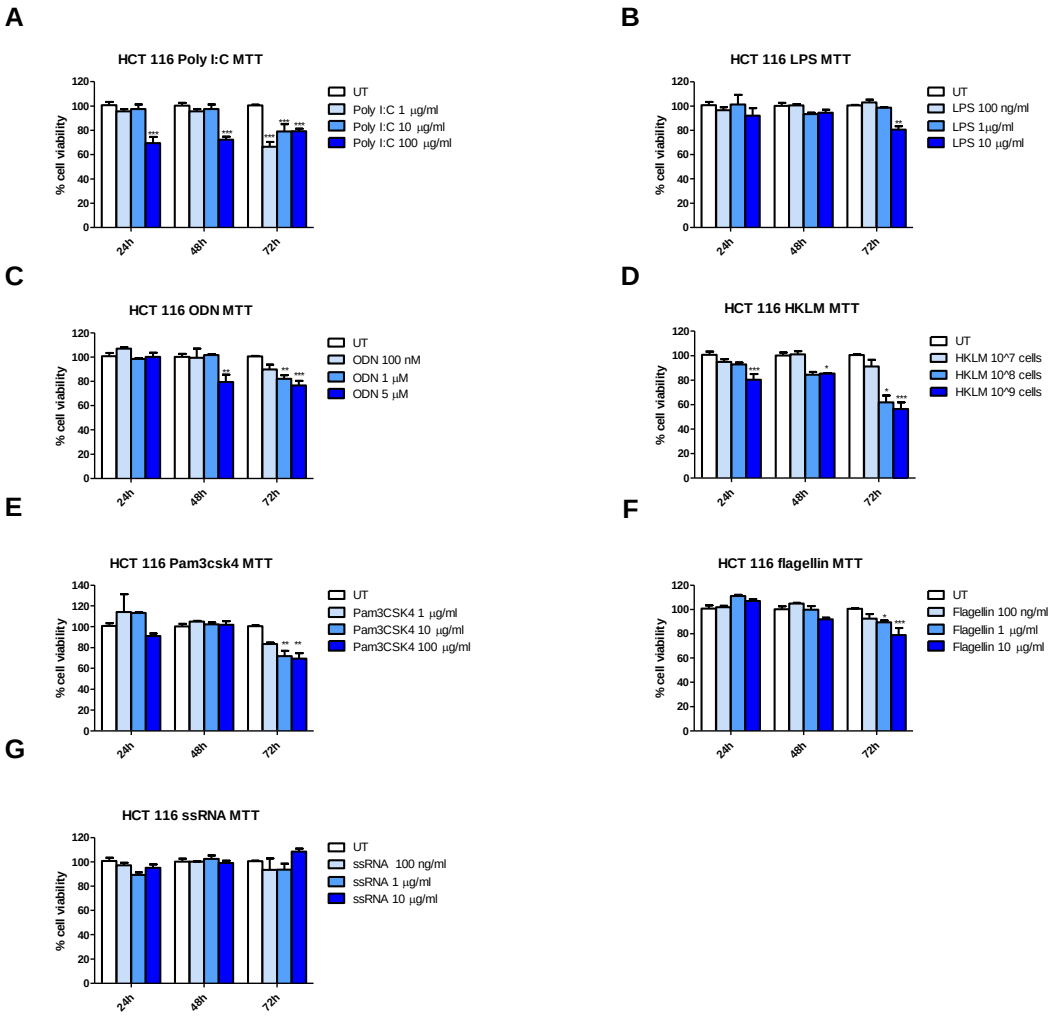


Figure 3.1. MTT cell viability assay in HCT 116 cells following TLR ligand treatment.

(A-G) Cell viability assays were performed in HCT 116 cells to measure the effect of the TLR ligands on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. The % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT.

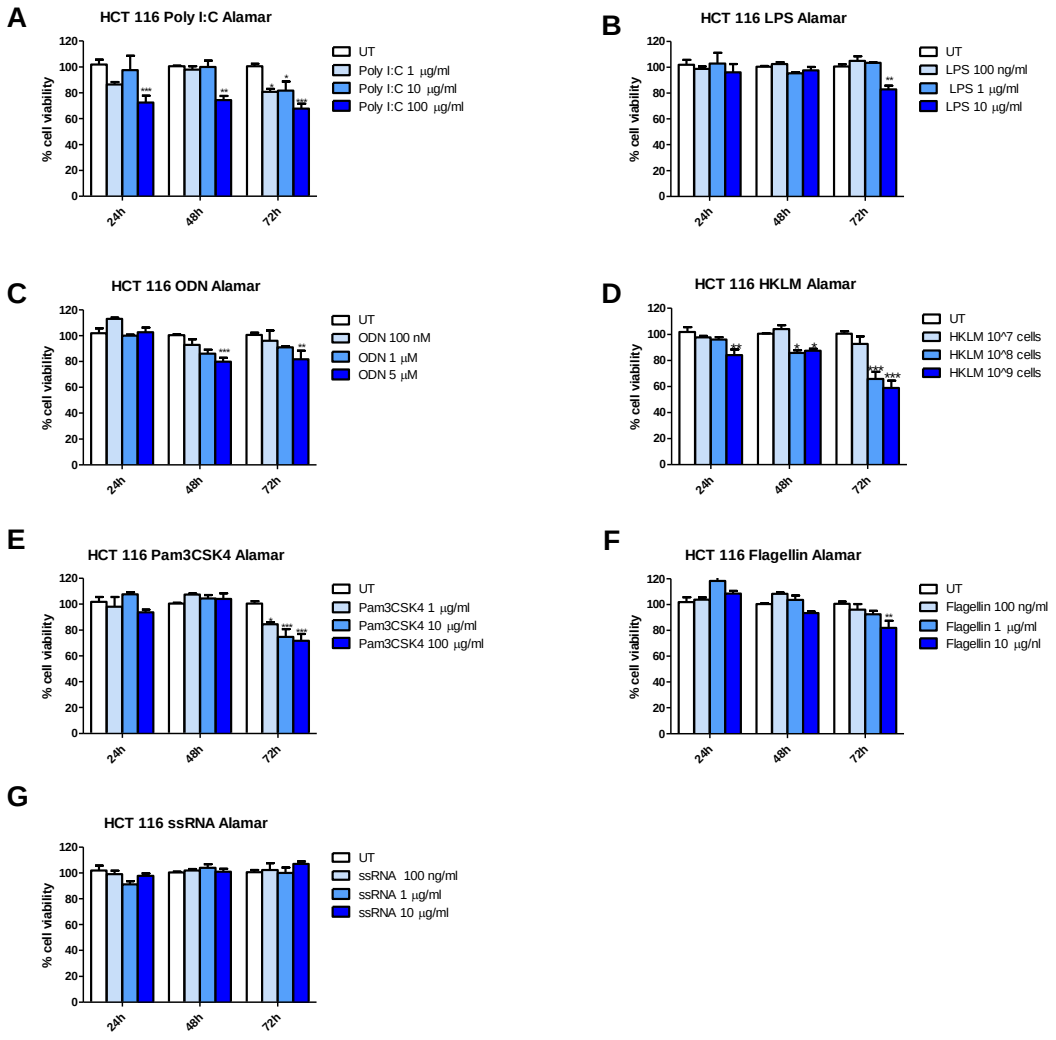


Figure 3.2. Alamar Blue™ cell Viability assay in HCT 116 cells following TLR ligand treatment.

(A-G) Cell death assays were performed in HCT 116 cells to measure the effect of the TLR ligands on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. The % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT.

3.4.1.2 Cell Viability in THP-1 cells was measured following administration of TLR ligands.

Following the observations made in the HCT 116 cell line, we then sought to examine the effect that the administration of TLR ligands would have on our other cell line, the THP-1 cells. The same dosing and time course regimen that was used in the HCT 116 cells was also utilised in the cell viability studies in the THP-1 cells. As shown in Figure 3.3A, there was a reduction in THP-1 cell viability seen with poly I:C treatment, most notably at the 100 μg/ml dose at 72hr (86% ± 3.4, $P < 0.05$). The 100 μg/ml dose also reached significance at the 48hr timepoint (87% ± 4.4, $P < 0.001$). The only other significance seen with poly I:C was at the 10 μg/ml dose at 72h (72% ± 3.4, $P < 0.02$). In Figures 3.3B LPS caused significant reduction in cell viability after 72h, with significant changes being seen in the 1 μg/ml group (82% ± 2.8, $P < 0.01$) and the 10 μg/ml group (82% ± 3.4, $P < 0.01$). Figures 3.3C shows the effect of ODN treatment on THP-1 cell viability, with significant reductions in viability seen in all treatment groups at 72h (100 nM (76% ± 5.3, $P < 0.001$), 1 μM (76% ± 4.6, $P < 0.001$), 5 μM (82% ± 2.3, $P < 0.05$). There were no other significant reductions seen in the ODN treatment groups, however it's worth noting that there was significant increases in cell viability seen in all ODN treatment groups in the MTT assay at the 24hr timepoint (ODN 100 nM (112% ± 3.8, $P < 0.05$), ODN 1 μM (117% ± 1.2, $P < 0.01$), ODN 5 μM (114% ± 6.4, $P < 0.05$). The effects of HKLM were examined, the results of which can be seen in Figures 3.3D. We observed significant reductions of cellular viability at all concentrations at the 72hr timepoint (10^7 cells MTT (77% ± 3.9, $P < 0.001$), 10^8 cells MTT (77% ± 1.1, $P < 0.001$), 10^9 cells MTT (83% ± 5.4, $P < 0.01$). The results depicted in (E-G) in Figure 3.3

shows the effects of Pam3csk4, flagellin and ssRNA on THP-1 cell viability. There were no significant reductions or increases in cell viability observed in any of these treatment groups across all timepoints when they were compared to their relevant untreated controls.

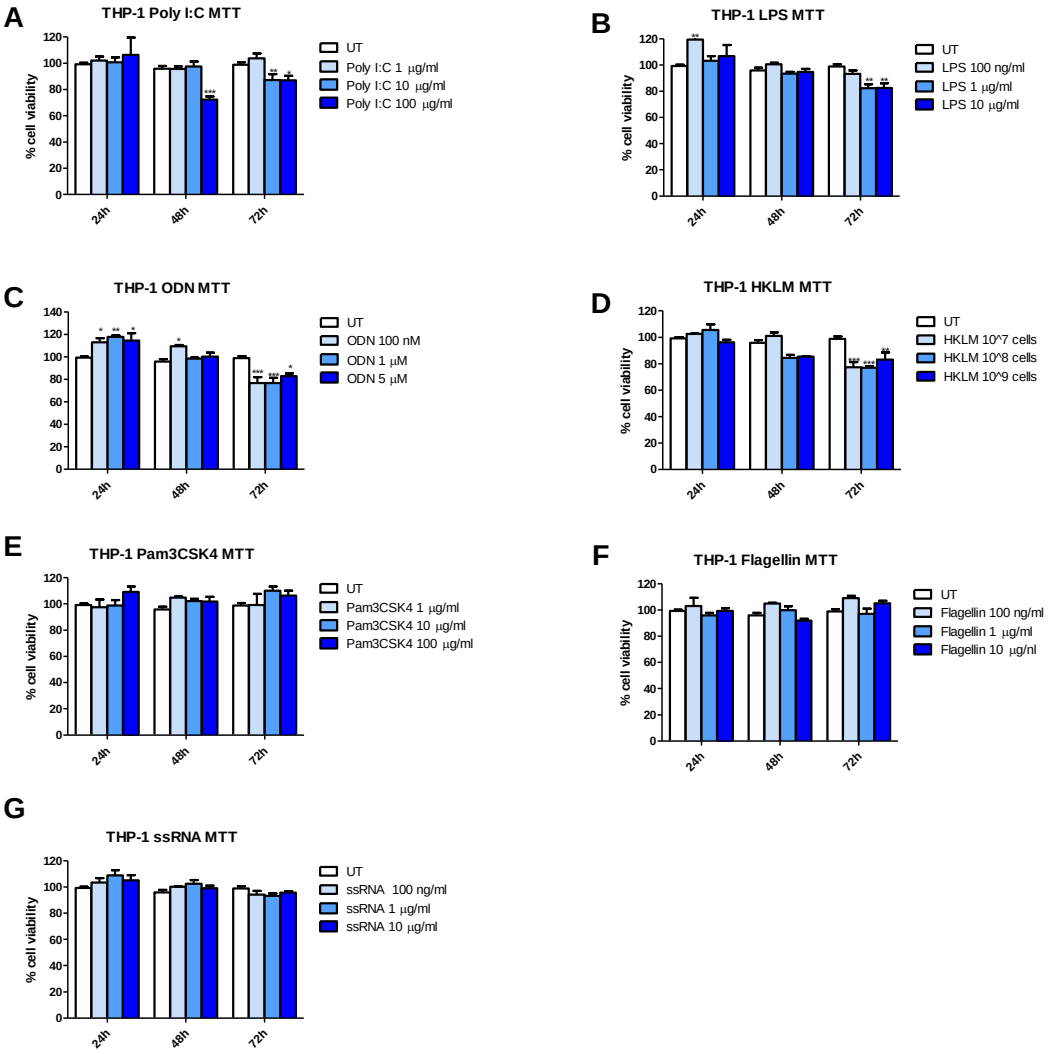


Figure 3.3. MTT cell viability assay in THP-1 cells following TLR ligand treatment. **(A-G)** MTT viability assays were performed in THP-1 cells to measure the effect of the TLR ligands on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. The % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT.

3.4.1.3 Effects of Epigenetic modifying drugs on cell viability in HCT 116 cells.

Following the examination of all the effects of the TLR ligands we next sought to determine whether the pharmacological compounds used in the studies were cytotoxic, and if so, to what degree. In Figure 3.4 we see the effect of 5-azacytidine, 5-aza-2-deoxycytidine, SAHA and TSA on cell viability. Each assay involved three doses of each ligand at 24, 48 and 72 hours, with data being calculated as percentage cell viability relative to the untreated control which is set as 100% cell viability. Data shown is mean % cell viability ± SEM. In Figures 3.4A the effect of 5-azacytidine treatment on HCT 116 cell viability are shown. There was no significant decrease in cell viability in any of the treatment groups used at all of the timepoints examined. In all cases data was compared to the relevant untreated control and in each case two-way ANOVAs were used, with the results showing that $P > 0.05$ for every treatment condition. Similar results were seen with the 5-aza-2-deoxycytidine treatment (Figure 3.4B), with little cell death seen in any of the treatment groups. As with the 5-azacytidine treatments, two-way ANOVAs did not show any significant effects of either dose or time, with $P > 0.05$ in all cases. The HDAC inhibitors however showed much greater cell death. In Figure 3.4C we see the results of SAHA treatment in the HCT 116 cell line. The MTT assay showed a significant decrease in viability at 72h following 1 μM SAHA (84% ± 0.9, $P < 0.001$). The 10 μM dose of SAHA was seen to cause a significant reduction in cell viability at 48hr (92% ± 1.3, $P < 0.05$), and 72hr (68% ± 2.4, $P < 0.001$). The highest dose used of 100 μM also produced significant changes at 48hr (92% ± 2.6, $P < 0.05$ and 72hr (MTT (66% ± 1.2, $P < 0.001$). In concordance with the SAHA results, treatment with TSA also resulted in significant reductions in cell viability. The 1 μM dose of TSA

proved to be cytotoxic at 72h in the MTT (91% ± 0.6, $P < 0.01$). The 10 μM dose of TSA however resulted in significant reductions in viability 48h (MTT (90% ± 2.9, $P < 0.01$) and 72h (MTT (63% ± 0.7, $P < 0.001$). The highest dose of TSA used proved to be extensively cytotoxic, with significant cell death seen at 48h (MTT (82% ± 4.2, $P < 0.001$), and even higher rates of death seen at 72h (MTT (36% ± 1.1, $P < 0.001$). Thus the results highlight that, in HCT 116 cells at least; the methyltransferase inhibitors are relatively benign, with very little cell death seen across the range of doses and concentrations used. However the HDAC inhibitors prove to cause significant reductions in viability, with TSA being more cytotoxic than its counterpart SAHA.

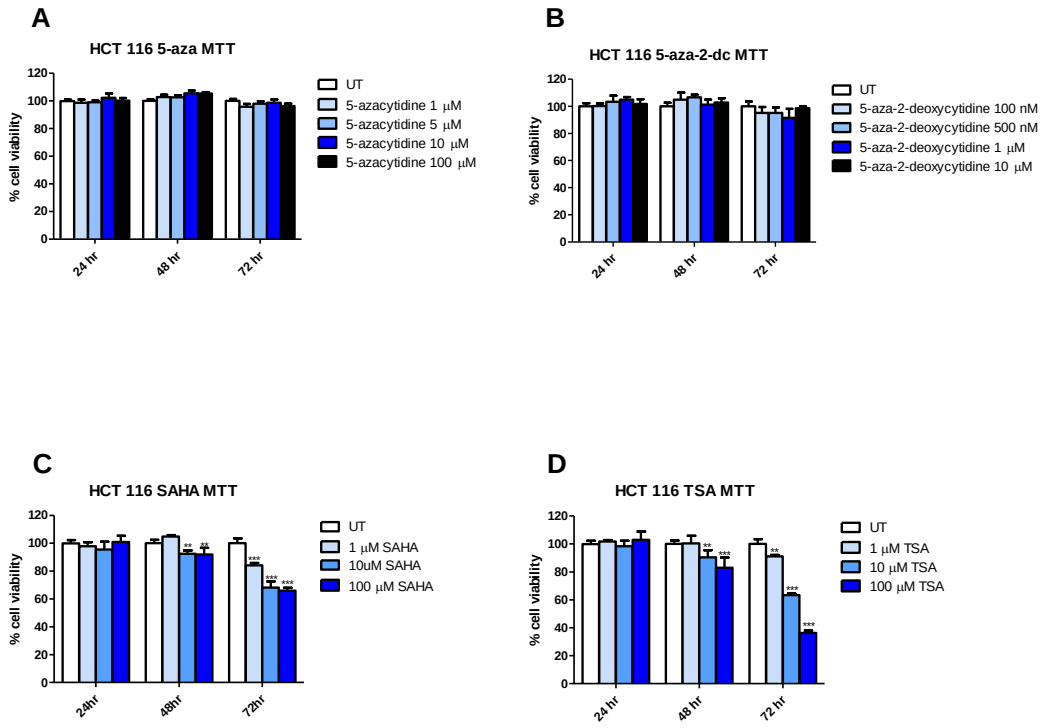


Figure 3.4. MTT cell viability assay in HCT 116 cells following treatment with Epigenetic modifying drugs

(A-D) Cell death assays were performed in HCT 116 cells to measure the effect of 5-azacytidine, 5-aza-2-deoxycytidine, SAHA and TSA on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. The % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT.

3.4.1.4 Effects of Epigenetic modifying drugs on cell viability in THP-1 cells.

Following the examination of the effects of the pharmacological treatments on the HCT 116 cells we sought to examine the effect the same compounds would have on the THP-1 cell line. In Figure 3.5 we see the effects of 5-azacytidine, 5-aza-2-deoxycytidine, SAHA and TSA on cell viability as measured by the MTT assay. As with the HCT 116 studies, each assay involved three doses of each ligand at 24, 48 and 72 hours, with data being calculated as percentage cell viability relative to the untreated control which is set as 100% cell viability. Data shown is mean % cell viability ± SEM. Similarly to what was seen in the HCT 116 cells, we see in figure 3.5A, that treatment with 5-azactidine did not lead to significant cell death at the doses and time points used. We did observe a decrease in cell viability in the 100 μM treatment group at 24h (88% ± 4.2, $P < 0.01$); however this effect was not seen at 48 or 72h. Treatment with 5-aza-2-deoxycytidine however did result in significant reductions in cell viability, with all treatment groups showing reductions in cell viability in the MTT assay (Figure 3.5B) at 72h (100 nM (82% ± 3.9, $P < 0.001$), 500 nM (81% ± 1.4, $P < 0.001$), 1 μM (80% ± 4.09, $P < 0.001$), 10 μM (79% ± 1.01, $P < 0.001$). Following the examination of the effects of the DNMT inhibitors, we sought to examine what impact the HDAC inhibitors had on THP-1 cells. Upon examination of the impact of SAHA (Figure 3.5C) on cell viability, we observed that it proved to be more cytotoxic than the DNMT inhibitors, with significant cell death observed. All doses of SAHA were seen to cause significant reductions in cell viability in the MTT assay at 48h (1 μM (85% ± 2, $P < 0.01$), 10 μM (69% ± 2.3, $P < 0.001$), 100 μM (65% ± 4.2, $P < 0.001$)) and 72h (1 μM (83% ± 1.6, $P < 0.001$), 10 μM (73% ± 1.1, $P < 0.001$), 100 μM (71% ± 1.2, $P < 0.001$). The highest dose of SAHA was also seen to cause reductions in cell viability at 24h (80% ± 2.1, $P < 0.001$), with none of the other

doses showing reductions at this time point. Following the examination of the effects of SAHA, we examined the last drug used, TSA, the results of which are visible in Figure 3.5D. In the HCT 116 cells TSA proved to be the most cytotoxic of all the compounds tested and the same was true for the THP-1 cells. Significant reductions were seen in the MTT assay at 24h and 48h with the 10 μM dose (24h (78% ± 3, $P < 0.001$), 48h (64% ± 3.7, $P < 0.001$)) and 100 μM dose (24h (85% ± 5.6, $P < 0.05$), 48h (69% ± 5, $P < 0.001$)), and at 72h there were significant reductions in cell viability observed in all treatment groups (1 μM (84% ± 3.6, $P < 0.01$), 10 μM (64% ± 3.4, $P < 0.001$), 100 μM (69% ± 1.1, $P < 0.001$)). Thus from the cell viability results shown it would seem that treatment with both 5-azacytidine and its more potent relative 5-aza-2-dexoycytidine is quite innocuous in both cell lines, with very little cell death observed across the range of doses and timepoints used. The HDAC inhibitors are different however, with the higher doses having quite pernicious effects at later timepoints. Fortunately however, the cell viability hovers around the 70% mark in even the higher doses, suggesting that while cytotoxic to a certain extent, the compounds used are not completely destructive.

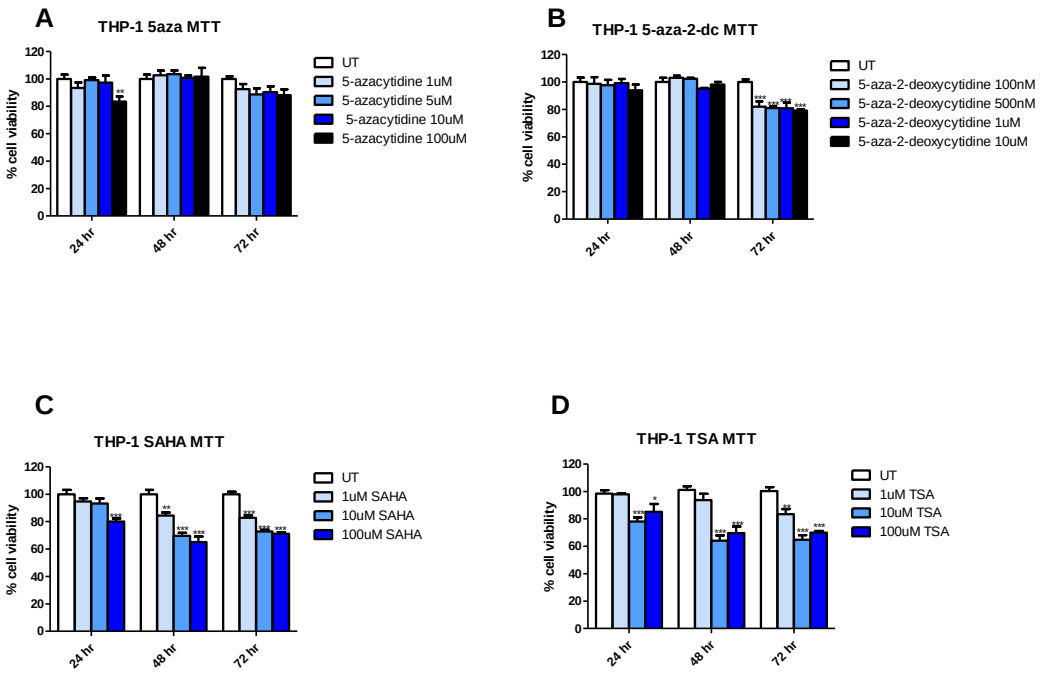


Figure 3.5. MTT cell viability assay in THP-1 cells following treatment with Epigenetic modifying drugs.

(A-D) Cell death assays were performed in HCT 116 cells to measure the effect of 5-azacytidine, 5-aza-2-deoxycytidine, SAHA and TSA on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. For both assays % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT.

3.4.1.5 Effects of cytokine and PMA treatments on HCT 116 cell viability

The cytokine and PMA treatments are the next and final set of treatments which were examined for their impact on cell viability. Each assay involved three doses of each of the cytokines/PMA at 24, 48 and 72 hours, with data being calculated as percentage cell viability relative to the untreated control which is set as 100% cell viability. Data shown is mean % cell viability ± SEM. The cytokines used were IL6, TNFα and IFNγ. Each cytokine was used at three concentrations; 10 ng/ml, 50 ng/ml and 100 ng/ml. PMA was used at 1 μg/ml, 10 μg/ml and 100 μg/ml respectively. In Figure 3.6A we see the effect of IL6 treatment on HCT 116 cell viability as measured by the MTT assay. Treatment with IL6 has no effect on cell viability across all the timepoints and doses measured. The treatment seems to be completely non-cytotoxic at the concentrations we have used. In Figure 3.6B, we observe the effects of TNFα on HCT 116 cell viability. Similar to what we observed with the IL6 treatment, administration of TNFα is relatively innocuous at the times and doses used in our studies. No significant changes or even trends towards changes were observed. Figure 3.6C shows the effects of IFNγ on cell viability in the HCT 116 cells, and as was seen with the previous two cytokine treatments, IFNγ is also observed to be non-toxic. When all timepoints and doses were examined, there were no significant changes in any group when compared to their untreated counterparts. Finally, in Figure 3.6D, we observed the results of PMA treatment on HCT 116 cell viability. We observed no significant reductions in cell viability in the MTT assay with any of the doses of PMA used, at all the time points examined. Thus, we determined the cytokine and PMA treatments used in the course of our studies are non-toxic in this cell line and do not adversely affect cell viability.

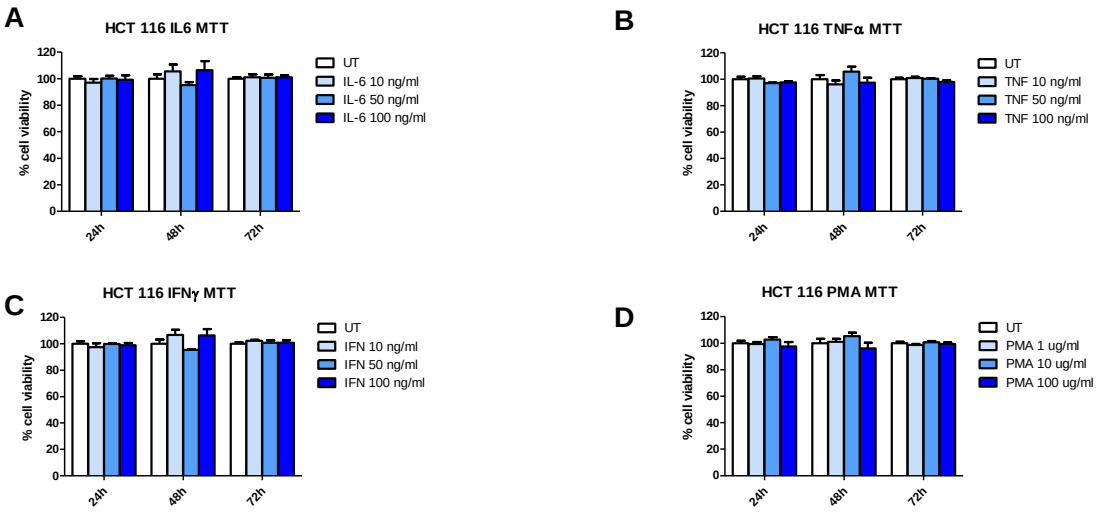


Figure 3.6. MTT and Alamar Blue™ cell viability assays in HCT 116 cells following cytokines and PMA treatments.

(A-D) MTT assays were performed in HCT 116 cells to measure the effect of IL6, TNFα, IFNγ and PMA on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. The % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT.

3.4.1.6 Effects of cytokine and PMA treatments on THP-1 cell viability

Following the examination of the effects of the cytokine and PMA treatments on HCT 116 cell viability, we examined the effects of these treatments on the THP-1 cell viability. As previously, each assay involved three doses of each of the cytokines/PMA at 24, 48 and 72 hours, with data being calculated as percentage cell viability relative to the untreated control, with the data shown being mean % viability ± SEM. Similarly to our previous observations, as shown in Figure 3.7A the administration of IL6 to the THP-1 cells seems relatively benign, with no reductions in cell viability seen across the doses and times examined. Interestingly however, the 10 ng/ml dose of IL6 lead to a significant increase in cell viability after 72h (118% ± 4.2, $P < 0.05$), the reason for which is unclear. TNFα continued the trend set by the previous examples, with no significant differences in cell viability seen in any groups across all the timepoints measured. In Figure 3.7B we observed that all of the treatment groups show cell viability values similar to the untreated control at all timepoints. The final cytokine examined was again IFNγ, and there were trends towards decreases in cell viability at the higher doses at 48h and 72h. In Figure 3.7C we see that there are no significant differences in any of the treatment groups at 24h or 48h, however we observed that at 72h there was a significant reduction in cell viability with the 100 μg/ml dose of IFNγ (83% ± 5.6, $P < 0.05$). Finally in Figure 3.7D, we observed the effects of PMA treatment on THP-1 cell viability. There was no discernible decrease in cell viability with any of the doses of PMA used, at all the timepoints we examined. This would suggest that PMA is a non-toxic method of inducing THP-1 cell differentiation. Furthermore, all of the compounds examined in Figure 3.7 do not produce any significant reductions in

THP-1 cell viability, which indicates that a treatment with the cytokines is a relatively harmless procedure.

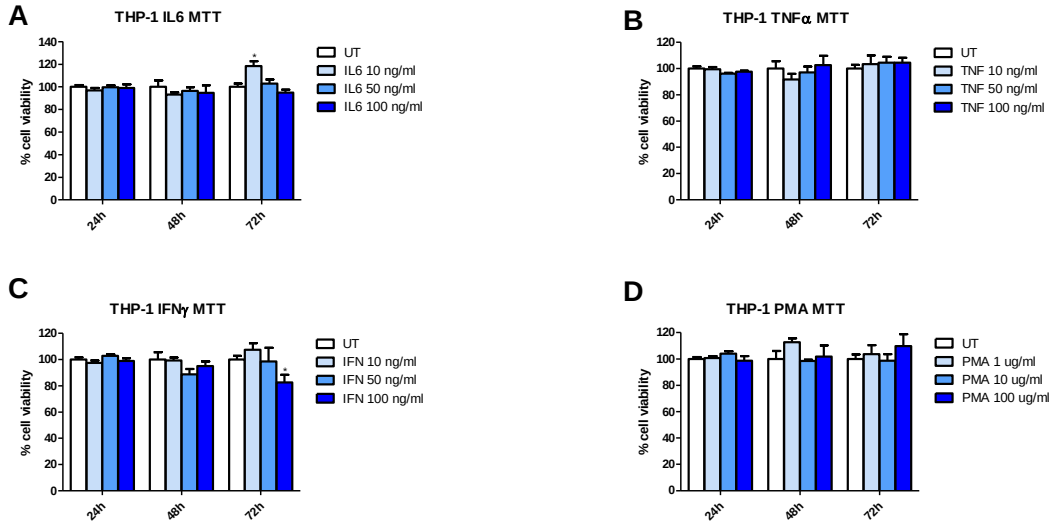


Figure 3.7. MTT and Alamar Blue™ cell viability assays in THP-1 cells following cytokines and PMA treatments.

(A-H) Cell death assays were performed in HCT 116 cells to measure the effect of IL6, TNFα, IFNγ and PMA on cell viability at different concentrations and timepoints. Cells were treated with the doses shown for 24, 48 and 72h. For both assays % viability was calculated relative to the UT controls, with data shown expressed as mean % viability ± SEM. Statistical analysis was performed using a 2-way ANOVA. $P < 0.05$ was considered significant. * = $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** $P < 0.001$ vs UT

3.4.2 Effect of DNMT 1/3b DKO on Basal expression of TLR1-9

At the outset of these studies, in order to determine which of the TLR family we would investigate further, we examined which of the members were affected by methylation, or in the case of the DKO cells, a complete lack of methylation. To determine the basal levels of TLR1-9 mRNA in HCT 116 cells, qPCR was performed on WT and DKO cell lines. Relative mRNA expression was quantified using β-Actin as an endogenous control, with the WT untreated group being set to 1. The fold change relative to the WT group was calculated using the $2^{-\Delta Ct}$ method. A student's t-test was used to compare the means. A P value of $P < 0.05$ was considered statistically significant. Figure 3.8 shows the results we observed upon examination of the effects of DNMT 1/3b DKO. In Figure 3.8A we see a difference in TLR1 expression in the WT and DKO cells. We found that in the DKO cells there was a significant reduction in expression (0.349 ± 0.046, $P < 0.01$) when compared to the WT group (1.00 ± 0.171). In Figure 3.8B we see differing expression of TLR2 in the WT and DKO cells, and while there appears to be a slight change in expression, there are no significant alterations in mRNA expression seen between the two groups (WT (1 ± 0.317), DKO (1.86 ± 1.1, $P > 0.05$)). The same is not true however for TLR3, which responds rather dramatically to the removal of methylation. In Figure 3.8C we observe that the knockout of DNMT 1/3b leads to a massive decrease in TLR3 mRNA expression when compared to the WT (WT (1 ± 0.098), DKO (0.007 ± 0.004, $P < 0.001$)). This amounts to a decrease in expression well in excess of 100-fold, and is the most dramatic response seen in the TLR family to hypomethylation. Following TLR3, we examined TLR4, and similarly to its predecessor there was a significant decrease in expression seen in the DKO (0.503 ± 0.801, $P < 0.001$) when compared to the WT (1 ± 0.492), however the change was not nearly as

dramatic as that seen previously, with the difference amounting to roughly a two-fold decrease in expression. TLR5 was the next TLR examined, and as is evident in Figure 3.8E, there is a slight trend towards a decrease in expression, however there was no significant differences between the expression of the WT group (1 ± 0.223) and the DKO group (0.681 ± 0.119, $P > 0.05$). TLR6 also showed some interesting changes when the methylation machinery is removed. In Figure 3.8F, we see that there is a significant increase in TLR6 expression in the DKO cells when compared to their WT counterparts (WT (1 ± 0.1), DKO (2.18 ± 0.8, $P < 0.001$)), with the difference amounting to a two-fold increase in expression. There were no significant changes seen with the remaining TLRs (Figure 3.8 G, H, I) with TLR7 (WT (1 ± 0.36), DKO (1.1 ± 0.17, $P > 0.05$)), TLR8 (WT (1 ± 0.17), DKO (1.2 ± 0.204, $P > 0.05$)) and TLR9 (WT (1 ± 0.26), DKO (0.992 ± 0.12. $P > 0.05$)) all showing similar expression in the WT and DKO cells. In order to confirm that the DKO cells were indeed knockout cells, we analysed the change in expression of CDKN2A, a gene which is used as a positive marker for inhibition of methylation. In figure 3.8J, we observed the comparison of CDKN2A expression in the WT and DKO cell line. This result confirms the knockout, as CDKN2d expression is significantly higher in the DKO cells (29.2 ± 3.25, $P < 0.001$) when compared to the WT cells (1 ± 0.156). As a result of these preliminary studies, which examined the basal effects of DNMT deletion on TLR expression, we chose to focus our attention on TLR3, given the scale of the change seen in mRNA expression.

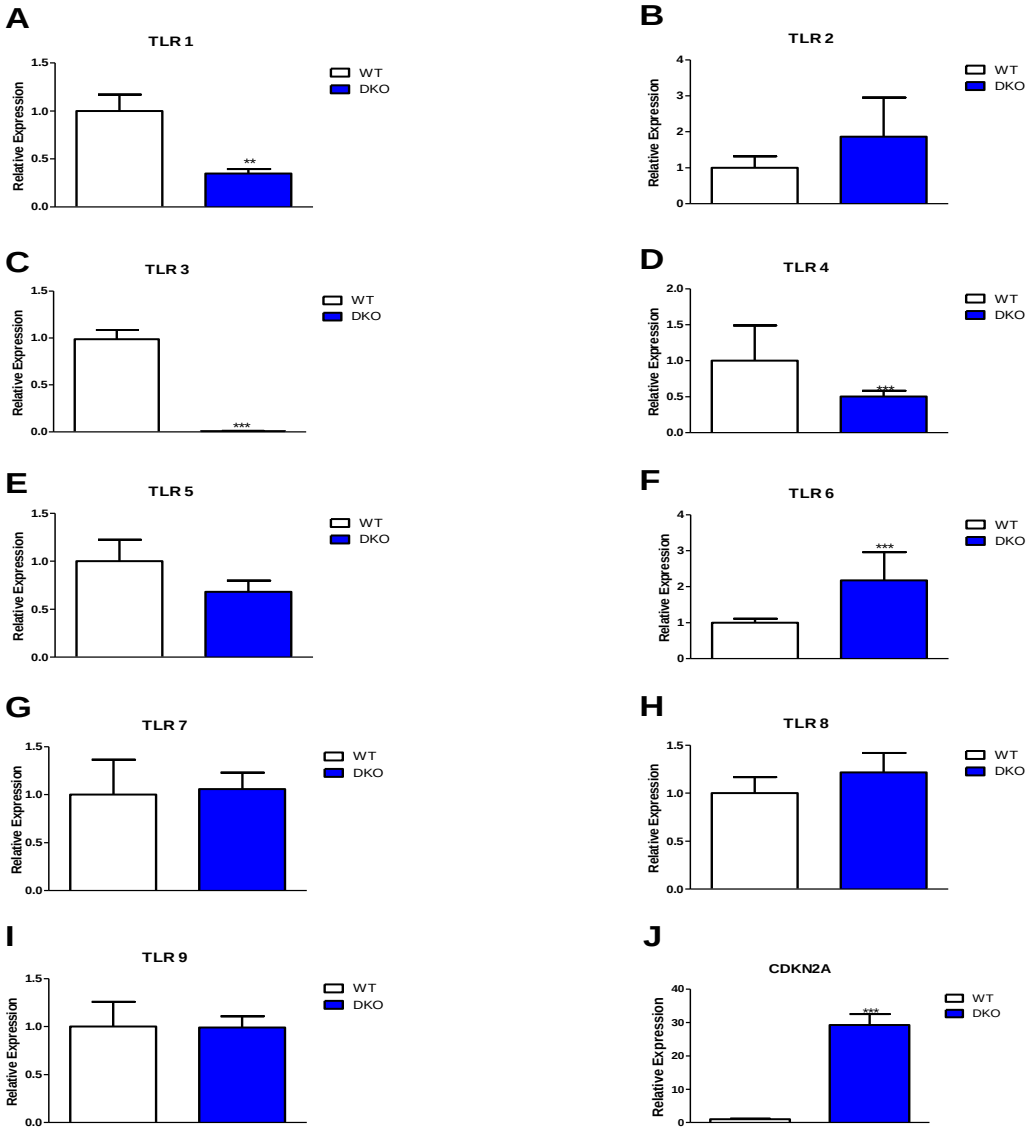


Figure 3.8.TLR 1, 3, 4 and 6 show significantly altered expression in DNMT1/3b knockout cells.

(A-I) Real-time PCR analysis was performed to examine the basal levels of TLR1-9 mRNA expression in both the WT and DKO cell lines. **J)** Real-time PCR was performed to analyse changes in CDKN2A, which is a positive control for inhibition of DNA methylation. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$

was considered significant. Data shown is mean ± SEM, n = 3,* = P < 0.05 vs WT, ** = P < 0.01 vs WT, *** = P < 0.001 vs WT.

3.4.3 INVESTIGATION OF THE EFFECTS OF CYTOKINE TREATMENTS ON TLR3 EXPRESSION

3.4.3.1 Effect of cytokine treatment on TLR3 expression in HCT 116 cells.

In order to examine the potential effect existing in an inflammatory environment would have on TLR3 receptor function; we performed studies to examine the effects of IL6, TNFα and IFNγ on TLR3 expression. HCT 116 cells were treated with 50 ng/ml of each of the cytokines on their own as well as all three together, for a period of 24h. Changes in mRNA expression were analysed using qPCR, and relative mRNA expression was quantified using β-Actin as an endogenous control, with the WT untreated group being set to 1. The fold change relative to the WT group was calculated using the $2^{-\Delta Ct}$ method, and a student's t-test was used to compare the means. A P value of $P < 0.05$ was considered statistically significant. Changes in the TLR3 protein level were examined using intracellular flow cytometry, with data shown being mean RFI (Relative Fluorescence Intensity) ± SEM. For the purpose of the cytokine treated HCT 116 flow cytometry, poly I:C was used as a positive control as it reliably showed significant upregulation of TLR3 protein expression in HCT 116 cells. In Figure 3.9A the effect of IL6 treatment on TLR3 mRNA expression is shown. We observed that treatment with IL6 for 24 hours significantly reduced expression of TLR3 when compared to the UT group (UT (1 ± 0.195), IL6 (0.267 ± 0.044. $P < 0.001$)). However when we examined whether there were any resultant changes in TLR3 protein level (Figure 3.10A) we found that there was no difference in TLR3 protein level in the UT (1 ± 0.010) and the IL6 treated groups (0.96 ± 0.07, $P > 0.05$). Next we investigated the effect of TNFα on TLR3 expression, and found that, at the mRNA level, there was no significant change in

expression between the WT (1 ± 0.2) and TNFα treated (0.78 ± 0.1, $P > 0.05$) groups (Figure 3.9B). When we examined the TLR3 protein level (Figure 3.10B), we found that there was no change in expression when the UT (1 ± 0.01) and TNFα (0.966 ± 0.05, $P > 0.05$) groups were compared. In Figure 3.9C, we observe that IFNγ treatment has a slight effect on TLR3 mRNA expression, with treatment resulting in a significant decrease in TLR3 expression (WT (1 ± 0.2), IFNγ (0.443 ± 0.04, $P < 0.05$)). Once again, as we see in Figure 3.10C, the change in mRNA expression was not followed by a subsequent alteration in TLR3 protein level, with UT (1 ± 0.01) and IFNγ treated (1.1 ± 0.5, $P > 0.05$) being similar. Finally, the effect of the combination of all three cytokines on both TLR3 mRNA and protein expression was measured, the results of which are visible in Figures 3.9D and 3.10D, respectively. The triple treatment resulted in a significant decrease in TLR3 mRNA expression (UT (1 ± 0.2), Triple (0.29 ± 0.03, $P < 0.001$)). However, following the trend set by the single cytokine treatments, the combination has no effect of TLR3 protein expression level, with the UT (1 ± 0.1) and the Triple treatment (0.929 ± 0.05, $P > 0.05$) being almost identical.

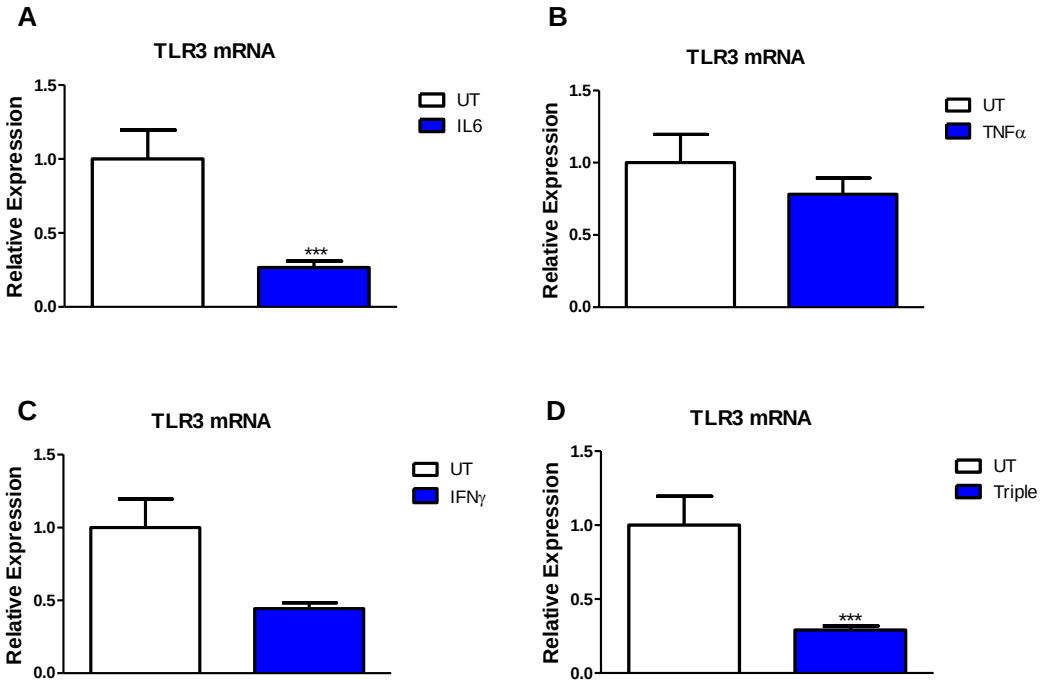


Figure 3.9. IL6 and triple cytokine treatments reduced TLR3 mRNA expression significantly in HCT 116 cells.

(A-D) Real-time PCR analysis was performed to examine effect of cytokine treatments on TLR3 mRNA expression in the WT cell line. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, n = 3,*= $P < 0.05$ vs WT, ** = $P < 0.01$ vs WT, *** = $P < 0.001$ vs WT.

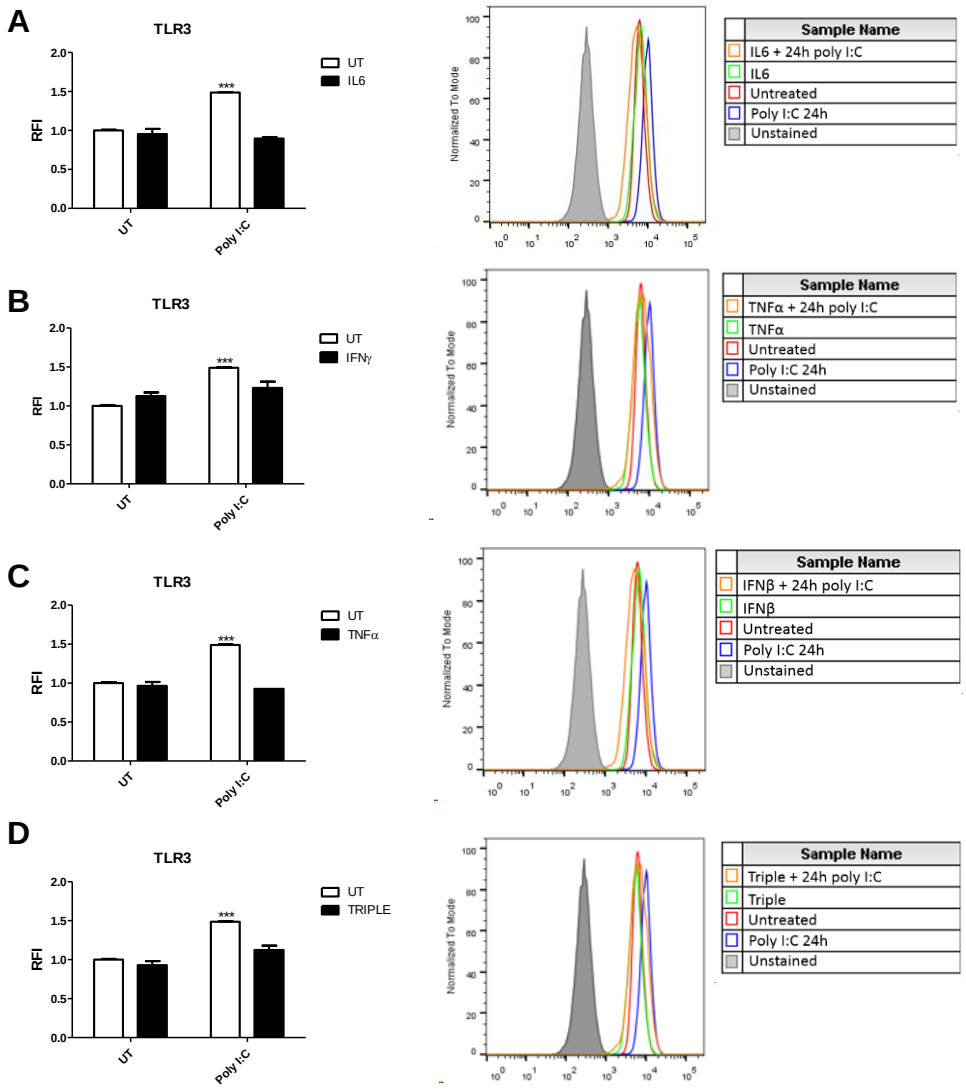


Figure 3.10. Cytokine treatments did not alter TLR3 protein expression significantly in HCT 116 cells.

(A-D) Intracellular flow cytometry was performed using the BD FACS canto II flow cytometer. Data shown is RFI which is relative to the UT group, with histograms shown to the right. The unstained group is shown in grey. Data was analysed using a 2-way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, n = 3,*= $P < 0.05$ vs WT, ** = $P < 0.01$ vs WT, *** = $P < 0.001$ vs WT.

3.4.3.2 Effect of cytokine treatment in THP-1 cells on TLR3 Expression

Following the examination of the effect of cytokine treatments on the HCT 116 cells we then examined the effects of these treatments on the THP-1 cells. As with the HCT 116 cells, cells were treated with 50 ng/ml of each of the cytokines on their own as well as all three together, for a period of 24h. Changes in mRNA expression and TLR3 protein levels were measured using qPCR and intracellular flow cytometry, as described in the previous section. In Figure 3.11A we see the effect of IL6 treatment on TLR3 mRNA expression in the THP-1 cells. Interestingly, the treatments lead to a significant increase in TLR3 expression (UT (1 ± 0.2), IL6 (7.1 ± 1.4, $P < 0.001$)). However, when we examined the effect of IL6 treatment on TLR3 protein level (Figure 3.12A) we found that there was no change in expression ((UT (1 ± .1), IL6 (1.02 ± 0.018, $P > 0.05$)). As seen in Figure 3.11B, treatment with TNFα did not result in a significant increase in TLR3 mRNA expression (UT (1 ± 0.2), TNFα (1.5 ± 0.4, $P > 0.05$)), and in Figure 3.12B we see that there was also no change in TLR3 protein level following the IL6 treatment (UT (1 ± 0.2), TNFα (1.02 ± 0.05, $P >0.05$)). In Figure 3.11C, we observed the effects of IFNγ treatment on TLR3 mRNA expression, and we found that, interestingly, the treatment led to a significant upregulation of TLR3 mRNA (UT (1 ± .2), IFNγ (4.3 ± 0.8, $P < 0.01$)). However, as with IL6, this increase in mRNA was not followed by a resultant increase in TLR3 protein, with no difference in the levels between the UT and IFNγ treated groups (UT (1 ± 0.2), IFNγ (1.05 ± 0.03, $P > 0.05$)). Finally, the last group we examined was the group which received all three treatments in combination. We observed that the triple treatment resulted in a significant increase in TLR3 mRNA in the THP-1 cells (Figure 3.11D), with an increase in expression

exceeding 90-fold as a result of the treatment (UT (1 ± 0.2), Triple (91.5 ± 18.4)). However, in spite of this large increase in mRNA expression, it was not followed by a resultant increase in protein, with TLR3 being similar in both the UT (1 ± 0.1) and Triple treated (0.94 ± 0.01, $P > 0.05$) groups (Figure 3.12D). Thus it was evident that, while cytokine treatment may alter the levels of TLR3 mRNA expression, we did not observe any resultant changes in protein levels of TLR3.

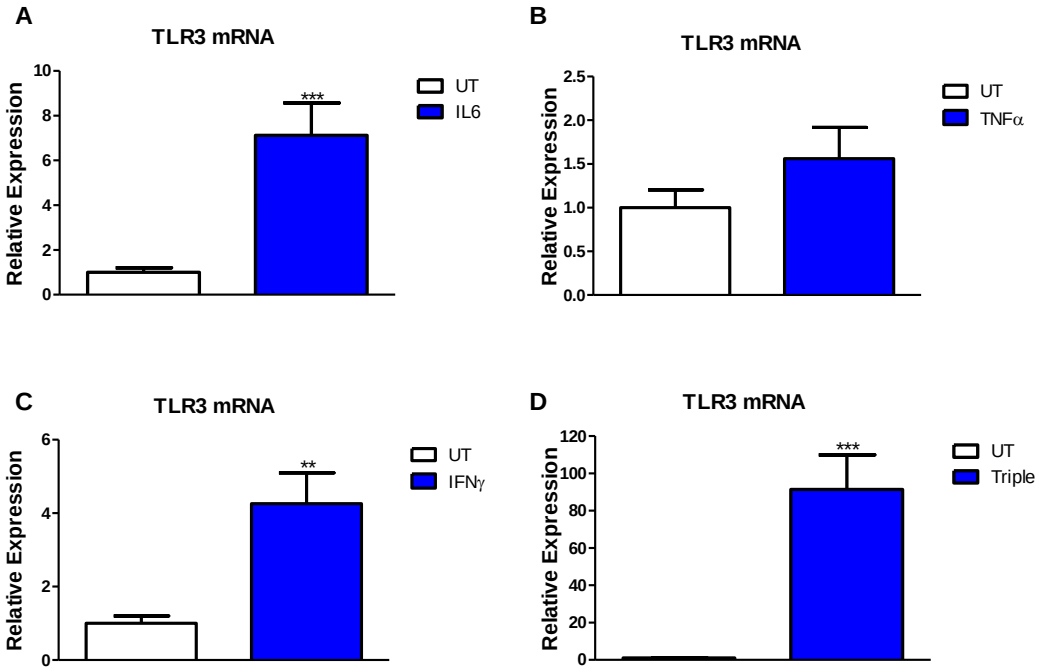


Figure 3.11. IL-6, IFNγ and triple cytokine treatments resulted in significant increase in TLR3 mRNA expression in THP-1 cells

(A-D) Real time PCR analysis was performed to examine effect of cytokine treatments on TLR3 mRNA expression in the THP-1 cell line. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, n = 3,*= $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** = $P < 0.001$ vs UT.

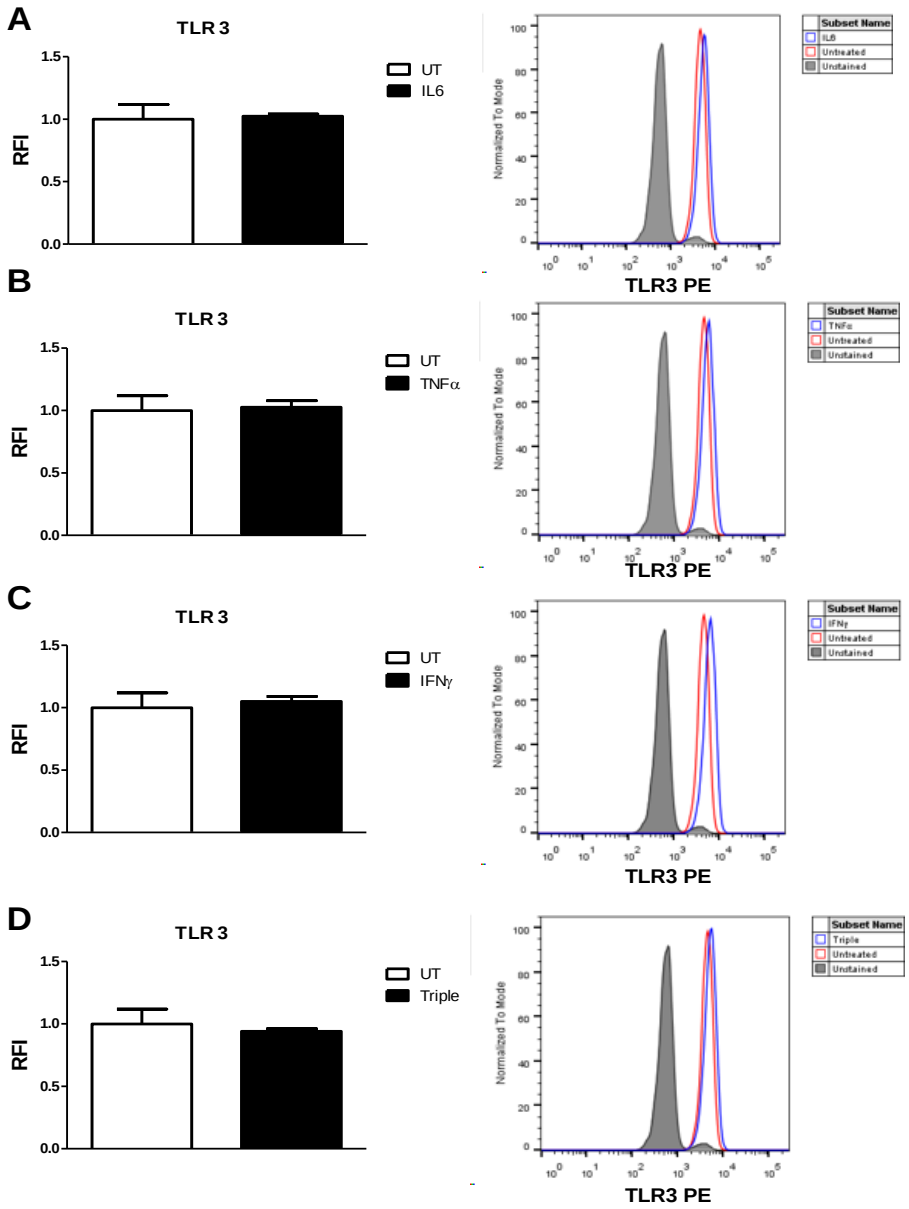


Figure 3.12. Cytokine treatments did not significantly alter TLR3 protein expression in THP-1 cells

(A-D) Intracellular flow cytometry was performed using the BD FACS canto II flow cytometer. Data shown is RFI which is relative to the UT group, with raw data shown as histograms . Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, n = 3,*= $P < 0.05$ vs UT, ** = $P < 0.01$ vs UT, *** = $P < 0.001$ vs UT.

3.4.4 INVESTIGATION OF THE TIME RESPONSE OF SIGNALLING PROTEINS TO POLY I:C

3.4.4.1 Poly I:C time response in HCT 116 cells.

In order to determine what time points HCT 116 cells experience protein phosphorylation following poly I:C stimulation, we performed a dose response in the WT cells. Cells were treated with 10 μg/ml poly I:C at 0, 15, 30, 60, 90, 120 and 180 minutes. Blots were performed as describe in the methods and images were obtained using the Licor Odyssey western blot scanner. The blots were analysed using densitometry, with values shown being fold increase relative to the untreated which is set to 1. The phosphorylated proteins examined were P-ERK 1/2, P-SAPK/JNK and P-P-38 from the MAPK family and P-NF-κB, P-IκBα and P-IRF 3 as the transcription factors activated by signalling (see figure 1.4). The Western blot images for the phosphorylated protein response in HCT 116 cells are shown in Figure 3.13. P-ERK (fig 3.13B) is the first row in the image, and we observed that it is strongest at 120 minutes (UT ()15 (3.82), 30 (3.25), 60 (1.14), 90 (7.67), 120(7.67), 180(5.69)). P-SAPK/JNK (fig 3.13C), was also elevated at 90-120 minutes, most notably so at 120 minutes post stimulation (UT (1),15 (0.79), 30 (0.4), 60 (0.93), 90 (1.56), 120(2.57), 180(0.67)). P-P38 (fig 3.13 D) does not appear to undergo any discernible changes in phosphorylation following poly I:C treatment (UT (1),15 (0.66), 30 (1.04), 60 (0.56), 90 (0.61), 120(0.67), 180(0.98)). P-IκBα (fig 3.13E) appears strongly phosphorylated at 180 minutes post stimulation (UT (1), 15 (0.91), 30 (1.6), 60 (1.85), 90 (1.69), 120(3.05), 180(5.6)). T- IκBα (fig 3.13) appears reduced at 90 minutes, reductions in which are indicators of increases in the phosphorylated form of the protein. P-NF-κB provided an

interesting result, with a biphasic response being elicited in the HCT 116 cells. We see a sharp rise in the levels of P-NF-κB (fig 3.13F) 30 minutes post stimulation increase, however they begin to taper off at 90 minutes, however, it rises significantly at 180 minutes (UT (1),15 (2), 30 (3.76), 60 (3.9), 90 (2.7), 120(0.6), 180(5.4)). The last protein examined was P-IRF3 (fig 3.13G), which we found to have no response at the early timepoints of 15 and 30 minutes post stimulation. P-IRF3 levels increased at 60 minutes post stimulation, with the greatest increase detected at 180 minutes post stimulation (UT (1),15 (0.74), 30 (0.09), 60 (3.83), 90 (3.24), 120(2.89), 180(5.42)). Thus, from these results its evident that the HCT 116 cells are very responsive to poly I:C stimulation, with sharp increases in phosphorylated signalling proteins being observed at different timepoints, providing strong evidence of activation of the inflammatory signalling cascade in response to the stimulus.

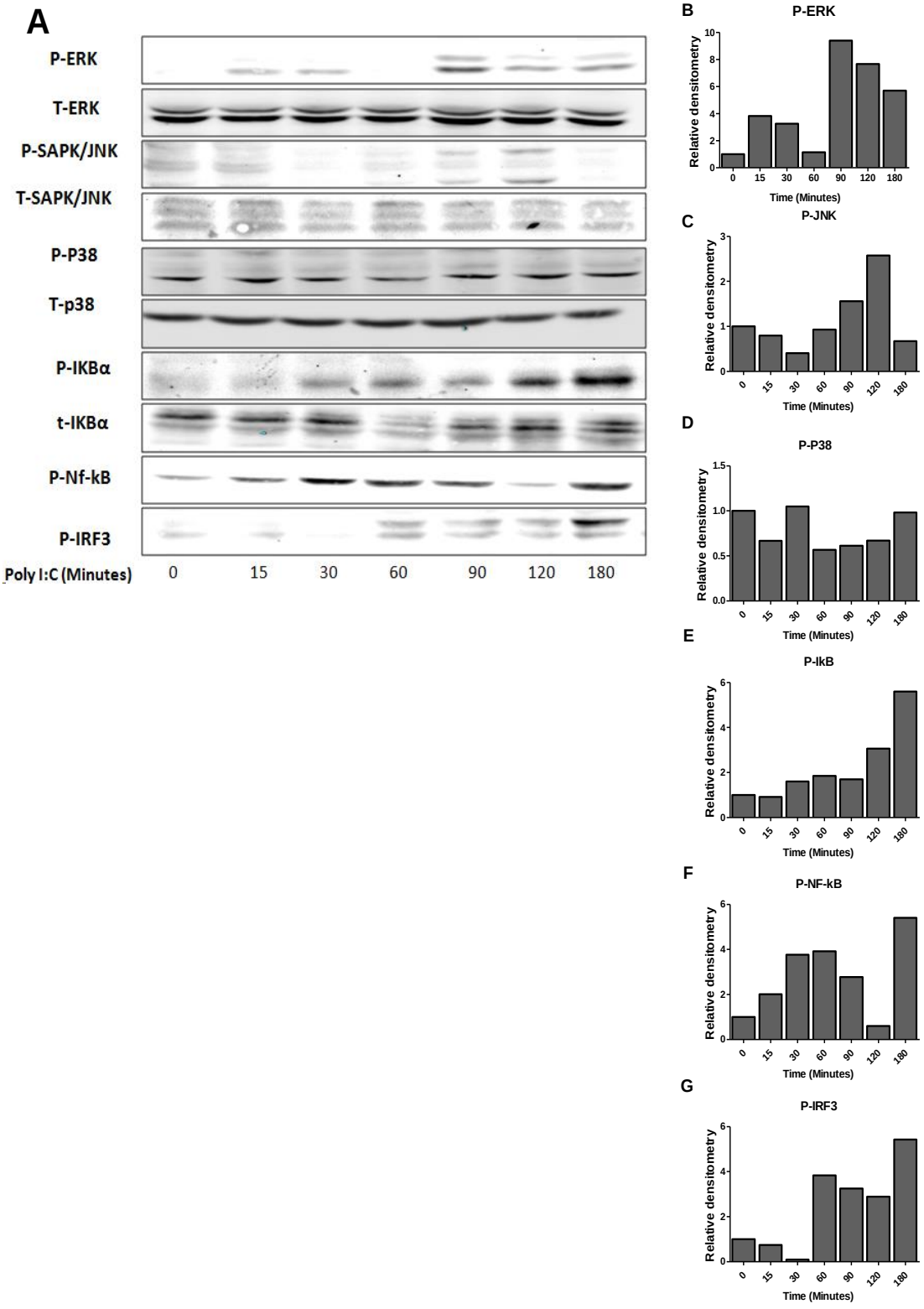


Figure 3.13. Time response effect of poly I:C treatment on protein phosphorylation in HCT 116 WT cells.

A) In order to determine the optimal time to examine each of the phosphorylation events, a time response was performed with poly I:C. Cells were treated with 10 μg/ml of poly I:C for 0, 15, 30, 60, 90, 120 and 180 minutes. Cell lysates were run on 10% gels and transferred to nitrocellulose membranes and imaged on the Licor Odyssey following incubation overnight with primary antibodies for the protein of interest. **B)** Densitometric analysis was performed in image studio.

3.4.4.2 Poly I:C time response in THP-1 Cells.

Following examination of the poly I:C time response in the HCT 116 cells, we sought to examine whether THP-1 cells were responsive to poly I:C, and if so, to what extent. As with the HCT 116 cells, cells were treated with 10 μg/ml poly I:C at 0, 15, 30, 60, 90, 120 and 180 minutes. Blots were performed as described in the methods, and images were obtained using the Licor Odyssey western blot scanner. As before, the phosphorylated proteins examined were P-ERK 1/2, P-SAPK/JNK and P-P-38 from the MAPK family, P-NF-κB, P-IκBα and P-IRF3. The analysis of the responses of the signalling proteins in the THP-1 cells (Figure 3.14) showcased the complete absence of a response to the stimulation with poly I:C. There was no measurable increase in P-ERK (fig 3.14B) levels at any of the timepoints measured (UT (1),15 (0.73), 30 (0.98), 60 (1.12), 90 (0.93), 120(1.27), 180(0.97)). Furthermore, P-SAPK/JNK (fig 3.14C) was similarly expressed across all treatment groups, with no discernible difference observed relative to the UT cells (UT (1),15 (0.83), 30 (1.31), 60 (1.30), 90 (0.76), 120 (1.07), 180 (1.16)). P-P38 (fig 3.14D) was similar, with no change in expression observed across groups (UT (1),15 (1.13), 30 (1.02), 60 (1.08), 90 (1.07), 120(0.93), 180(0.90)). Neither members of the NF-κB signalling pathway examined, P- IκBα (fig 3.14E) (UT (1),15 (0.26), 30 (0.44), 60 (0.72), 90 (0.38), 120 (0.79), 180 (1.02)) and its counterpart P-NF-κB (fig 3.14F) (UT (1),15 (0.48), 30 (0.60), 60 (0.59), 90 (0.98), 120 (0.93), 180 (0.86)), showed any change in expression following poly I:C stimulation across the timepoints measured. Finally, examination of P-IRF3 (fig 3.14G) levels revealed no significant changes in its expression (UT (1),15 (0.91), 30 (0.62), 60 (0.60), 90 (0.58), 120(0.49), 180(0.73)).. Thus, it appears the THP-1 cells are completely devoid of any measurable signalling response to TLR3 stimulation.

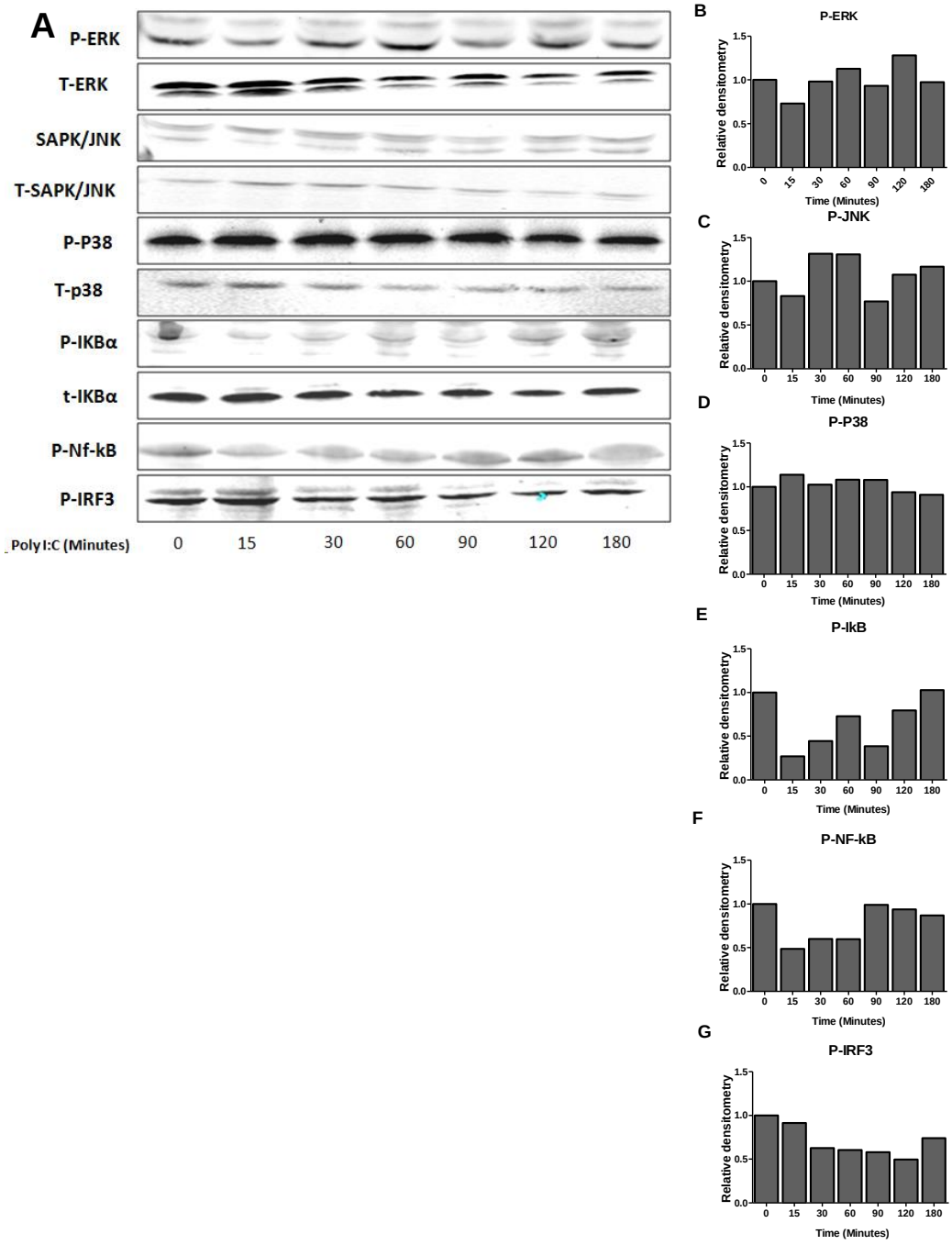


Figure 3.14. Effect of poly I:C treatment on protein phosphorylation in THP-1 cells.

A) In order to determine the optimal time to examine each of the phosphorylation events, a time response was performed with poly I:C. Cells were treated with 10 μg/ml

of poly I:C for 0, 15, 30, 60, 90, 120 and 180 minutes. Cell lysates were run on 10% gels and transferred to nitrocellulose membranes and imaged on the Licor Odyssey following incubation overnight with primary antibodies for the protein of interest. **B)** Densitometric analysis was performed in image studio.

3.4.4.3 Poly I:C time response in Macrophages

The last cell line which we examined the signalling cascade in was the THP-1 derived macrophages. We wished to examine whether the process of differentiation altered the responsiveness of the cell line to our ligand of interest. As with the other studies cells were treated with 10 μg/ml poly I:C for 0, 15, 30, 60, 90, 120 and 180 minutes and the phosphorylated proteins examined were P-ERK 1/2, P-SAPK/JNK and P-P38 from the MAPK family, P-NF-κB, P-IκBα and P-IRF3. The images showcasing the outcomes of these time response studies are visible in Figure 3.15. We observed no significant change in ERK (fig 3.15B) (0 (1),15 (1.03), 30 (0.96), 60 (0.53), 90 (0.96), 120(1.68), 180(0.54))or SAPK/JNK (fig 3.15C) (UT (1),15 (1.04), 30 (0.79), 60 (0.64), 90 (0.40), 120(0.62), 180(0.71)) phosphorylation in response to poly I:C stimulation. The same was true of P38 (fig 3.15D), with no measurable change in the expression present (UT (1),15 (0.96), 30 (1.14), 60 (1.23), 90 (1.07), 120(1.01), 180(1.07)).. When we examined the components of the NF-κB pathway however, we found that there was increased phosphorylation of IκBα (fig 3.15E) at 120 minutes post stimulation (UT (1),15 (1.02), 30 (1.19), 60 (1.03), 90 (0.83), 120(1.54), 180(0.32)).. This was followed by increased levels of P-NF-κB (fig 3.15F) at 120-180 minutes post poly I:C stimulation (UT (1),15 (0.54), 30 (0.69), 60 (1.09), 90 (1.02), 120(2.03), 180(1.33)).. Finally, P-IRF3 (fig 3.15G) was expressed highly at 120 minutes post stimulation (UT (1),15 (1.75), 30 (2.95), 60 (3.11), 90 (3.30), 120(3.5), 180(1.21)).. Thus, while the responses were not on par with those seen in the HCT 116 cells, it's obvious that there is significantly more signalling activity present upon differentiation of the THP-1 cells into macrophages.

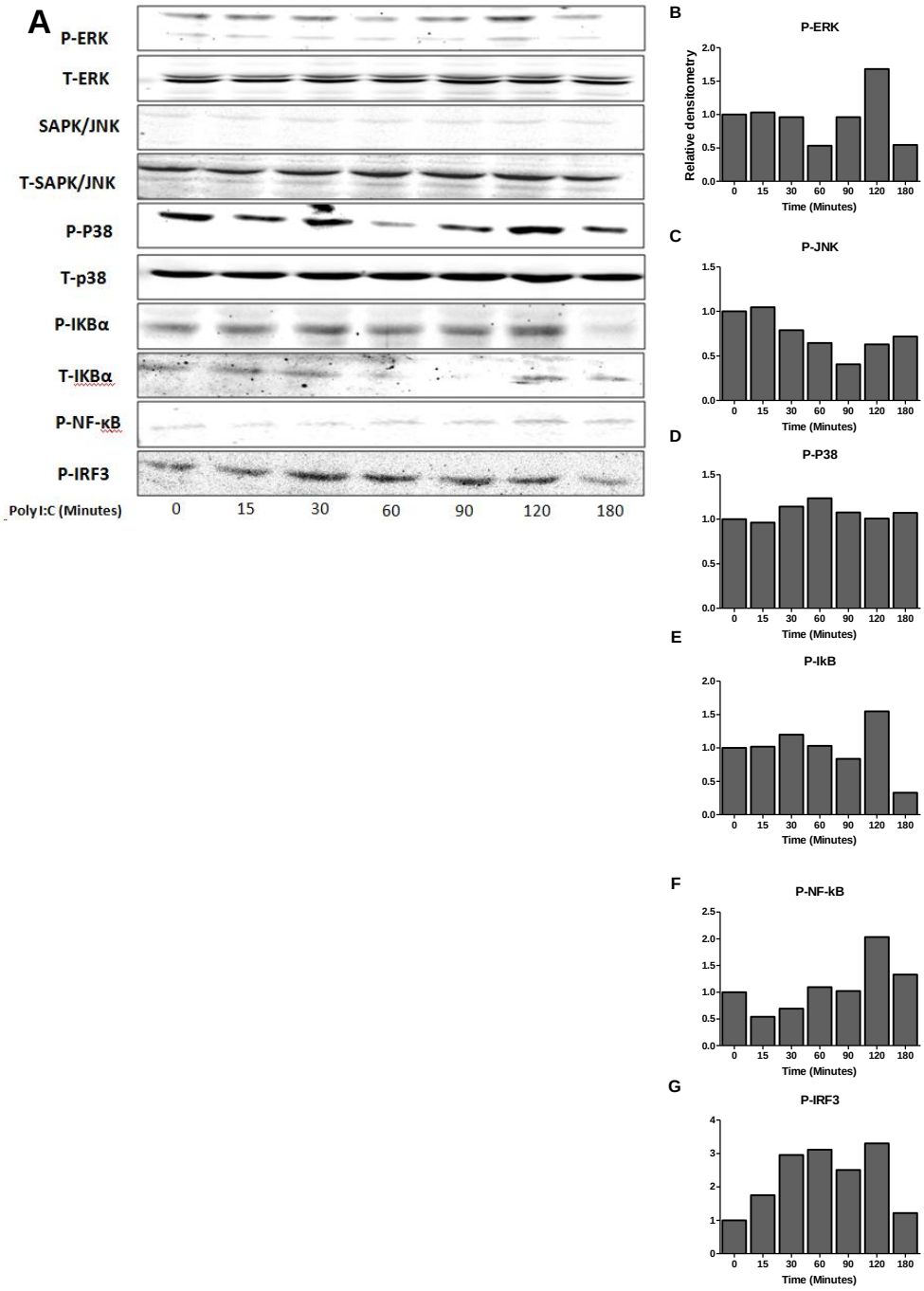


Figure 3.15. Effect of poly I:C treatment on protein phosphorylation in macrophage cells.

A) In order to determine the optimal time to examine each of the phosphorylation events, a time response was performed with poly I:C. Cells were treated with 10 μg/ml of poly I:C for 0, 15, 30, 60, 90, 120 and 180 minutes. Cell lysates were run on 10% gels

and transferred to nitrocellulose membranes and imaged on the Licor Odyssey following incubation overnight with primary antibodies for the protein of interest. **B)** Densitometric analysis was performed in image studio.

3.4.4.4 TLR3 Expression in HCT 116 cells, monocytes, and macrophages.

Following the results which outlined the differences in signalling protein activation to TLR3 stimulation, we examined whether TLR3 expression was different across the three cell lines used. In Figure 3.16A, we see that THP-1 cells have very low TLR3 expression relative to the other cell lines. The THP-1 derived macrophages have over 8 fold higher expression (THP-1 (1 ± 0.186), Macrophages (8.843 ± 1.6, $P < 0.001$)) when compared to the THP-1 cells. Expression in the HCT 116 cells is greater again, with over 60 fold increase in expression observed in these cells. (THP-1 (1 ± 0.186), HCT 116 (63.5 ± 6.3, $P < 0.001$)). Thus, this lagre disparity in TLR3 expression seen could explain the differences in responses to poly I:C stimulation observed in the different cells.

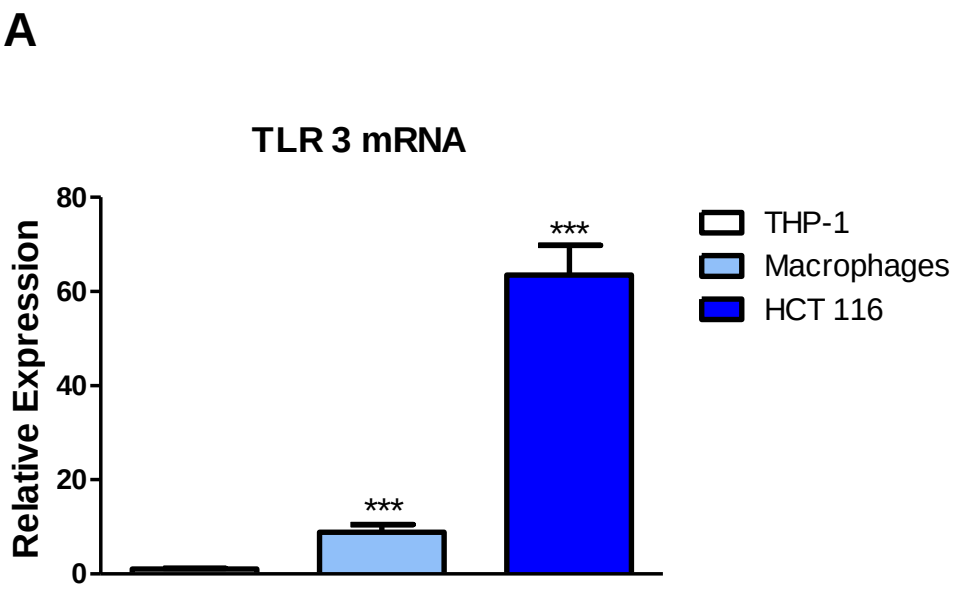


Figure 3.16. Relative TLR3 expression is lowest in the THP-1 cells, with increased expression seen in the macrophages and HCT 116 cells.

A). qPCR was performed to measure differences in TLR3 expression in the monocytes, monocyte derived macrophages, and HCT 116 cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a one way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. UT.

3.5 DISCUSSION

This initial results chapter was set to serve multiple purposes. Firstly, we wished to examine whether any of the drugs and compounds used in the course of the studies were cytotoxic, and if so, to what extent they affected cell viability. Secondly, we wished to investigate the basal expression of the TLR family, and how this expression was altered by epigenetic changes, such as those seen in the DKO HCT 116 cells. Following these studies, our observations led us to focus on TLR3, given the drastic change in expression seen in the HCT 116 cells upon genetic knockout of the methylation machinery. Once our focus had shifted to TLR3, we wished to perform some preliminary studies to determine the effects of cytokines on the expression of this receptor, as it may have proved important in the context of other changes seen as a result of stimulations or drug treatments. And finally we wished to examine the responsiveness of the TLR3 signalling pathway to various epigenetic modifications, which necessitated performing dose response studies in each cell line to determine the optimal points of examination for each of the signalling proteins of interest.

To determine the cytotoxicity of the various compounds used in our studies, we performed cell viability assays. Initially we used both the MTT and the Alamar Blue™ cell viability assays. Following the results of the initial study which showed comparable results in both assays, we decided to proceed with the MTT assay as the sole cell viability assay. Although there are cases in the literature which suggest that use of Alamar blue might be more appropriate (Rampersad, 2012), comparisons between the two assays showed near identical results.Upon examination of the effects of the numerous drugs and other compounds used in cell treatments in these studies, we were able to determine the effects of each treatment across different time points and

concentrations. Thus, we were able to select the most suitable concentration, which would result in a positive effect with minimal effects on cell viability. We selected the 10 µM dose for TSA and SAHA going forward, and the 5 µM and 500 nM doses were selected for 5-azacytidine and 5-aza-2-deoxycytidine, respectively. For our ligands, namely poly I:C, we selected the 10 µg/ml dose as the most suitable for our experiments. Our findings highlighted that,while certain TLR ligands were cytotoxic, the ligand of choice going forward, poly I:C, was relatively benign. This was in line with findings that showed previously that poly I:C did not induced significant cell death across a range of does, albeit in a cancer cell line (Stier et al., 2013). We decided to use all the cytokines at the same final concentration of 50 ng/ml. And finally, PMA, which was used to differentiate the THP-1 cell line into macrophages, was used at the lowest concentration shown of 1 ng/ml. This was sufficient to induce differentiation and as such did not necessitate the use of higher concentrations.

Following the examination of the effects of our various treatments on cell viability, we then sought to determine the effects of our epigenetic drugs on the expression of the TLRs. We examined the differences in mRNA expression of each of the TLRs in the WT HCT 116 cells, and the DKO cells which lack the DNA methyltransferases. As shown in the results we noticed significant changes in the expression of TLR3 between the two groups, with a decrease of over 100 fold seen in the DKO cell line. This dramatic result led us to focus our attention on TLR3, with its expression and regulation ultimately becoming the focus of this thesis. This was an interesting results, as there is no information currently direct effect of epigenetic modifications on TLR3 expression or function. There appears to be relatively little information in the literature regarding the regulation of TLR3, with other TLRs, such as

TLR4, often the focus of research. Thus, going forward, the focus of the studies was on TLR3, with much of it revolving around responses to stimulation with the TLR3 ligand poly I:C, and how treatment with the epigenetic modifying drugs alters cellular responses to stimulation of the receptor.

However before we proceeded with our studies with the epigenetic modifying drugs in our cell lines of interest, we first performed some preliminary studies. We wished to examine whether the presence of cytokines would have an effect on the expression of our receptor of interest, especially given that many of our treatment conditions were expected to lead to the production and release of cytokines. As the results showed, we did notice changes in TLR3 expression at the mRNA level, but these did not result in changes at the protein level. This suggests that the presence of cytokines was causing changes in mRNA level of TLR3, but the lack of carryover into protein expression would suggest that it was not altering the function of the receptor.

Finally, we wished to determine the best time point at which to measure each of the various signalling proteins, in order to determine whether the phosphorylation events involved in the signalling cascade can be altered by the addition of epigenetic modifying drugs. We examined the times at which we saw phosphorylation, with our initial studies being performed in the HCT 116 WT cell line. We determined that stimulation with poly I:C in the HCT 116 cells resulted in the phosphorylation of a wide array of signalling proteins at different time points, time points which have been observed in other cell lines where the poly I:C time response was measured (Ko et al., 2015; Mastri et al., 2012). However, upon examination of the response of the THP-1 cells to poly I:C, it was observed that the treatment did not result in any change in phosphorylation of proteins, yet, when the cells were differentiated into macrophages, they developed a response to

poly I:C, with changes in the levels of certain signalling proteins observed. Thus, going forward we had time points at which to examine the signalling cascade in the HCT 116 cells and the THP-1 derived macrophages. The absence of a response in the THP-1 cells was itself interesting, and led us to examine the difference in TLR3 expression across the different cell lines. TLR3 expression was the lowest in the THP-1 cells, which echoes the findings of (Carpenter et al., 2011), which highlighted that TLR3 expression was almost non-existent in THP-1 cells. Expression increased in the macrophages cells, and was highest in the HCT 116 cells. This finding makes sense from a physiological standpoint, with TLR signalling important in innate barrier function, one would expect a signalling response to stimulation with a viral ligand. The lack of response in the monocytes is not surprising, as the THP-1 cells lack TLR3, and the increased response of the differentiated THP-1 cells also makes sense from a physiological standpoint, as a macrophage response to viral infection is an important component of the innate immune response.

3.6 CONCLUSION

In this chapter we established the optimal dose a time points for the compounds we will used in the rest of the studies in this thesis. However, the main conclusion is the results observed with TLR3 expression and stimulation. We determined that removal of the DNMTs resulted in a significant decrease in TLR3 mRNA, a finding which has not been shown in the literature to date. Furthermore we established the different responses of the three cell lines to stimulation with the TLR3 ligand, poly I:C. and showed that THP-1 cells are extremely unresponsive to stimulation, which has foundation in the

literature, but we also showed that differentiation of THP-1 results in a more responsive cell state.

Chapter 4: The effect of epigenetic modifications on TLR3 expression and signalling in HCT 116 cells

4.1 INTRODUCTION

Toll-like Receptors (TLRs) are a family of innate immune receptors, which are part of a larger family of pattern recognition receptors (PRRs). This family of receptors recognise structurally conserved molecules known as pathogen associated molecular patterns (PAMPs) present on the surface of bacteria and viruses (Akira et al., 2006). Activation of these receptors results in the phosphorylation of cytoplasmic nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or interferon regulatory factors (IRF) 3/5/7 transcription factors, which, following activation translocate to the nucleus of the cell and induce transcription of inflammatory cytokines such as interferon-α/β, interleukin-6 and tumour necrosis factor α (Akira et al., 2006). The receptor family is accepted as having a large role in the innate immune response to microbial challenges as well as in the pathogenesis of autoimmunity (Maximiliano Javier Jiménez-Dalmaroni et al., 2015).

Toll-like receptor 3 is an extremely important member of the TLR family yet there is currently very little research into the regulation of this receptor. TLR3 was the first identified intracellular TLR, and it was originally shown to recognise the structure of polyinosinic-polycytidylic acid (poly I:C), a synthetic analogue of the double stranded RNA that is a structurally conserved component of viruses (Alexopoulou et al., 2001). Poly I:C mimics viral infection by inducing the production of type I interferons and other inflammatory cytokines. TLR3 is important physiologically as it has an essential role in the sensing of viruses such as West Nile virus, respiratory syncytial

virus (RSV), encephalomyocarditis virus and certain small interfering RNAs (Akira et al., 2006; Kawai and Akira, 2006). However, despite the importance of TLR3 in the innate immune system we are still unsure as to how its expression is regulated. Certain studies have shown that polymorphisms in TLR3 are associated with dysregulation of the receptor and poorer disease outcomes, with *tlr3* dysregulation linked to the development of Crohn's disease (Cario, 2010; Cario and Podolsky, 2000) and the development of type one diabetes (Assmann et al., 2014). With the knowledge that altered expression of TLR3 can lead to disease states, this suggested a possible role for epigenetic induced changes in the regulation of this receptor.

Rather than focusing on traditional changes in gene expression induced by altered nucleotide sequences, epigenetics is concerned with changes in gene expression induced by the remodelling of chromatin (Berger, 2002; Boyes and Bird, 1991; Sterner and Berger, 2000; Struhl, 1998). DNA methylation and histone acetylation are two of the main methods by which the cell regulates chromatin structure and function. DNA methylation generally results in epigenetic silencing, either by directly inhibiting transcription factor binding to chromatin, as seen in the case of the transcription factor erythroblastosis 1 (ETS1), (Bell and Felsenfeld, 2000; Maier et al., 2003) or by recruiting histone deacetylase complexes (HDACs), which remodel chromatin by removing histone bound acetyl groups which leads to the formation of a closed chromatin-complex which is inaccessible to transcription factors (Kuo and Allis, 1998). Given our initial results in chapter 3, we hypothesize that epigenetic mechanisms play a role in the regulation of *tlr3* expression. Thus, in this chapter we sought to investigate if epigenetic modification would alter the expression of TLR3 and interfere with its subsequent signalling pathway.

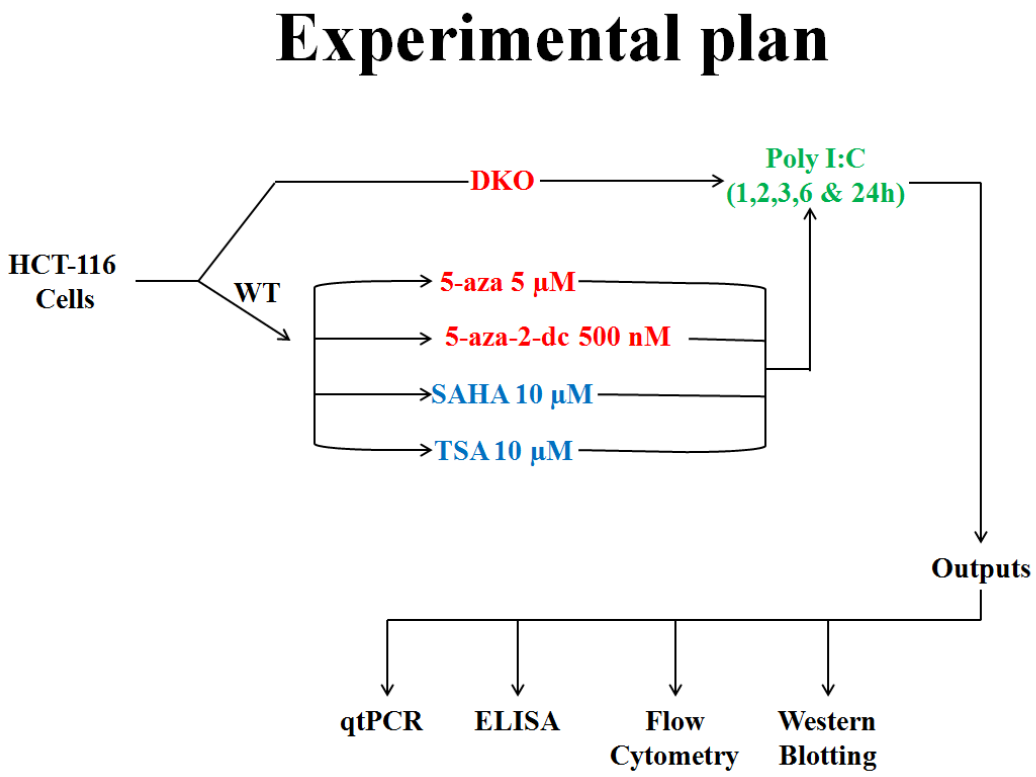
Epithelial cells are one of the most basic units of self defence against invading pathogens, acting as a physical and biomechanical barrier to external microorganisms. Dysregulated barrier epithelial cells have been implicated in the development of intestinal inflammation and IBD (Mankertz and Schulzke, 2007). There is also mounting evidence that suggests that prober barrier function is essential in host defence against viral infections, with the loss of epithelial barrier function shown to contribute to the development of systemic immune activation, which can propagate infections such as hepatitis and HIV (Cani et al., 2007). For these studies we used HCT 116 cells, which are an immortalised human colonic epithelial cell line. Using HCT 116 cells we examined the effect of inhibition of the two main epigenetic modifying mechanisms. DNA methylation was inhibited by either genetic knockout of the DNA methyltransferase enzymes or by pharmacological inhibition by 5-aza-2-deoxycytidine (Decitibine). HDAC enzymes were inhibited pharmacologically by the drug suberoylanilide hydroxamic acid (SAHA), which is a pan-HDAC inhibitor. The effect of these epigenetic modifications on stimulated TLR3 was measured by examining changes in TLR3 expression and activity as well as changes in the expression of important signalling proteins and inflammatory cytokines.

Thus, we hypothesize that TLR3 expression in HCT 116 cells will be altered by epigenetic modifications. Furthermore, we hypothesize that these epigenetic modifications will alter the signalling of TLR3 in response to stimulation, as well as altering the expression and release of cytokines.

4.2 METHODS

The methods used in this chapter did not differ from those outlined in materials and methods chapter.

4.3 EXPERIMENTAL DESIGN



The experimental design was followed as outlined in the graphic above. HCT 116 WT cells were treated with the epigenetic modifying drugs 5-aza-2-deoxycytidine and SAHA, following which they were treated with poly I:C. The effect of poly I:C on gene expression, and subsequently the effect of pretreatment with the epigenetic modifying drugs on gene expression, was measured using qPCR, western blotting,

ELISAS and flow cytometry. The concentrations and timepoints used for each of the drugs are as follows

Treatment	Concentration	Timepoints
Western Blotting		
poly I:C	10 µg/ml	30min, 60min, 90min, 120min, 240min
ELISAs		
poly I:C	20 µg/ml	24hr
qPCR		
poly I:C	10 µg/ml	6hr, 24hr
Flow Cytometry		
poly I:C	10 µg/ml	24hr
Ep**igenetic Drugs**		
5-aza-2-deoxycytidine	500 nM	72hr
SAHA	10 µM	48hr

Table 4.1. Concentrations and time points of ligands and drug treatments used in these studies.

4.4 RESULTS

4.4.1 TLR3 EXPRESSION VARIES IN DIFFERENT INTESTINAL EPITHELIAL REGIONS

Our interest in TLR3 expression led us to investigate TLR3 expression in a human context. We were able to obtain pinch biopsy samples (containing an majority of epithelial cells) from patients attending an endoscopy clinic, which allowed us access to both healthy controls, as well as patients who were diagnosed with IBD/Crohn's disease. To measure changes in TLR3 mRNA level we performed qPCR. Relative mRNA expression was quantified using **β**-Actin as an endogenous control. The fold change relative to the normal ileum group was calculated using the $2^{-\Delta Ct}$ method. A 2-way ANOVA was used to compare the means and p value of $p < 0.05$ was considered statistically significant. We observed (fig 4.1A) that there were differences in TLR3 expression across the different regions of the healthy controls, however these did not reach significance. We did notice however, that there was a significant difference in expression in the rectum of the IBD/Crohns patients (0.257 ± 0.043, $P < 0.01$ vs. normal ileum and IBD/Crohns ileum) when compared to the ileum of both the normal (1 ± 0.154) and the IBD/Crohns (1.216 ± 0.255) patients. Although there were no significant differences between the other groups, the data highlights the diversity of TLR3 expression across the different regions of the GI tract, as well as in disease states. These results led us to develop a further interest in TLR3, with a goal to understanding what exactly is regulating its expression.

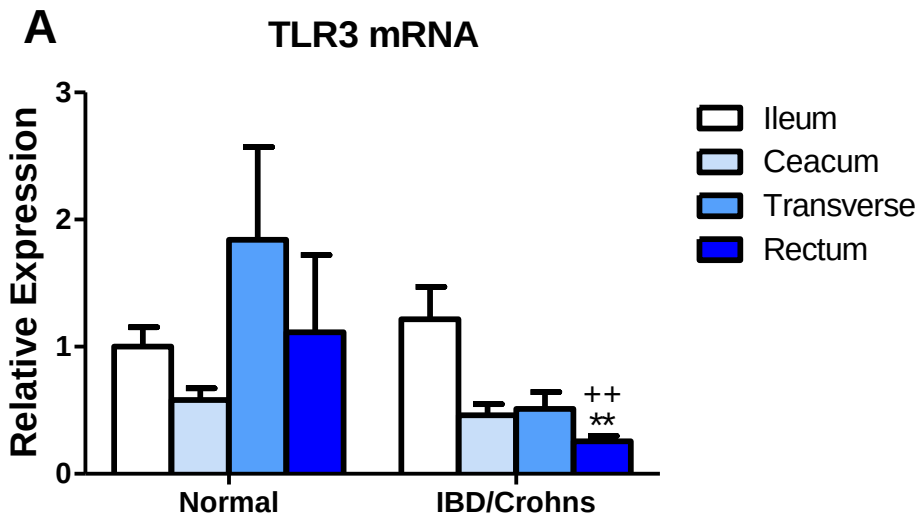


Figure 4.1 Variations in TLR3 expression were observed across intestinal epithelial regions

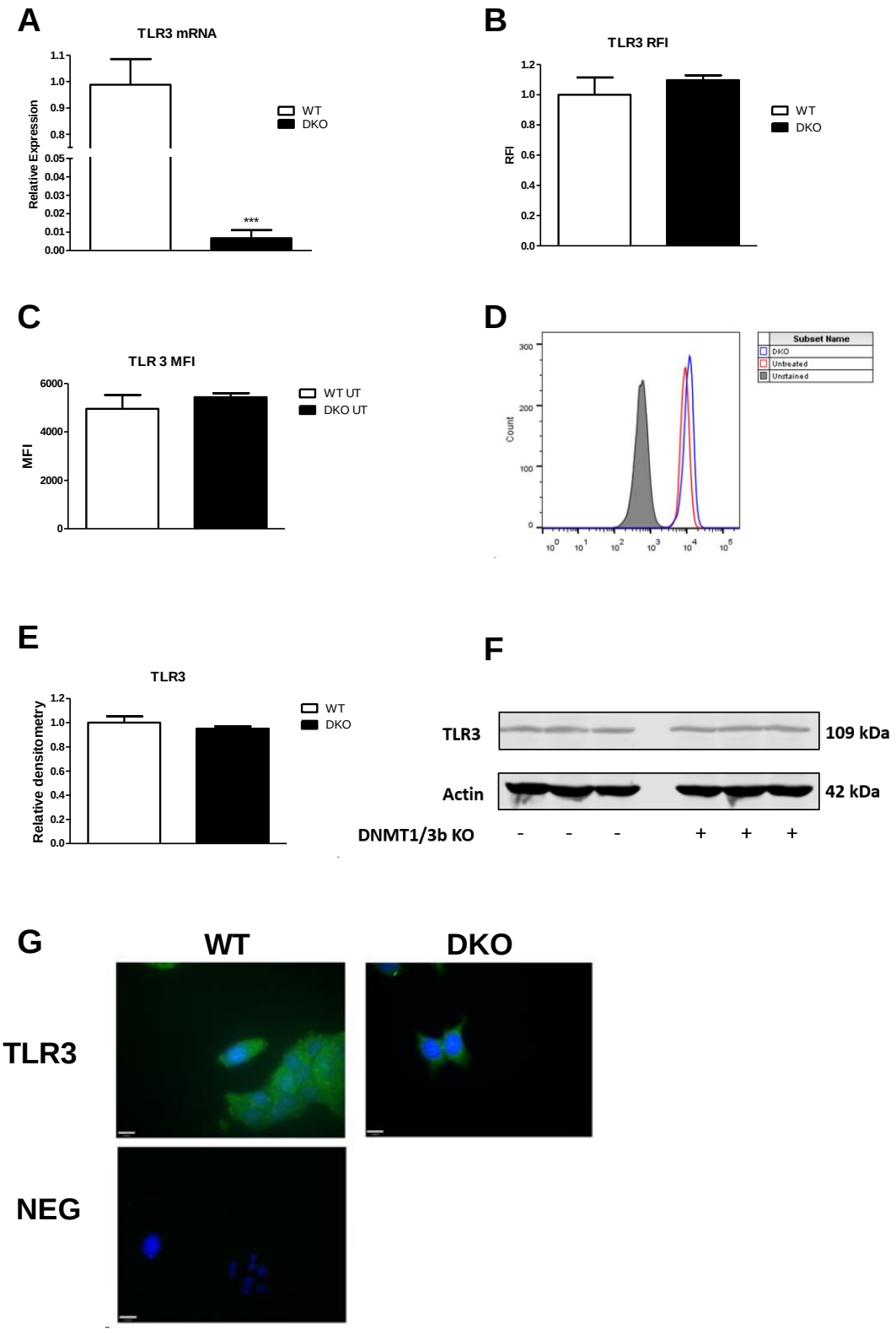
A) Real time PCR analysis was performed to examine the basal levels of TLR3 mRNA in biopsy samples taken from both normal (n = 9) and IBD/Crohn's (n = 5) patients. RNA expression was quantified using the Roche LightCycler 480 and the fold change relative to the normal ileum group was calculated. A 2 way ANOVA was used to examine differences between groups. $P < 0.05$ was considered significant. ** = $P < 0.01$ vs Ileum normal, ++ = $P < 0.01$ vs Ileum IBD/Crohns

4.4.2 Basal TLR3 mRNA and protein expression was measured in HCT 116 WT and DKO cells.

To determine the basal levels of TLR3 mRNA in HCT 116 cells, qPCR was performed on WT and DKO cell lines. Relative mRNA expression was quantified using β -Actin as an endogenous control. The fold change relative to the WT group was calculated using the $2^{-\Delta Ct}$ method. A students t-test was used to compare the means and P value of $P < 0.05$ was considered statistically significant. TLR3 mRNA expression was significantly lower in the DKO cells (0.0023 ±0.0061, $P < 0.001$) when compared to the WT cells (1 ± 0.0980), which amounted to almost a 500 fold decrease in expression in the DKO cells.

Following the analysis of the mRNA levels, we sought to examine if a similar decrease in TLR3 protein level would be seen in the DKO cells when compared to the wild type. To examine this, we performed intracellular flow cytometry to determine the expression of TLR3 protein in both cell lines. Shown in Figure 4.2 B and C are the RFI and MFI values respectively. Statistical analysis showed no difference between the WT (1 ± 0.114) and DKO (1.096 ± 0.032, $P > 0.05$) protein levels as shown by RFI. The raw MFI values are also shown and similarly there was no difference between the WT (4962±566.65) and DKO (5441.33±159.63, $P > 0.05$). The change observed in mRNA expression in the DKO cells when compared to the WT cells led to suspicions that the antibody used for TLR3 protein detection may not be function as intended. In order to address this, we performed a number of other protein based studies in the HCT 116 cell line. In figure 4.2E, the densitometry for western blots (Images shown in fig 4F) of TLR3 protein expression in WT and DKO cells is shown. The densitometry values were similar across both groups (WT (1 ± 0.052), DKO (0.95 ± 0.02, $P > 0.05$). This result

echoed the result seen with the flow cytometry. We then performed immunocytochemistry in WT and DKO cells, to determine if there were any observable changes in expression or localisation of TLR3 protein. As shown in figure 4.2G, there was no difference in expression or localisation observed between the two cell lines. Finally, in Figure 4.2H and Figure 4.2I, we performed flow cytometry again, with a different antibody, and the same result was observed as seen previously. There was no observable difference in TLR3 protein in the DKO cells when compared to the WT cells. Thus, across four separate instances of analysing TLR3 protein levels, we could not detect and difference in protein expression between the WT and DKO cells. This data shows that, although the mRNA expression of TLR3 is affected by the knockout of DNMT1/3b, it appears the same isn't true for the protein expression.


A
TLR3 mRNA
Relative Expression
WT
DKO
***
B
TLR3 RFI
RFI
WT
DKO
C
TLR 3 MFI
MFI
WT UT
DKO UT
D
Subset Name
DKO
Untreated
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E
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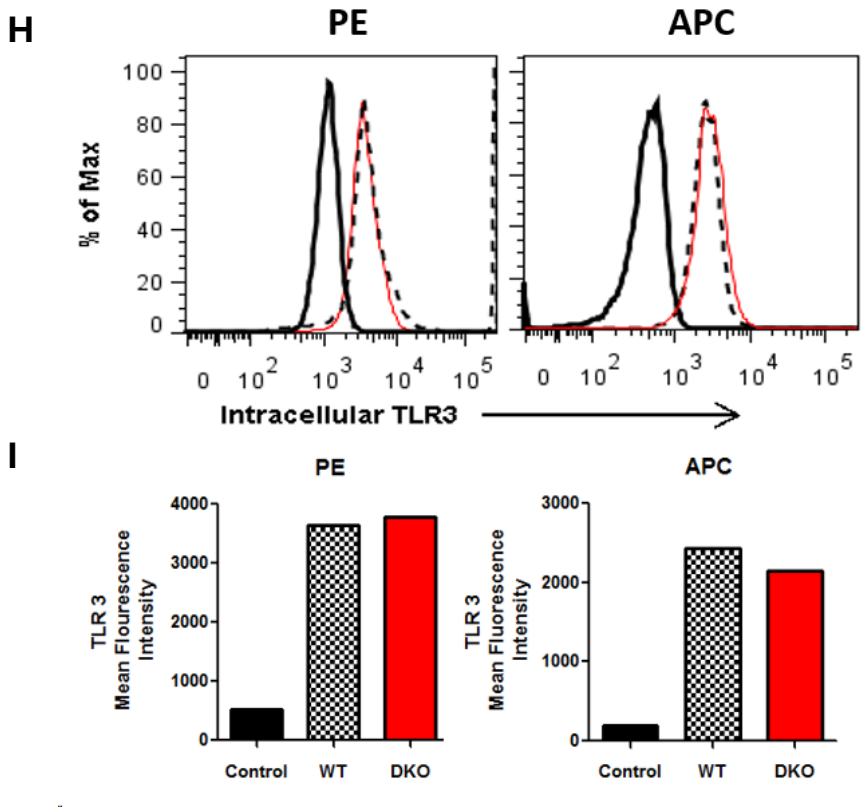

Figure 4.2. Genetic knockout of DNMT1/3b decreases TLR3 mRNA with no effect on protein

A) Real time PCR analysis was performed to examine the basal levels of TLR3 mRNA expression in both the WT and DKO cell lines. RNA expression was quantified using the Roche Light Cycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. WT. **B)** Shown is the relative fluorescence intensity (RFI) of the TLR3 PE (Miltenyi) stain in both WT and DKO HCT 116 cells. The mean fluorescence intensity is shown in **C)**, and histograms of raw values are shown in **D). E)** Densitometry was performed on western blot images (shown in **F)**) of TLR3 in WT and DKO HCT 116 cells. B-actin was used as an endogenous control and data was relative to WT UT. **G)** Immunocytochemistry was performed on WT and DKO

HCT 116 cells to examine whether there was observable changes in TLR3 expression or localisation. Antiboy used was Invivogen TLR3 antibody (Mab-hTLR3). **H-I)** TLR3 expression was analysed by flow cytometry using an Invivogen TLR3 antibody to confirm the flow findings seen with the miltenyi antibody. All flow cytometry data was generated using the BD Facs Canto II Flow Cytometer and analysed using FlowJo software. Immunocytochemistry images were generated using the Olympus IX81 Microscope with optigrid slider. Western blot mages were acquired using the Licor Odyssey with images generated using Image studio lite.

4.4.3 KNOCKOUT OF DNMT 1/3B PREVENTS POLY I:C INDUCED INCREASES IN TLR3 AND INFLAMMATORY CYTOKINE MRNA EXPRESSION.

We next sought to examine the effect of DNMT knockout on the response to poly I:C stimulation. QPCR was performed to examine changes in mRNA levels of TLR3 and the relevant cytokines, with relative expression being quantified using β-Actin as an endogenous control. The fold change relative to the WT UT group was calculated using the $2^{-\Delta Ct}$ method. . Using ELISAs we measured the cytokine release from HCT 116 WT and DKO cells. Data shown is in pg/μl of the cytokine being measured. For both the PCR and ELISA data, statistical significance was quantified using a 2 way ANOVA followed by a Bonferroni post hoc test. All data is expressed as mean ± SEM.

The effect of TLR3 stimulation by poly I:C on TLR3 mRNA levels was examined as shown in Figure 4.3A. Following 24 hour poly I:C stimulation , there was a significant increase in TLR3 mRNA expression in the WT cells (5.347 ± 0.735, $P<0.001$) when compared to the WT untreated group (1.00 ± 0.117). This was not the case in the DKO cells, where the administration of poly I:C for 24 hours did not result in an increase in TLR3 mRNA, with the DKO UT cells (0.002 ± 0.003) showing similar expression to the poly I:C treated DKO cells (0.008 ± 0.0012, $P>0.05$).

Upon examination of the changes in TLR3 protein levels (Fig 4.3B), as measured by flow cytometry, we found that, although there was a significant increase in the levels of TLR3 in the poly I:C treated WT group (WT UT (1.00 ± 0.114) vs. WT poly I:C (1.51 ± 0.07, $P < 0.001$), no such change was seen in the DKO group, with similar expression seen in both the DKO UT (1.09 ± 0.032) and the DKO + poly I:C groups (0.942 ± 0.16, $P > 0.05$).

When we examined the expression of the inflammatory cytokines, a similar pattern was seen (Figure 4.3C-H). Following 6hr poly I:C stimulation, there was a significant increase in the mRNA expression of IL6 (WT UT (1.00 ± 0.231) vs WT poly I:C (67.020 ± 6.826, P<0.001)), TNFα (WT UT (1.00 ± 0.090) vs WT poly I:C (53.066 ± 3.638, P<0.001)) and IFNβ (WT UT (1.00 ± 0.208) VS WT poly I:C (11.386 ± 0.473, P<0.001)), and similarly to the result seen with the TLR3 data, there was no significant change in expression in any of the cytokines in the DKO cells (IL6 (DKO UT (3.165 ± 0.553) vs DKO poly I:C (3.146 ± 0.635, P>0.05)), TNFα (DKO UT (0.924 ± 0.253) vs DKO poly I:C (1.738 ± 0.244, P>0.05)) and IFNβ (DKO UT (0.927 ± 0.275) VS DKO poly I:C (0.078 ± 0.009, P<0.001)).

The ELISA data showed similar findings to what was seen at the mRNA level. Following stimulation with poly I:C for 24 hours there was a significant increase in the release of IL6 (fig 4.3D) from the WT poly I:C treated cells (14.057 pg/µl± 1.819, P<0.001) when compared to the untreated WT cells (1.168 pg/µl ±0.251). Similarly to the mRNA results seen above, there was no such response in the DKO cells, with no significant difference seen between the DKO UT (0.243 pg/µl ±0.032) and the DKO poly I:C (1.313 pg/µl ±0.490, P>0.05) treatment groups. Similar results were also seen with TNFα release (fig 4.3F), with poly I:C treated WT cells (81.97 pg/µl ±7.686, P<0.001) showing a significant increase in TNF release when compared to the WT UT Cells (15.987 pg/µl ± 1.778). Again this was not the case in the DKO cells with poly I:C treatment having no significant effect on TNF release when compared to the DKO UT group (DKO poly I:C (21.55 pg/µl ± 0.651) VS DKO UT (14.87 pg/µl ± 1.304, P .0.05). The largest cytokine release observed was with IFNβ (fig 4.3H), with poly I:C treatment resulting in a significant increase in cytokine release when compared to the WT UT cells

(WT UT (91.8 pg/ml ± 13.9), WT poly I:C (1512 pg/ml ± 289, $P < 0.01$). However, in keeping with the trends set by the other cytokines, no effect of poly I:C was observed on the DKO cells, with no significant difference in cytokine release seen between the DKO UT (182 pg/ml ± 16) and the DKO poly I:C group (149 pg/ml ± 15.96, $P > 0.05$). This data would suggest that, similarly to what was seen in the mRNA data, the removal of DNMT 1/3b prevents the poly I:C induced cytokine response.

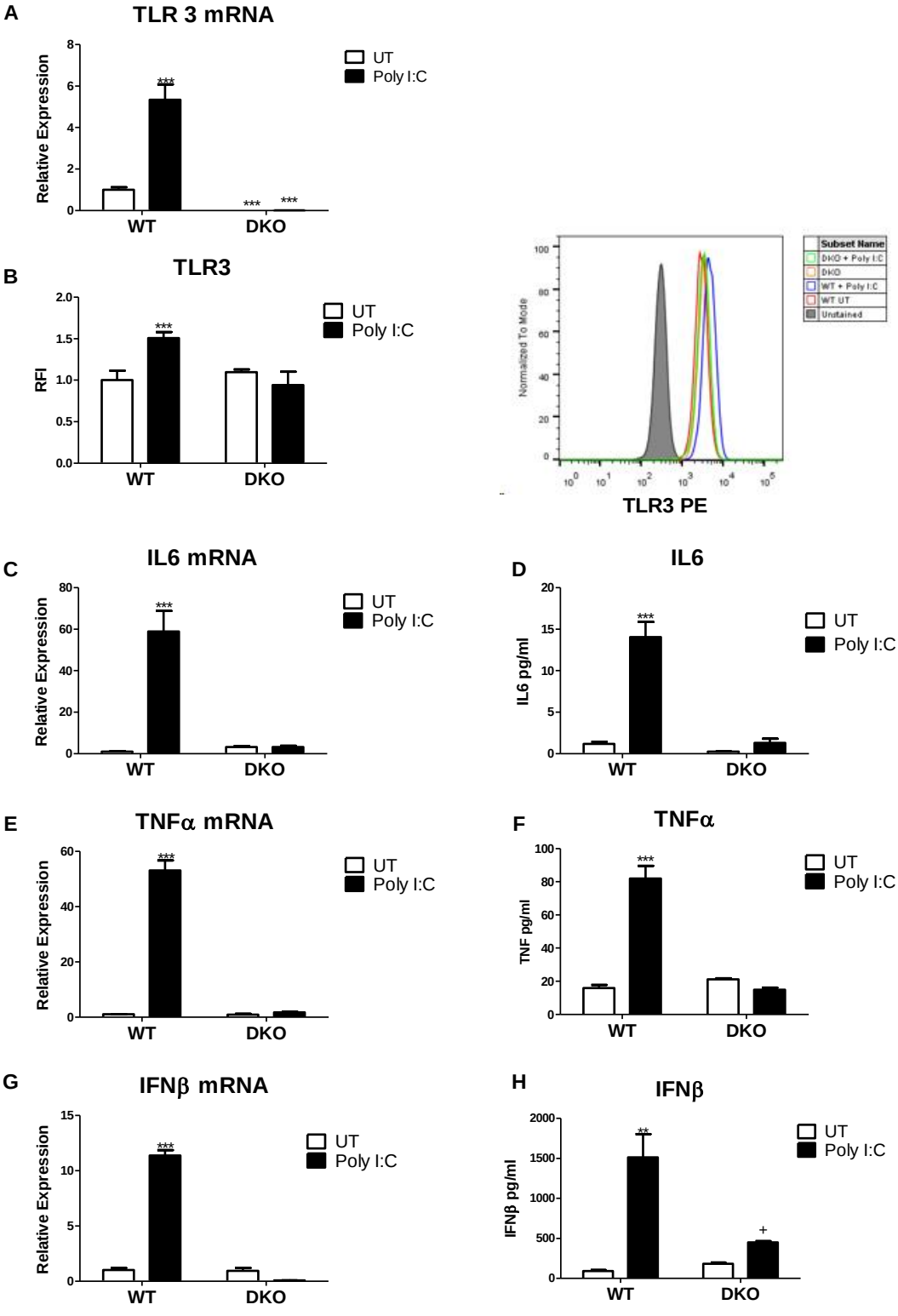

Figure 4.3. DNMT 1/3b Knockout prevents poly I:C induced increases in TLR3 expression, and cytokine expression and release.

A) TLR3 mRNA levels in WT & DKO cells as measured by qPCR. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. WT untreated. **B)** TLR protein expression was measured using flow cytometry. Fluorescence data was generated using the BD FACS Canto II Flow Cytometer and analysed using FlowJo software. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. WT untreated. Flow cytometry histograms are shown on right **C, E, G)** IL6, TNFα, & IFNβ mRNA levels in WT & DKO cells as measured by qPCR. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs WT untreated. ### = $P < 0.001$ vs WT poly I:C Treated. **D, F, H)** IL6, TNFα & IFNβ protein levels as measured by ELISA. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3,** = $P < 0.01$ vs WT untreated, *** = $P < 0.001$ vs. WT untreated.

4.4.4 DNMT 1/3B KNOCKOUT PREVENTS POLY I:C INDUCED SIGNALLING PROTEIN PHOSPHORYLATION.

Following the results seen with the TLR3 mRNA and protein changes in response to DNMT1/3b DKO, as well as the effects on cytokine release we observed, we wished to delve into the signalling cascade involved in transducing signals from TLR3 activation. In order to investigate this, we performed western blots to examine changes in the levels of phosphorylated signalling proteins form the MAPK family, namely P-ERK 1/2 , P-JNK, P-P38, as well as the components of the P-NF-κB signalling pathway as well as P-IRF3. The timepoints used for each protein were as follows, P-ERK (120 min), P-JNK (120 min), P-P38 (90 min), P-IκBα (180 min), P-NF-κB (180 min), and P-IRF3 (180 min). Blots were performed for three independent experiments, with a representative example shown in figure 4.4A. In figure 4.4B-G we show densitometry of each phospho-protein relative to β-Actin controls. The data is expressed as mean relative densitometry ± SEM.

In lane one of figure 4.4A we see the response of P-ERK to each of the different treatment groups. From both the image in lane 1, and the densitometry (3B), we saw a significant increase in ERK phosphorylation in the WT + poly I:C group (6.582 ± 0.25, $P < 0.001$) when compared to the WT UT group (1 ± 0.15). However no such effect was seen in the DKO cells, with the DKO UT and DKO + poly I:C groups showing similar expression of P-ERK relative to the WT UT group (DKO UT (0.62 ± 0.01, $P > 0.05$), DKO + poly I:C (0.875 ± 0.03, $P > 0.05$). In a similar vein to what we observed with P-ERK, there was also a significant increase in the levels of SAPK/JNK phosphorlyation in response to poly I:C treatment (4.4A Lane 3 and 4.4C). When the densitometry values were compared, there was a significant increase in the WT + poly I:C group (2.76 ± 0.1,

$P < 0.05$) when compared to the WT UT group (1 ± 0.433). The same was not evident in the DKO cells, with no significant difference between the DKO UT (0.6 ± 0.2) and the DKO + poly I:C group (1.1 ± 0.3, $P > 0.05$). Thus, it was apparent that knockout of the DNMTs was inhibiting both ERK and JNK/SAPK phosphorylation in response to poly I:C treatment. Upon examination of the final member of the MAPK family we investigated, P-P38 (Fig 4.4A lane 5, 4.4 D), we did not find and differences across any of our treatment groups. It was apparent that poly I:C treatment was not inducing P-38 phosphorylation, with values being similar to those seen in the WT UT group (WT UT (1 ± 0.05), WT poly I:C (0.9 ± 0.03, $P > 0.05$). There was also no significant changes in the DKO UT (0.95 ± 0.08) and the DKO + poly I:C (0.94 ± 0.08, $P > 0.05$) groups. P-IκBα (Fig 4.4A lane 7, 4.4E) was the first member of the NF-κB family examined, and we observed an significant increase in its phosphorylation in the poly I:C treated WT cells (4.4 ± 0.94, $P < 0.01$) when compared to the WT UT group (1 ± 0.15). However no such increase was observed in the DKO cells, with DKO UT (1.17 ± 0.03) and the DKO + poly I:C (0.984 ± 0.08, $P > 0.05$) showing similar protein levels. We observed a similar result pattern with P-NF-κB (Fig 4.4A lane 9, 4.4F), with poly I:C treatment of the WT cells leading to a significant increase in levels of the phosphorylated protein when compared to the WT UT cells (WT UT (1 ± 0.17), WT + poly I:C (2.25 ± 0.35, $P < 0.05$). Once again however, we observed no increase in expression in the DKO group with or without poly I:C treatment (DKO UT (0.9 ± 0.28), DKO + poly I:C (0.94 ± 0.3, $P > 0.05$).

The last protein we examined using densitometry was IRF 3 (Fig 4.4A lane 10, 4.4 G). Given its importance in TLR3 signalling, we were interested in its response to poly I:C treatment, and how this response may differ in the DKO cells. We observed a

significant increase in the levels of P-IRF 3 in the WT cells treated with poly I:C (WT UT (1 ± 0.16), WT + poly I:C (2.1 ± 0.2, $P < 0.01$). In keeping with the trend seen so far, this increase in phosphorylation following poly I:C treatment was not present in the DKO cells (DKO UT (1.3 ± 0.1), DKO + poly I:C (1.2 ± 0.1, $P > 0.05$). Thus, it appears that in the cases examined here, where poly I:C has the ability to induce phosphorylation of signalling proteins involved in the TLR3 signalling cascade, the removal of DNMT 1/3b prevents this poly I:C induced phosphorylation.

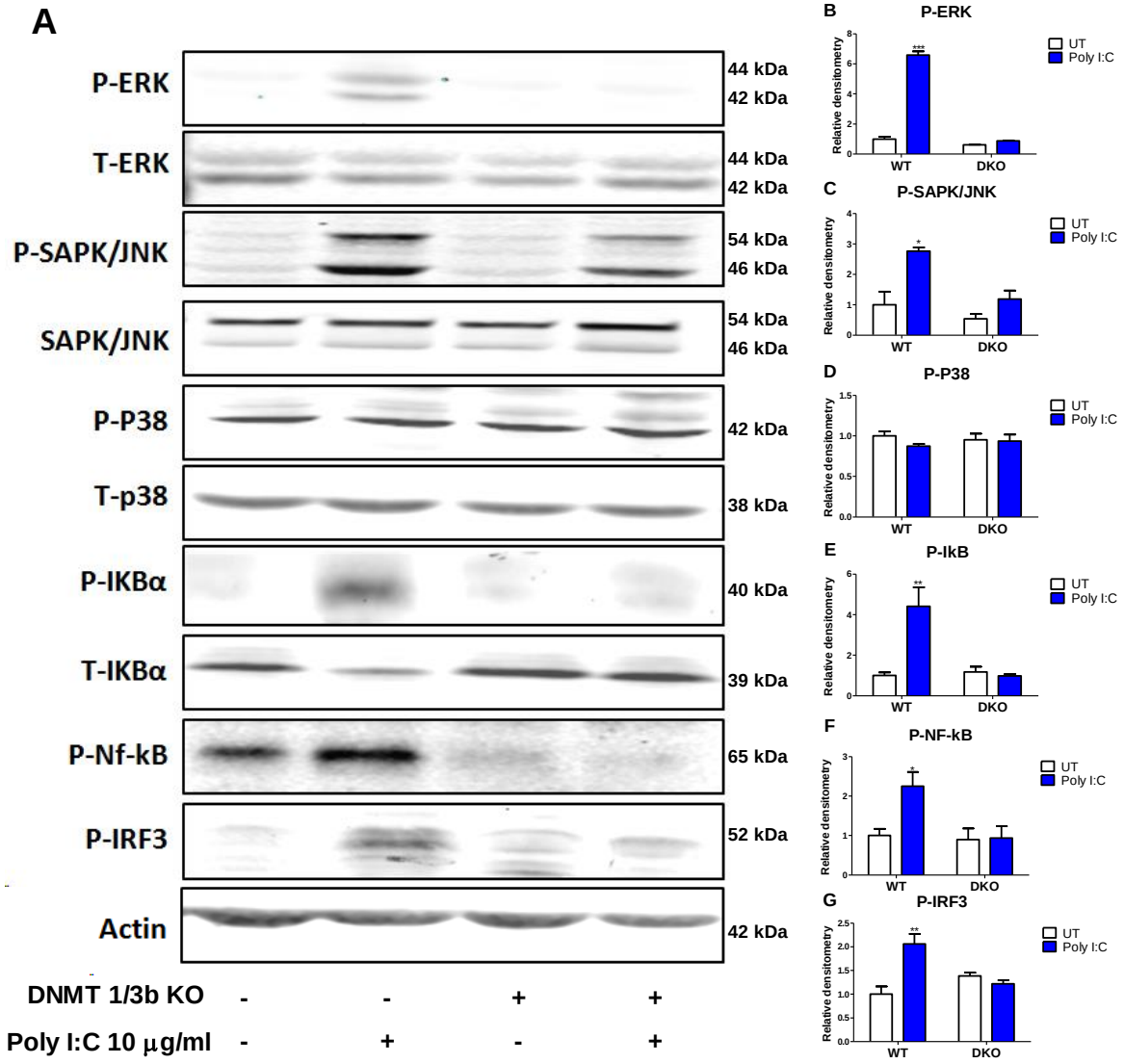

Figure 4.4. DNMT1/3b knockout prevented poly I:C induced phosphorylation of signalling proteins

A) Cell lysates were analysed using western blot analysis using phospho-specific antibodies. Images were acquired using the Licor Odyssey with images generated using Image studio lite. Blots shown are representative images. N=3 for all groups. **B-G)** Using analysis software in image studio lite, blots were analysed for densitometry, with each band being set relative to β-Actin loading control. Values

were then set relative to the UT group such that data is expressed as Mean relative densitometry ± SEM. Data was analysed using a 2 way ANOVA, with Bonferroni pots hoc test being used. $P < 0.05$ was considered significant. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, vs WT untreated.

4.4.5 5-AZA-2-DEOXYCYTIDINE DECREASES BASAL TLR3 MRNA EXPRESSION BUT DOES NOT ALTER BASAL TLR3 PROTEIN EXPRESSION.

We next sought to determine if pharmacological inhibition of DNMT1/3b would have a similar effect as the knockout of the enzymes, on TLR3 mRNA in HCT 116 cells. qPCR was performed on WT UT and WT 5-aza-2-dc treated cell lines. Relative mRNA expression was quantified using β-Actin as an endogenous control. The fold change relative to the UT group was calculated using the $2^{-\Delta Ct}$ method. A student's t-test was used to compare the means. A P value of $P < 0.05$ was considered statistically significant.

TLR3 mRNA (fig 4.5A) expression was significantly lower in the 5-aza-2-dc treated cells (0.673 ± 0.045, $p < 0.01$) when compared to the UT cells (1.01 ± 0.170), which amounted to a twofold decrease in expression in the 5-aza-2-dc treated cells. To confirm the effect seen with DNAM inhibition, we used another DNMT inhibitor, 5-azacytidine (fig 4.5B). We observed a similar result to that seen in the 5-aza-2-dc treated cells, with a significant decrease in mRNA expression in the 5-aza treated cells (0.538 ± 0.138, $P < 0.05$) when compared to the UT group (1 ± 0.336).

Following the analysis of the mRNA levels, we sought to examine if a similar decrease in TLR3 protein level would be seen in the 5-aza-2-dc treated cells when compared to the UT group. To examine this, we performed intracellular flow cytometry to determine the expression of TLR3 protein in both cell lines. Shown in Figure 4.5 C and 4.5D are the MFI and RFI values respectively. Statistical analysis showed no difference between the WT UT (2153 ± 178) and WT 5-aza-2-dc (2450 ± 121, $P > 0.5$)

protein levels as shown by MFI. The data is also expressed as RFI, and similarly there was no difference between the WT UT (1.00 ± 0.083) and WT 5-aza-2-dc treated cells (1.138 ±0.057, $P > 0.05$). This data shows that although the mRNA expression of TLR3 is affected by the inhibition of DNMT 1/3b, similarly to what was seen when we compared the WT and DKO cells, it appears the same isn't true for the protein expression

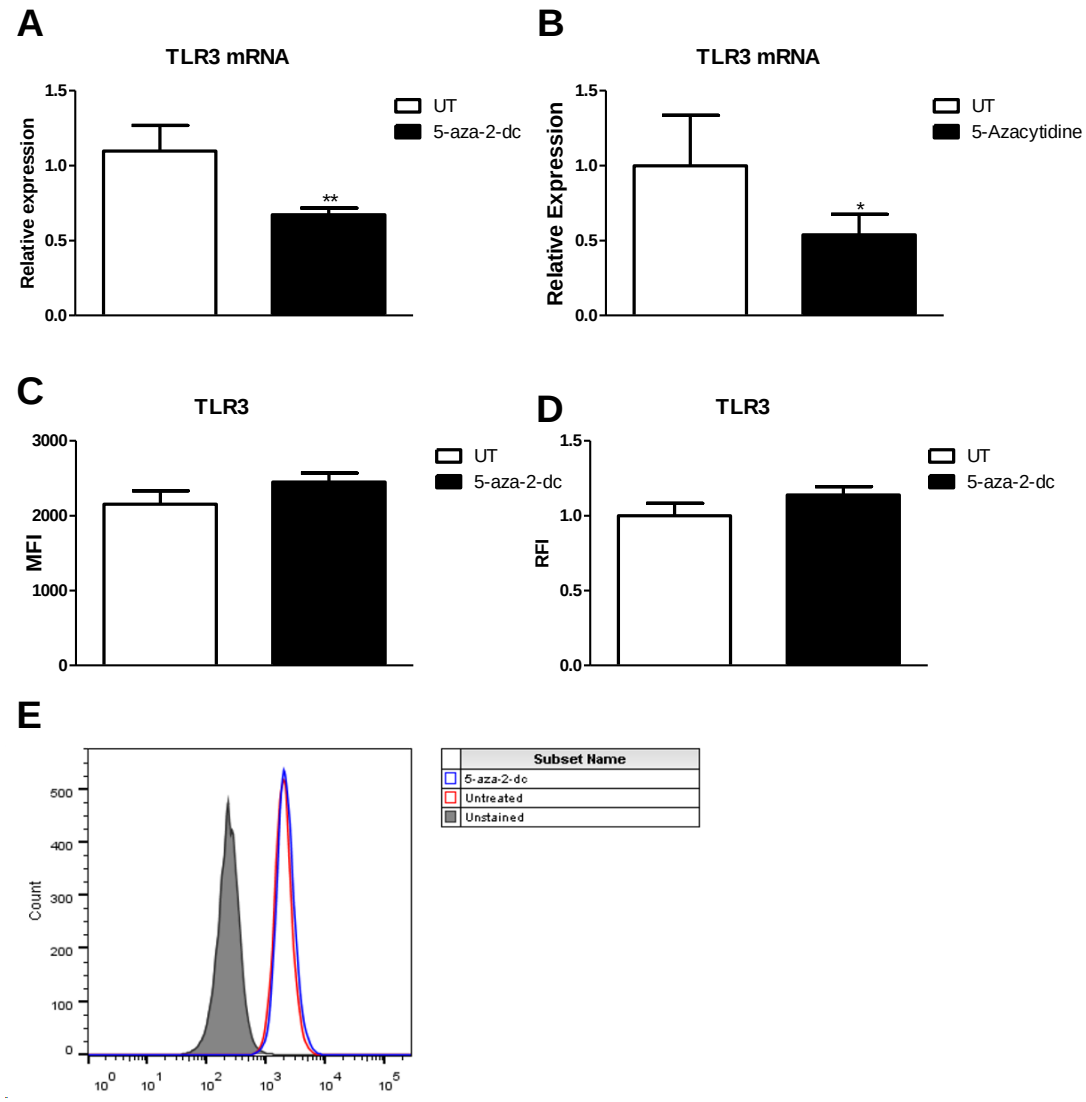

Figure 4.5. Treatment of HCT 116 cells with 5-aza-2-deoxycytidine decreases TLR3 mRNA expression but not TLR3 protein level.

A-B) Real time PCR analysis was performed to examine the levels of TLR3 mRNA expression in the UT, 5-aza-2-dc treated, and 5-azacytidine treated cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3,* = $P < 0.05$ vs UT ** = $P < 0.01$ vs UT. **C)** Shown is the relative fluorescence intensity (RFI) of the TLR3 PE stain in both UT and 5-aza-2-dc treated cells. The Mean fluorescence intensity is shown in **D)**, and the

histograms of the raw data are shown in **E).** All fluorescence data was generated using the BD Facs Canto II Flow Cytometer and analysed using FlowJo software**.**

4.4.6 PHARMACOLOGICAL INHIBITION OF DNMT 1/3B WITH 5-AZA-2-DEOXYCYTIDINE PREVENTS POLY I:C INDUCED INCREASES IN TLR3 AND INFLAMMATORY CYTOKINE EXPRESSION AND RELEASE.

We next sought to examine the effect of 5-aza-2-deoxycytidine on the response to poly I:C stimulation. qPCR was performed to examine changes in mRNA levels of TLR3 and the relevant cytokines, with relative expression being quantified using β-Actin as an endogenous control. The fold change relative to the WT UT group was calculated using the $2^{-\Delta Ct}$ method. ELISAs were used to examine cytokine release in response to poly I:C stimulation, and flow cytometry was used to examine changes in TLR3 protein levels. Statistical significance was quantified using a 2 way ANOVA followed by a Bonferroni post hoc test. All data is expressed as mean ± SEM.

We first examined the effect of 5-aza-2-dc on the response to poly I:C in the HCT 116 cells, with the results being graphically represented in figure 4.6. Similarly to what was seen in the DKO cells, the pharmacological inhibition of the DNMT enzymes resulted in the prevention of the poly I:C induced increase in TLR3 mRNA (Fig 4.6A). When The WT UT group (1.01 ± 0.170) was compared to the WT poly I:C treated group (2.727 ± 0.332, $P < 0.001$) there was clear significant increases in TLR3 mRNA following poly I:C stimulation. However, when we compared the 5-aza-2-dc treated groups there was a clear effect of inhibition of the DNMTs, as TLR3 mRNA expression was similar in both the 5-aza-2-dc (0.720 ± 0.038) and the 5-aza-2-dc + poly I:C groups (1.082 ± 0.082, $P > 0.05$).

A similar outcome was seen with the TLR3 protein level (Fig 4.6B), where it was evident that there was a significant increase in TLR expression in the poly I:C group (1.764 ± 0.1, $P < 0.001$) when compared to the UT group (1 ± 0.08). When the cells

were treated with 5-aza-2-dc for 72hr prior to the stimulation with poly I:C however, we saw no such increase in TLR3 protein level, with similar expression seen in the 5-aza-2-dc (1.1 ± 0.06) and the 5-aza-2-dc + poly I:C groups (1.2 ± 0.1, $P > 0.05$). Neither 5-aza-2-dc group differed from the UT group either; suggesting that 5-aza-2-dc alone was having no effect on TLR3 protein expression.

We then looked at the cytokine data and found similar expression patterns to those seen in the WT vs DKO cells. When we examined IL6 mRNA levels (Fig 4.6C), we observed a large increase in IL6 mRNA following poly I:C treatment (IL6 (UT (1.00 ± 0.407), poly I:C (6.612 ± 2.223, $P < 0.001$)), with no such change being seen in the 5-aza-2-dc + poly I:C group (5-aza-2-dc (0.503 ± 0.245), 5-aza-2-dc + poly I:C (0.533 ± 0.311, $P > 0.05$)). The findings seen at the mRNA level seemingly carried over to the protein level, with IL6 ELISAs (Fig 4.6D) showing that there was a significant release of IL6 in the poly I:C treated cells (UT (1.16 pg/ml ± 0.25), poly I:C (14 pg/ml ± 1.8, $P < 0.001$) which was inhibited by pre-treatment with 5-aza-2-dc (5-aza-2-dc (0.3 pg/ml ± 0.1), 5-aza-2-dc + poly I:C (1.3 pg/ml ± 0.8, $P > 0.05$)). A similar effect was seen with TNFα mRNA (Fig 4.6E), with treatment with poly I:C leading to significant increases in expression when compared to the UT group (UT (1.00 ± 0.365), poly I:C (9.58 ± 2.058, $P < 0.001$)). However, as with IL6, no such increase in TNFα expression was seen in the 5-aza-2-dc treated cells (UT (5-aza-2-dc (1.637 ± 0.354), 5-aza-2-dc + poly I:C (1.702 ± 0.240, $P > 0.05$)). Once again, the mRNA data was seemingly representative of the changes occurring at the protein level, as TNFα ELISAs (Fig 4.6F) showed a significant increase in cytokine release following poly I:C treatment (UT (15.9 pg/ml ± 1.7), poly I:C (81.9 pg/ml ± 7.6, $P < 0.001$). The pre-treatment of the cells with 5-aza-2-dc once again seemingly prevented this poly I:C induced increase in cytokine release, with no

significant difference in release observed between the 5-aza-2dc (14 pg/ml ± 1.37) and the 5-aza-2-dc + poly I:C group (23.7 ± 2.58, $P > 0.05$), with neither group differing from the UT group also.

Lastly, the IFNβ response was similar to that observed in the WT vs DKO cells. There was a significant increase in mRNA expression (Fig 4.6G) in the poly I:C treated cells when compared to their untreated counterparts (UT (1 ± 0.28), poly I:C (10.9 ± 2.3, $P < 0.001$)). Much like what was seen with the DNMT1/3b knockout cells, inhibition of methyltransferases with 5-aza-2-deoxycytidine prevented the poly I:C induced upregulation of IFNβ (5-aza-2-dc (1.6 ± 0.4), 5-aza-2-dc + poly I:C (1.6 ± 0.2, $P > 0.05$)). Similarly to what was seen with IL6 and TNF, the changes seen at the mRNA level translated to changes in cytokine release (Fig 4.6H). There was a significant release of IFN following poly I:C stimulation (UT (91 pg/ml ± 13.9), poly I:C (1512 pg/ml ± 289, $P < 0.001$). However this increase in cytokine release was abrogated by pre-treatment with 5-aza-2-dc (5-aza-2-dc (202 pg/ml ± 115.9), 5-aza-2-dc + poly I:C (570 pg/ml ± 140, $P > 0.05$). Thus, the data shown here would suggest that, similar to what we observed in the DKO cells, pharmacological inhibition of the DNMTs is sufficient to inhibit the cytokine response initiated by poly I:C stimulation.

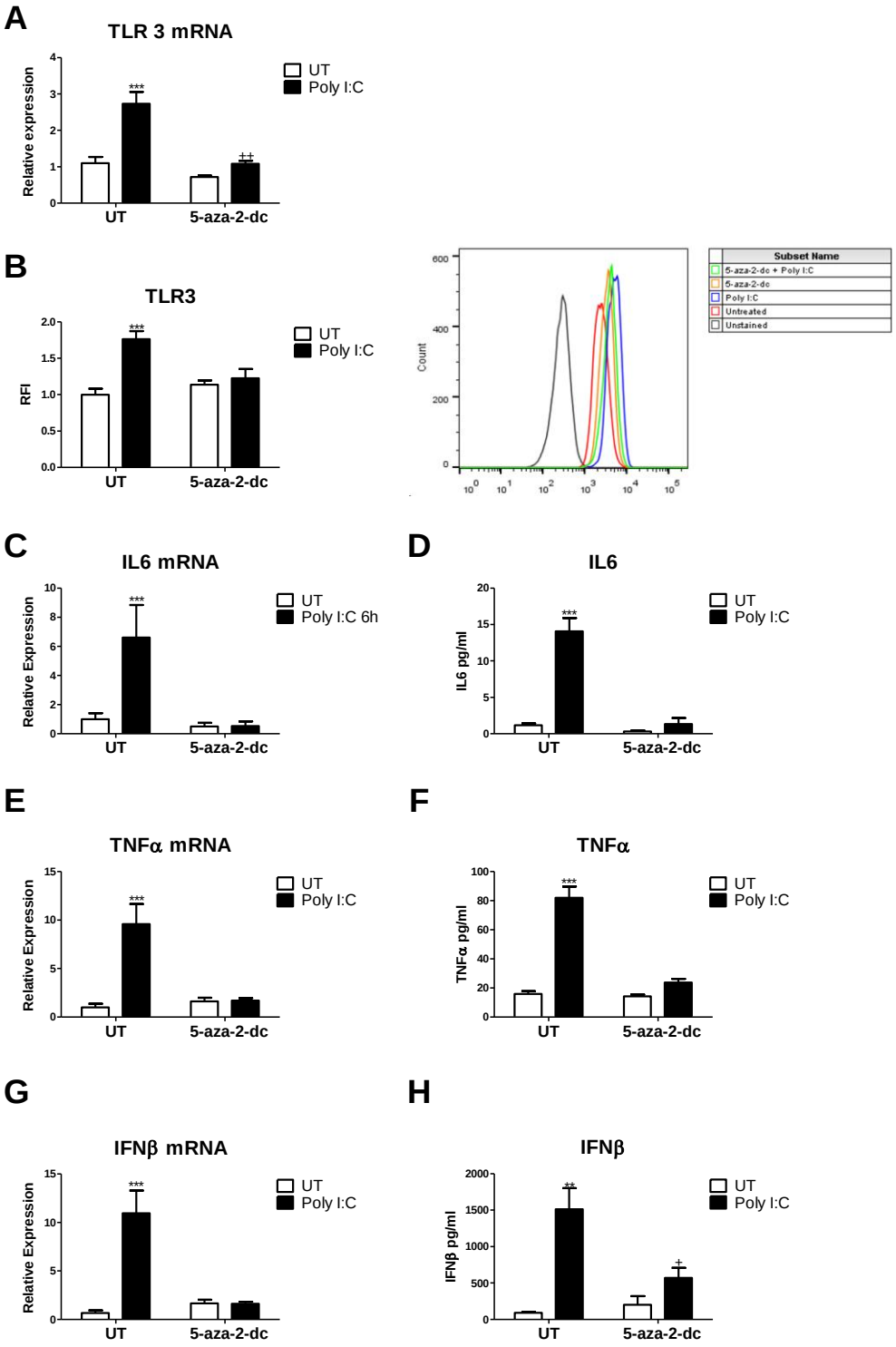

Figure 4.6. Pharmacological inhibition of DNMT 1/3b with 5-aza-2-dc decreases TLR3 mRNA expression and prevents poly I:C induced protein phosphorylation and cytokine release.

A) TLR3 mRNA levels in UT vs. 5-aza-2-dc treated cells as measured by qPCR. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. untreated. **B)** TLR protein expression was measured using flow cytometry. Fluorescence data was generated using the BD Facs Canto II Flow Cytometer and analysed using FlowJo software. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs untreated **C, E, G)** IL6, TNFα, & IFNβ mRNA levels in UT and 5-aza-2-dc treated cells as measured by qPCR. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. WT untreated. **D, F, H)** IL6, TNFα & IFNβ protein levels as measured by ELISA. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3,** = $P < 0.01$ vs. WT untreated, *** = $P < 0.001$ vs WT untreated.+ = $P < 0.05$ vs. 5-aza-2-dc + poly I:C treated

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4.4.7 PHARMACOLOGICAL INHIBITION OF DNMT 1/3B PREVENTS POLY I:C INDUCED SIGNALLING PROTEIN ACTIVATION.

Following our examination of the effect of pharmacological inhibition of DNMTs on TLR3 expression as well as cytokine expression and release, we sought to examine whether 5-aza-2-dc would have a comparable effect to DNMT1/3b knockout when it came to signalling proteins. The timepoints used for each protein were as follows, P-ERK (120 min), P-JNK (120 min), P-P38 (90 min), P-IκBα (180 min), P-NF-κB (180 min), and P-IRF3 (180 min). Blots were performed for three independent experiments, with a representative example shown in figure 4.7A. In figure 4.7B-G we show densitometry of each phospho-protein relative to β-Actin controls. The data is expressed as mean relative densitometry ± SEM.

In lane one of figure 4.7A, we see the response of P-ERK to each of the different treatment groups. From both the image in lane 1 and the densitometry (4.7B), we saw a significant increase in ERK phosphorylation in the poly I:C group (6.387 ± 0.551, $P < 0.001$) when compared to the UT group (1 ± 0.15). However, no such effect was seen in the 5-aza-2-dc treated cells, with the 5-aza-2-dc + poly I:C groups showing similar expression of P-ERK relative to the 5-aza-2-dc alone group (5-aza-2-dc (4.776 ± 0.22, $P > 0.05$), 5-aza-2-dc + poly I:C (5.675 ± 0.37, $P > 0.05$). It is worth noting however, that the level of P-ERK in the 5-aza-2-dc group alone was higher than in the UT (UT (1 ± 0.15), 5-aza-2-dc (4.67 ± 0.229)).

In a similar vein to what we observed with P-ERK, there was also a significant increase in the levels of SAPK/JNK phosphorylation in response to poly I:C treatment (figure 4.7A Lane 3 and 4.7C). When the densitometry values were compared there was

a significant increase in the poly I:C group (4.136 ± 0.1, $P < 0.05$) when compared to the UT group (1 ± 0.465). The same was not evident in the 5-aza-2-dc treated cells, with no significant difference between the 5-aza-2-dc alone (1.214 ± 0.37) and the 5-aza-2-dc + poly I:C group (0.97 ± 0.03, $P > 0.05$).

Upon examination of P-P38 expression (Fig 4.7A lane 5, 4.7D), we did not find and differences across any of our treatment groups. It was apparent, similar to what we observed in the WT vs DKO cells, that poly I:C treatment was not inducing P38 phosphorylation, with values being similar across all groups (UT (1 ± 0.04), poly I:C (0.9 ± 0.03, $P > 0.05$), 5-aza-2-dc, (0.95 ± 0.08), 5-aza-2-dc + poly I:C (0.825 ± 0.004, $P > 0.05$). P-IκBα (Fig 4.7A lane 7, 4.7E) showed a small, albeit significant, increase in its phosphorylation in the poly I:C treated cells (1.5 ± 0.2, $P < 0.05$) when compared to the UT group (1 ± 0.07). However, no such increase was observed in the 5-aza-2-dc treated cells, with 5-aza-2-dc alone (0.986 ± 0.09) and the 5-aza-2-dc + poly I:C (0.782 ± 0.03, $P > 0.05$) showing similar protein levels.

We observed a similar result with P-NF-κB (Fig 4.7A lane 9, 4.7F), with poly I:C treatment leading to a significant increase in levels of the phosphorylated protein when compared to the UT cells (UT (1 ± 0.107), poly I:C (10.02 ± 0.958, $P < 0.001$). Once again however, we observed no increase in expression in the 5-aza-2-dc treated group with or without poly I:C treatment (5-aza-2-dc (1.5 ± 0.229), 5-aza-2-dc + poly I:C (1.428 ± 0.3, $P > 0.05$).

The last protein we examined using densitometry was IRF 3 (Fig 4.7A lane 10, 4.7G), and we observed a significant increase in the levels of P-IRF3 in the cells treated with poly I:C (UT (1 ± 0.15), poly I:C (1.5 ± 0.2, $P < 0.05$). In keeping with the trend seen so far, this increase in phosphorylation following poly I:C treatment was not

present in the 5-aza-2-dc treated cells (5-aza-2-dc (0.812 ± 0.1), 5-aza-2-dc + poly I:C (0.6 ± 0.1, $P > 0.05$). Thus, similarly to what we observed in the WT vs DKO cells, pharmacological inhibition of the DNMTs prevents the phosphorylation of signalling proteins which is induced by poly I:C treatment.

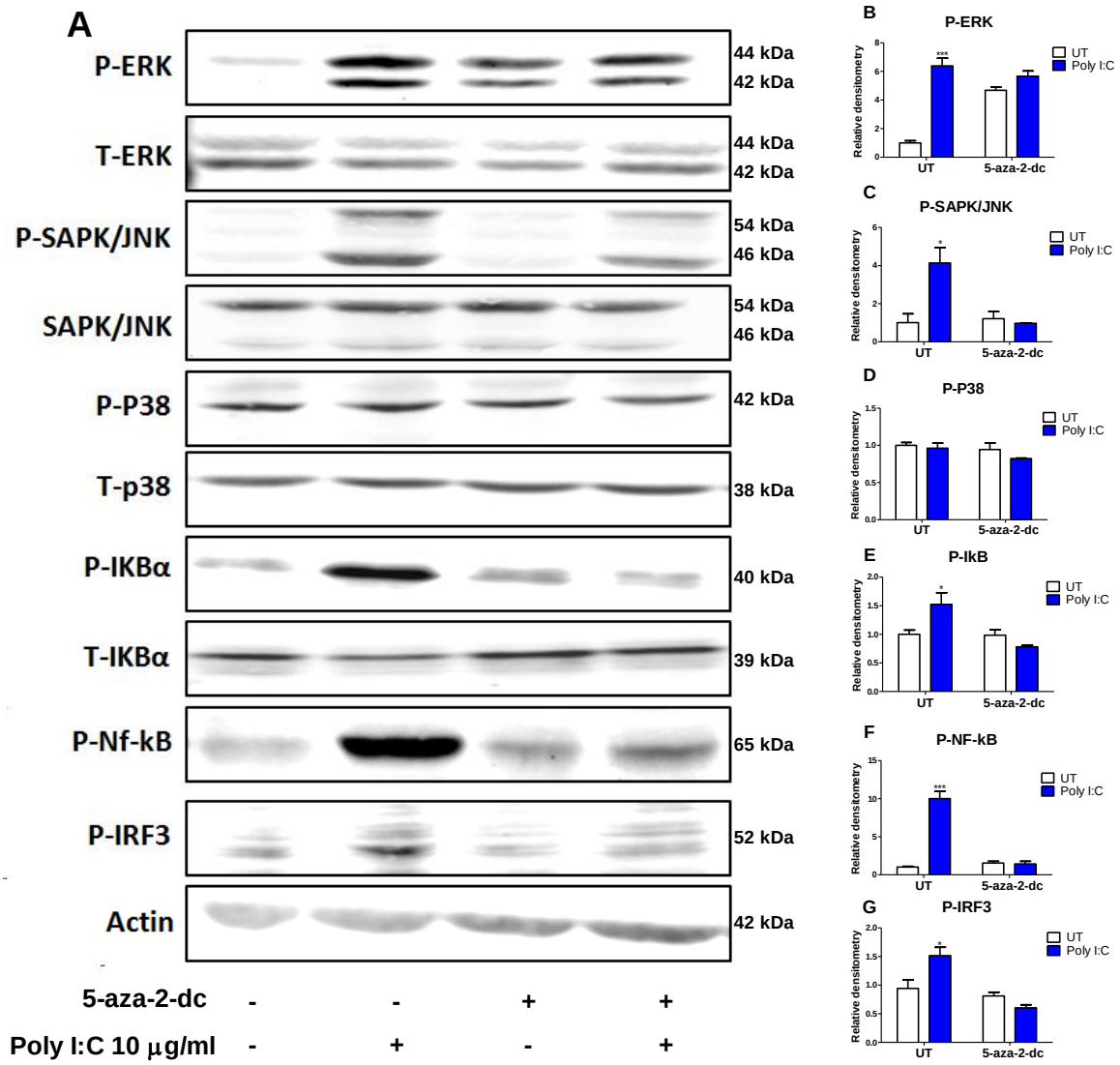


Figure 4.7. Treatment of HCT 116 cells with 5-aza-2-dc prevents poly I:C induced signalling protein phosphorylation

A) Cell lysates were analysed using western blot analysis using phospho specific antibodies. Images were acquired using the Licor Odyssey with images generated using Image studio lite. Blots shown are representative images. N=3 for all groups. **B-G)** Using analysis software in image studio lite, blots were analysed for densitometry, with each band being set relative to β-Actin loading control. Values were then set relative to the UT group such that data is expressed as Mean relative densitometry ± SEM. Data

was analysed using a 2 way ANOVA, with Bonferroni pots hoc test being used. $P < 0.05$ was considered significant. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, vs WT untreated.

4.4.8 PAN-HDAC INHIBITOR SAHA DECREASES TLR3 MRNA BUT NOT TLR3 BASAL PROTEIN LEVELS.

Following the results that DNMT knockout and DNMT inhibition alter TLR3 responses in HCT 116 cells, we examined the effects of HDAC inhibition on the expression of TLR3 and the subsequent signalling cascades and cytokine release. HDACs are recruited to deacetylate histones following the methylation of DNA so the two processes often go hand in hand. As before, qPCR was performed to examine changes in mRNA levels of TLR3 and the relevant cytokines, with relative expression being quantified using β-Actin as an endogenous control. The fold change relative to the UT group was calculated using the $2^{-\Delta Ct}$ method. Statistical significance was quantified using a 2 way ANOVA followed by a Bonferroni post hoc test. All data is expressed as mean ± SEM.

In concordance with what we had seen in the WT vs. DKO cells and the UT vs. 5-aza-2-dc treated cells, treatment with SAHA led to significant reductions in TLR3 mRNA levels. As seen in figure 4.8A, when compared to the WT UT group (1.00±0.226) there was a significant reduction in TLR3 mRNA expression following SAHA treatment (0.079±0.017, $P < 0.001$). The effect of the HDAC inhibitors was confirmed using another HDAC inhibitor, TSA (fig 4.8B). When we compared TLR3 mRNA expression in the UT and the TSA treated cells, we observed significantly lower TLR3 expression in the TSA treated cells (0.045 ± 0.01, $P < 0.001$) when compared to the UT cells (1 ± 0.072). This highlighted that the effects of SAHA are not drug specific.

Given the previous results seen, we expected that the large decrease in mRNA levels would not result in a decrease in TLR3 protein, and this was indeed the case. We again used flow cytometry to examine the changes in TLR3 protein expression and the results shown in figure 4.7C and 4.7D are expressed as RFI and MFI respectively. There

was no significant difference between the UT group (1.00 ± 0.042) and the SAHA treated group (1.184 ± 0.032, $P > 0.05$) RFI values for TLR3 expression. A similar result was seen when we compared the MFI values also, with no significance seen when the WT UT MFI (2632 ± 109.36) was compared to the WT SAHA MFI (3118 ± 84.96, $P > 0.05$). These results mirror the trend that was seen previously, with a large decrease in TLR3 mRNA not resulting in a decrease in TLR3 protein level.

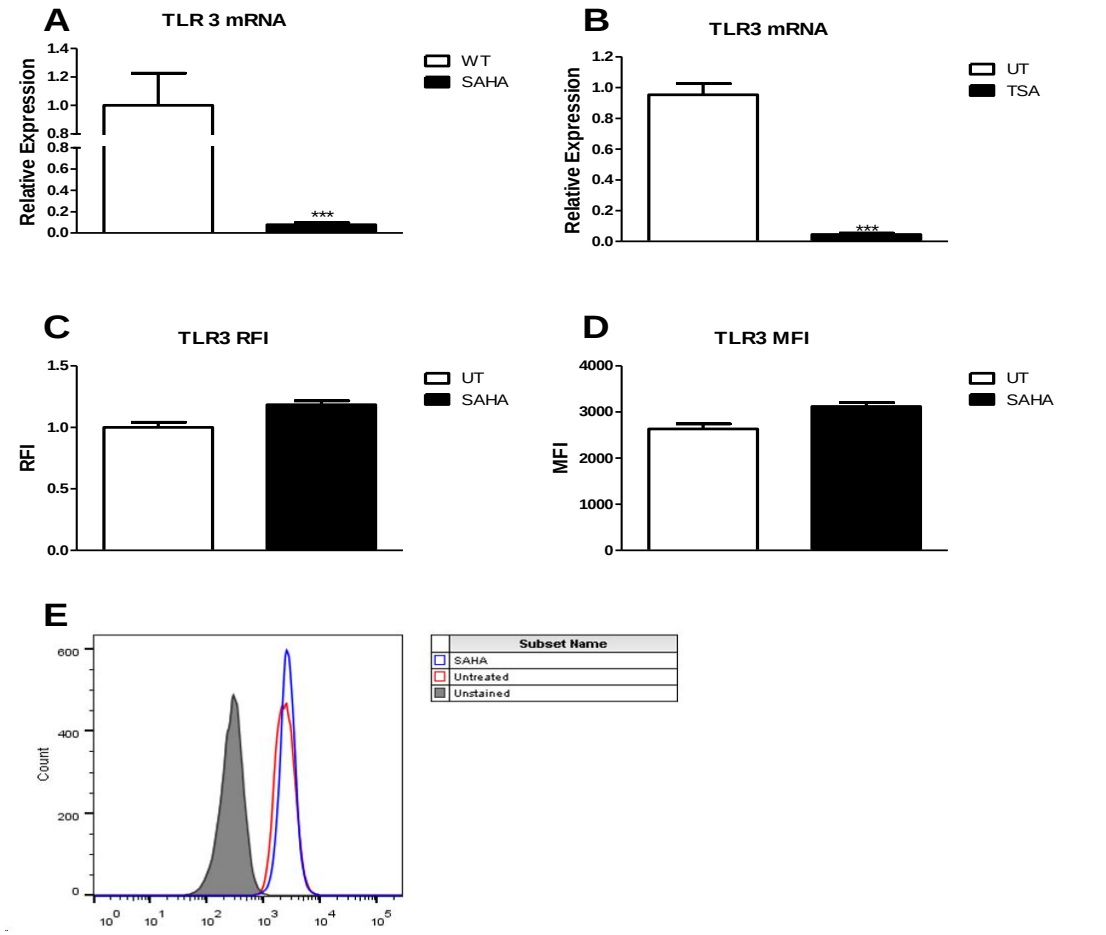


Figure 4.8. Treatment of HCT 116 cells with the HDAC inhibitor SAHA decreases TLR3 mRNA expression, but not TLR3 protein level.

A-B) Real time PCR analysis was performed to examine the levels of TLR3 mRNA expression in both the UT and SAHA treated cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a students t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. WT. **C)** Shown is the relative fluorescence intensity (RFI) of the TLR3 PE stain in both WT UT and WT SAHA treated cells. The Mean fluorescence intensity is shown in **D)**, and the histograms are shown in **E).** All fluorescence data was generated using the BD FACS Canto II Flow Cytometer and analysed using FlowJo software.

4.4.9 PAN HDAC INHIBITOR SAHA PREVENTS POLY I:C INDUCED INCREASES IN TLR3 EXPRESSION AND CYTOKINE EXPRESSION AND RELEASE.

We next sought to determine whether HDAC inhibition would have a similar effect on the poly I:C induced increases in TLR3 and cytokine mRNA as to those seen with the inhibition of DNMTs. qPCR was performed to examine changes in mRNA levels of TLR3 and the relevant cytokines, with relative expression being quantified using β-Actin as an endogenous control. The fold change relative to the WT UT group was calculated using the $2^{-\Delta Ct}$ method. Statistical significance was quantified using a 2 way ANOVA followed by a Bonferroni post hoc test. All data is expressed as mean ± SEM.

We first examined the effect of SAHA on the response to poly I:C in the HCT 116 cells. Similarly to what was seen with DNMT inhibition, the pharmacological inhibition of the HDAC enzymes resulted in the prevention of the poly I:C induced increase in TLR3 mRNA (figure 4.9A). As seen previously, poly I:C treatment results in a significant increase in TLR3 mRNA expression (8.172 ± 0.461, $P < 0.001$) when compared to the UT cells (1.00 ± 0.072). Treatment with SAHA prior to the poly I:C stimulation resulted in a prevention of this poly I:C induced upregulation, with TLR3 mRNA expression being significantly lower in the SAHA + poly I:C group (0.054 ± 0.007, $P < 0.001$ vs UT and poly I:C) when compared to the UT group (1.00 ± 0.072) as well as the poly I:C group (8.172 ± 0.461, $P < 0.001$).

The result at the protein level (figure 4.9B) was reflective of what we observed in the DKO cells and DNMT inhibitor treatment groups. Treatment with poly I:C resulted in a significant increase in TLR3 protein expression (UT (1± 0.04), poly I:C (1.5 ± 0.08, $P < 0.001$), however there was no difference in any of the SAHA treatment groups when compared to the UT group (SAHA (1.18 ± 0.03), SAHA + poly I:C (1.25 ±

0.01). The cytokine results followed a similar trend to those seen above, with SAHA treatment preventing poly I:C induced increases in IL6, TNFα and IFNβ . When we examined the expression of IL6 (fig 4.9C), there was a significant increase in IL6 mRNA following 6h poly I:C stimulation (WT UT (1.00 ± 0.215) vs poly I:C (19.094 ± 2.914, $P < 0.001$), but pre-treatment with SAHA prevented this significant increase (UT (1.00 ± 0.215) vs SAHA + poly I:C (3.662 ± 0.628), $P > 0.05$).

The mRNA results for IL6 seeming translated to what we observed at the protein level, when ELISAs were used to measure changes in IL6 secretion. Following poly I:C stimulation there is a significant increase in IL6 (fig 4.9D) release in the poly I:C treated cells (14.057 pg/µl ± 1.819, $P < 0.001$) when compared to the UT cells (1.168 pg/µl ± 0.251). However there was no significant difference between the SAHA group (0.841 pg/µl ± 0.161) and the SAHA + poly I:C group (1.621 pg/µl ± 0.437, $P > 0.05$), with the SAHA group being non responsive to poly I:C. The results for TNFα were similar, with a large increase in mRNA expression seen in the poly I:C group (33.72 ± 7.55, $P < 0.001$) when compared to the UT group (1.00±0.192), but again this increase was abated with SAHA pre-treatment (UT (1.00 ± 0.192) vs SAHA + poly I:C (3.740 ± 1.225, $P > 0.05$). TNFα release followed the same pattern, with the poly I:C group displaying a significant increase in cytokine release (81.976 pg/µl ± 7.686, $P < 0.001$) when compared to the UT group (15.987 pg/µl ± 1.778), but no significant response seen when we compared the SAHA group (13.427 pg/µl ± 0.578) with the SAHA + poly I:C group (22.130 pg/µl ± 4.607, $P > 0.05$).

Finally, the examination of IFNβ mRNA expression and cytokine release followed the trend set by the other cytokines, with SAHA pre-treatment preventing the poly I:C induced increase in mRNA expression of the cytokine. The poly I:C group

showed a huge increase in expression following 6h stimulation (poly I:C (128.65±13.512, $P < 0.001$) when compared to the UT group (1.00±0.183). This increase was not seen in the SAHA + poly I:C group however (1.291±0.140, $P > 0.05$), again showing that SAHA prevents the poly I:C response. This was seen at the protein level also, with poly I:C treatment resulting in a huge release of IFNβ (1512 pg/ml ± 289, $P < 0.001$) when compared to the UT group (91.8 pg/ml ± 13.9). No effect of stimulation was observed in the SAHA treated group (SAHA (165 pg/ml ± 45.3), SAHA + poly I:C (142.6 pg/ml ± 55.9, $P > 0.05$). Thus, these results suggest that both inhibition of DNMTs and HDACS have a similar effect, with both preventing any poly I:C induced increases in TLR3 or cytokine mRNA expression and release.

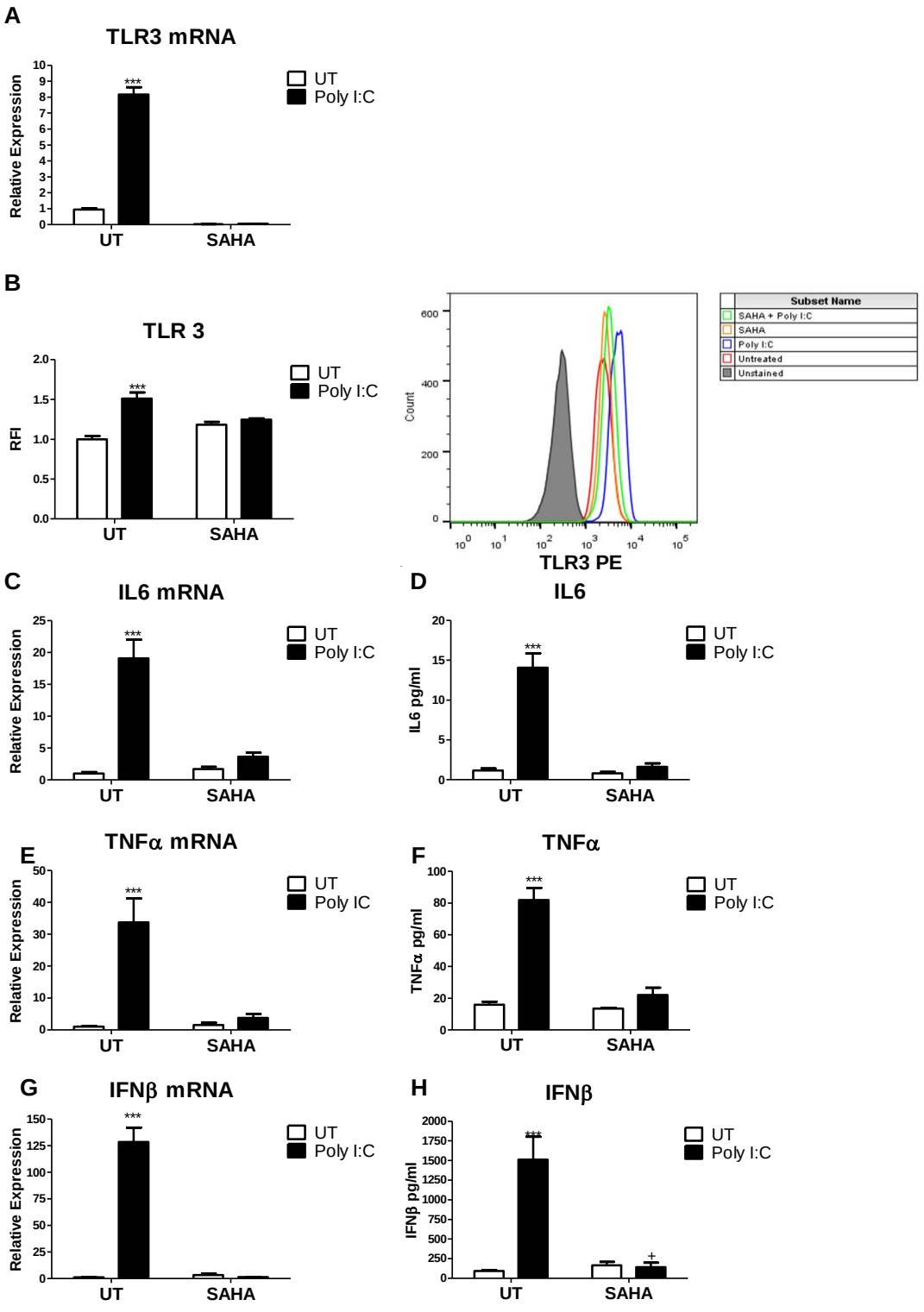


Figure 4.9. Treatment of HCT 116 cells with SAHA inhibits poly I:C induced increases in cytokine expression and release.

A) TLR3 mRNA levels in WT & DKO cells as measured by qPCR. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs WT untreated. **B)** TLR protein expression was measured using flow cytometry. Fluorescence data was generated using the BD Facs Canto II Flow Cytometer and analysed using FlowJo software. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs untreated **C, E, G)** IL6, TNFα, & IFNβ mRNA levels in UT and SAHA treated cells as measured by qPCR. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs untreated. **D, F, H)** IL6, TNFα & IFNβ protein levels as measured by ELISA. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3,** = $P < 0.01$ vs untreated, *** = $P < 0.001$ vs WT untreated.

4.4.10 PAN HDAC INHIBITOR SAHA PREVENTS POLY I:C INDUCED SIGNALLING PROTEIN ACTIVATION.

We wished to characterise the effect of SAHA treatment on the TLR3 signalling cascade following poly I:C treatment, to determine whether the HDAC inhibitor was having a comparable effect on the signalling cascade to that seen in the DNMT 1/3b knockout/inhibited cells. The timepoints used for each protein were as follows, P-ERK (120 min), P-JNK (120 min), P-P38 (90 min), P-IκBα (180 min), P-NF-κB (180 min), and P-IRF3 (180 min). Blots were performed for three independent experiments, with a representative example shown in figure 4.10A. In figure 4.10B-G we show densitometry of each phospho-protein relative to β-Actin controls. The data is expressed as mean relative densitometry ± SEM.

In lane 1 of figure 4.10A we see the response of P-ERK to each of the different treatment groups. From both the image in lane 1 and the densitometry (fig 4.10B), we saw a significant increase in ERK phosphorylation in the poly I:C group (3.652 ± 0.747, $P < 0.05$) when compared to the UT group (1 ± 0.15). However, no such effect was seen in the SAHA treated cells, with the SAHA + poly I:C groups showing similar expression of P-ERK relative to the SAHA alone group (SAHA (1.795 ± 0.1, $P > 0.05$), SAHA + poly I:C (1.774 ± 0.06, $P > 0.05$).

In a similar vein to what we observed with P-ERK, there was also a significant increase in the levels of SAPK/JNK phosphorylation in response to poly I:C treatment (fig 4.10 Lane 3 and fig 4.10C). When the densitometry values were compared there was a significant increase in the poly I:C group (2.105 ± 0.1, $P < 0.01$) when compared to the

UT group (1 ± 0.11). The same was not evident in the SAHA treated cells, with no significant difference between the SAHA alone (.810 ± 0.106) and the SAHA + poly I:C group (0.94 ± 0.36, $P > 0.05$).

Upon examination of P-P38 expression (fig 4.10A lane 5, fig 4.10D), we noted that, as seen previously, poly I:C treatment did not result in increased levels of P-P38 (UT (1 ± 0.5), poly I:C (1.4 ± 0.034, $P > 0.05$). However, treatment with SAHA did result in a significant increase in P-P38 expression, with the SAHA alone group (7.5 ± 0.05, $P < 0.001$) and the SAHA + poly I:C group (2.3 ± 0.25, $P > 0.05$) both showing increases in P38 phosphorylation. P-IκBα (fig 4.10A lane 7, fig 4.10E) showed a significant increase in its phosphorylation in the poly I:C treated cells (3.7 ± 0.94, $P < 0.05$) when compared to the UT group (1 ± 0.45). However, no such increase was observed in the SAHA treated cells, with SAHA alone (1.137 ± 0.06) and the SAHA + poly I:C (1.182 ± 0.941, $P > 0.05$) showing similar protein levels.

We observed a similar result with P-NF-κB (fig 4.10A lane 9, fig 4.10F), with poly I:C treatment leading to a significant increase in levels of the phosphorylated protein when compared to the UT cells (UT (1 ± 0.446), poly I:C (6.476 ± 0.378, $P < 0.001$). Once again however, we observed no increase in expression in the SAHA treated group with or without poly I:C treatment (SAHA (1.045 ± 0.09), SAHA + poly I:C (1.01 ± 0.3, $P > 0.05$). The last protein we examined using densitometry was IRF 3 (fig 4.10A lane 10, fig 4.10G), and we observed a significant increase in the levels of P-IRF3 in the cells treated with poly I:C (UT (1 ± 0.3), poly I:C (2.26 ± 0.38, $P < 0.05$). In keeping with the trend seen so far, this increase in phosphorylation following poly I:C treatment was not present in the SAHA treated cells (SAHA (0.89 ± 0.14), SAHA + poly I:C (0.67 ± 0.22, $P > 0.05$). Thus, similarly to what we observed in the WT vs DKO

cells, and in the 5-aza-2-dc treated cells, pharmacological inhibition of the HDACs seemingly prevents the phosphorylation of signalling proteins which is induced by poly I:C treatment.

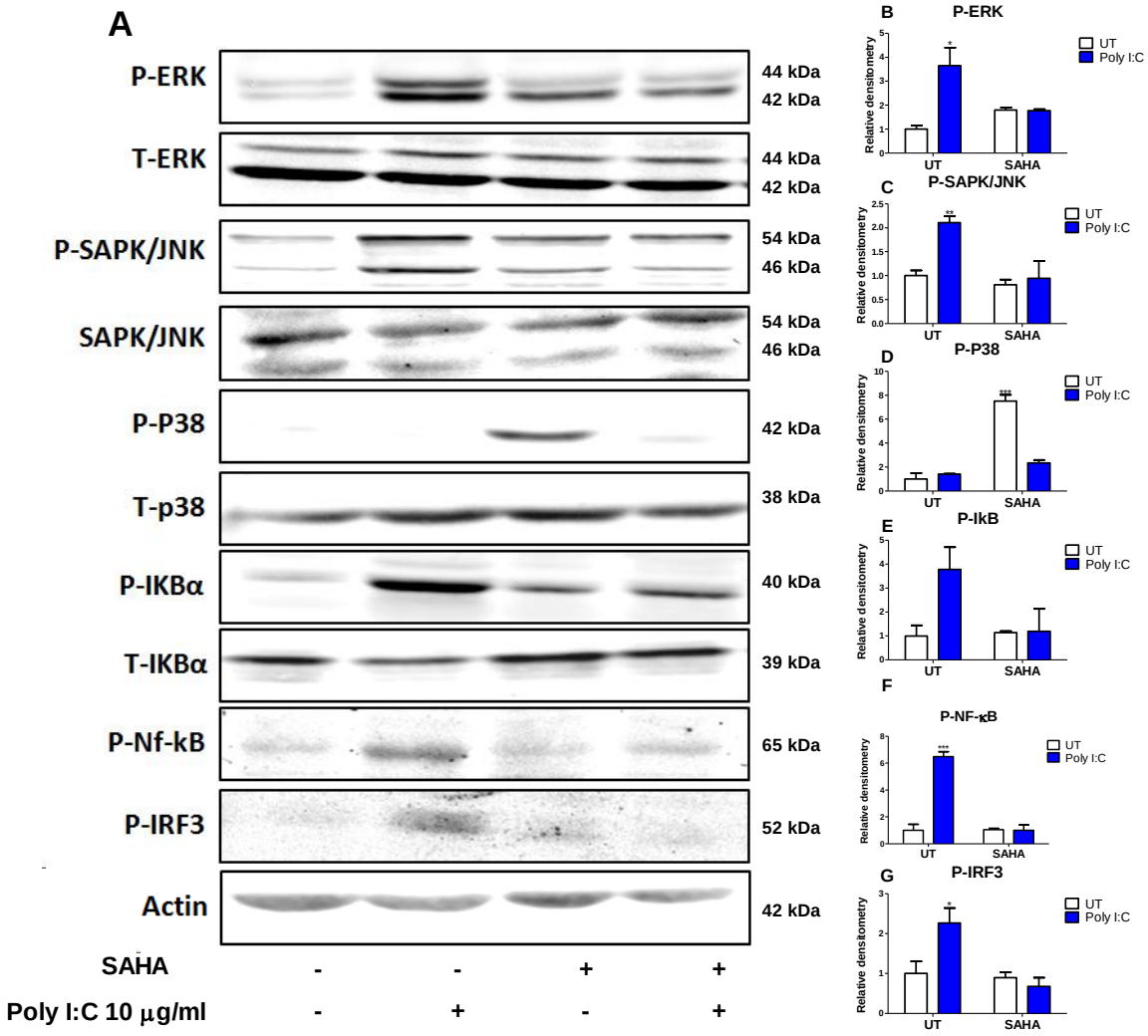


Figure 4.10. Treatment of HCT 116 cells with SAHA prevents poly I:C induced signalling protein phosphorylation.

A) Cell lysates were analysed using western blot analysis using phospho specific antibodies. Images were acquired using the Licor Odyssey with images generated using Image studio lite. Blots shown are representative images. N=3 for all groups. **B-G)** Using analysis software in image studio lite, blots were analysed for densitometry, with each band being set relative to β-Actin loading control. Values were then set relative to the UT group such that data is expressed as Mean relative densitometry ± SEM. Data

was analysed using a 2 way ANOVA, with Bonferroni pots hoc test being used. $P < 0.05$ was considered significant. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, vs untreated.

4.5 DISCUSSION

The overarching goal of this chapter was to investigate the TLR3 response in HCT 116 cells, and to examine the effects of epigenetic modifications on this response. Information on TLR3 regulation, and the role of epigenetics in TLR3 regulation, is lacking. There is certainly evidence to suggest that TLR3 is expressed differently in different cell types (Muzio et al., 2000), and based on our biopsy sample data, in different locations within the same organ structure. Yet there is little, if any, information on what exactly is regulating TLR3 expression.

We showed that TLR3 expression was greatly decreased in the DNMT 1/3b DKO cells, which was interesting and suggested that methylation may be playing a role in TLR3 regulation. Given that the inhibition of methylation generally has an epigenetic activation effect, leading to increased gene expression in hypomethylated states, this suggested to us that the effect of methylation seen on TLR3 expression was not a direct one, as it was decreasing expression as opposed to increasing it. We did discover however that the large decrease in TLR3 observed at the mRNA level, was not present at the protein level, with protein expression between the WT cells and DKO cells being almost identical. This result was replicated using different methods of protein analysis, including two different flow cytometry antibodies, western blotting, and immunocytochemistry. The results were consistent across all methods of analysis, with no observable difference in TLR3 protein level in the WT and DKO cells. However, the disparity seen in the mRNA level and the protein level of TLR3 led us to examine whether TLR3 responses were similar in the two cell lines, and despite the absence of any discernible difference in TLR3 protein expression, the results that followed showed that there was clearly differences between the WT and DKO cells.

We observed that stimulation of the DKO cells with poly I:C did not elicit any response whatsoever. While the WT cells showed increased TLR3 mRNA and protein, huge increases in cytokine expression and release, and clear changes in signaling protein phosphorylation following poly I:C stimulation. In line with previous studies, the response to poly I:C in the WT cells resulted in increases in cytokines, with IFNβ being the most notable example (Matsumoto and Seya, 2008). However, the same was not true in the DKO cells, with the complete absence of any response to TLR3 stimulation seemingly the hallmark of the knockout of the DNA methyltransferases.

Similar findings were seen with DNMT inhibition with 5-aza-2-deoxycytidine, and HDAC inhibition with SAHA. Inhibition of either set of enzymes seems to produce marked decreases in TLR3 mRNA expression, and inhibition of cytokine release. The findings in HDACs are supported by other studies also, with studies showing that HDAC inhibitors can inhibit the cytokine storm in lupus (Li et al., 2008), and can prevent LPS induced inflammation in mouse models also (Leoni et al., 2005). The effect the epigenetic modifications are having on TLR3 function could have interesting physiological implications. Using epigenetic agents such as 5-aza-2-dc or SAHA in instances where there is an abnormal or excessive viral response could regulate viral inflammation, by decreasing cytokine production and release.

Its worth considering the limitations of these studies however, especially given that we are looking at a single cell type that make up the epithelium. As mentioned in the introduction, the GIT is comprised of a multitude of cell types, and focusing on the effects in one cell line does not give an accurate representation of the effect in the whole microenvironment. Furthermore, the cell line we are using are an immortalized cell line, which are microsatellite instable (MSI) (Boland and Goel, 2010), meaning they are

prone to mutations. However, the merit of these studies lies in the identification of a role for epigenetic regulation in regulation TLR3 function.

4.6 CONCLUSION

Evidently, epigenetic modifications are altering TLR3 function, with a complete inhibition of responses to stimulation with poly I:C observed. However, despite the effect of epigenetic changes on TLR3 mRNA expression and function, no change was observed in TLR3 protein levels. We speculated that there may have been issues detecting TLR3 protein using flow cytometry, but using other analytical techniques produced similar results, leading us to believe that there was no change in protein in the DKO cells. This led us to theorise that another factor, which is altered by epigenetic changes, may be causing the changes in TLR3 function seen.

Chapter 5: Investigating TLR3 expression and signalling in THP-1 Cells and THP-1 derived macrophages

5.1 INTRODUCTION

There is a general consensus that TLR expression is different across many different tissues and cell types, with certain tissues expression at least one member of the TLR family, and some showing expression of all the TLRs (spleen, peripheral blood leukocytes) (Zarember and Godowski, 2002). Monocytes are one cell line which has an interesting TLR expression profile, with evidence suggesting that certain TLRs are highly expressed at the monocytic phase, and their expression decreases significantly upon differentiation of monocytes. Although there is limited research on TLR3 expression in monocytes, one study showed that TLR3 expression is almost nonexistent in the cell line under normal conditions, yet when they differentiate into dendritic cells, the expression profile changes and TLR3 shows marked increases following differentiation (Visintin et al., 2001). There is also evidence that differentiation of monocytes into macrophages also alters the expression profile of many genes, TLRs included. Some studies have shown that TLR3 is expressed in macrophages, and its function is essential in defence against viral infection (Zhu et al., 2015).

Mononuclear phagocytes play an important role in the defence of the host against viral infections, with recruitment of monocytes and the differentiation into macrophages providing a rapid response to limit the viral spread at the onset of infection. The elimination of the pathogen is typically by phagocytosis or the generation of factors which lead to viral degradation (Langermans et al., 1994). Given the important role of these mononuclear cells in the defence against pathogens, we wished

to further investigate the expression and regulation of TLR3 in these cells. To this end we used THP-1 cells, which are a well-established model for human primary monocytes (Tsuchiya et al., 1980), and we also differentiated them into macrophages, which allowed us to examine how the potential viral response and TLR3 in particular, responded to the differentiation process.

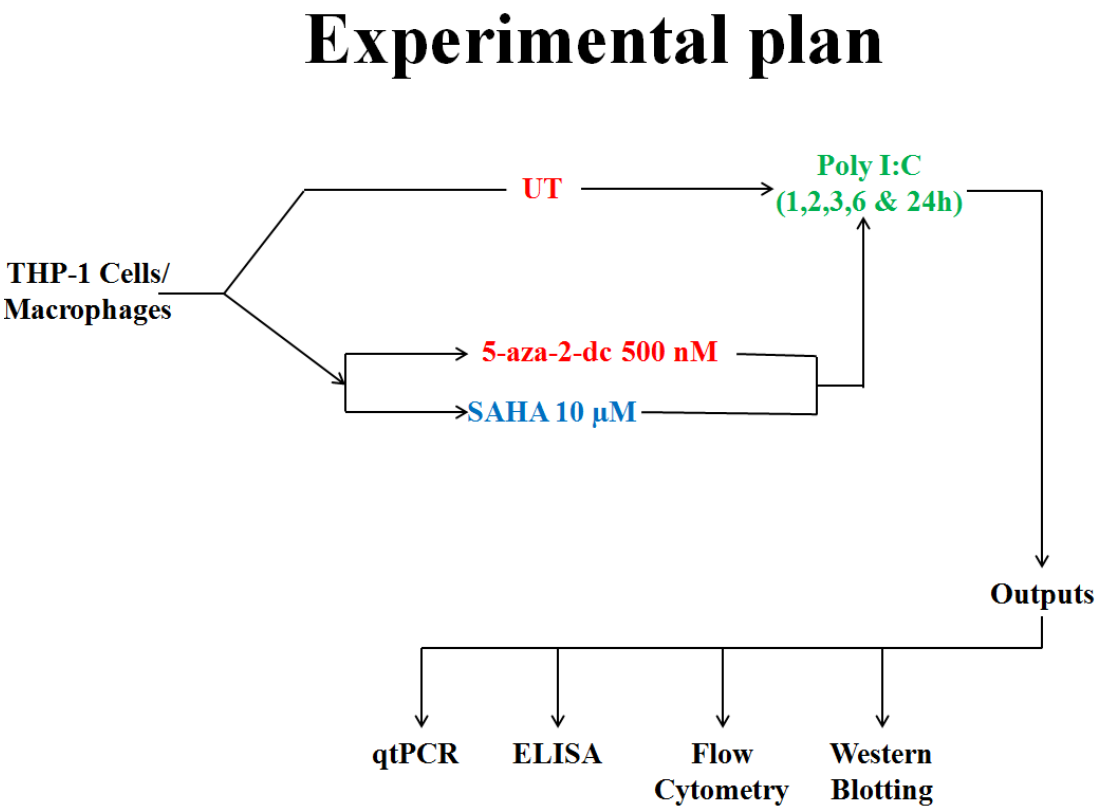
Thus, in this chapter we sought to first characterize the response of THP-1 cells to stimulation with poly I:C, and to determine whether epigenetic modifying drugs would have any effect on this. Following the characterization of the TLR3 response, encompassing changes in expression, intracellular signaling and cytokine release, we turned to their progeny, the macrophages. THP-1 differentiation into macrophages can be induced by 24h treatment with PMA, as described in chapter 2 (Smith et al., 2015). This allows us to then study the difference in responses between the precursor monocytes and the differentiated macrophages, from which we hope to further understand the regulation of our receptor of interest, TLR3.

Thus, we hypothesize that TLR3 expression in THP-1 cells will be altered by epigenetic modifications. Furthermore, we hypothesize that these epigenetic modifications will alter the signalling of TLR3 in response to stimulation, as well as altering the expression and release of cytokines. We also hypothesize that differentiation of THP-1 cells into monocytes will produced changes in TLR3 expression and signalling, and that epigenetic modifications of these monocytes will further alter TLR3 expression and signalling.

5.2 METHODS

The Methods used for these studies did not differ from those outlined in chapter 2.

5.3 EXPERIMENTAL DESIGN



The experimental design was followed as outlined in the graphic above. HCT 116 WT cells were treated with the epigenetic modifying drugs 5-aza-2-deoxycytidine and SAHA, following which they were treated with poly I:C. The effect of poly I:C on gene expression, and subsequently the effect of pretreatment with the epigenetic modifying drugs on gene expression, was measured using qPCR, western blotting, ELISAS and flow cytometry. The concentrations and time points used for each of the drugs are as follows:

Treatment	Concentration	Timepoints
Western Blotting		
poly I:C	10 µg/ml	30min, 60min, 90min, 120min, 240min
ELISAs		
poly I:C	20 µg/ml	24hr
qPCR		
poly I:C	10 µg/ml	6hr, 24hr
Flow Cytometry		
poly I:C	10 µg/ml	24hr
Ep**igenetic Drugs**		
5-aza-2-deoxycytidine	500 nM	72hr
SAHA	10 µM	48hr

Table 5.1. Concentrations and time points of ligands and drug treatments used in these studies.

5.4 RESULTS

5.4.1 Effect of poly I:C on TLR3 responses in THP-1 cells treated with 5-aza-2-deoxycytidine.

In light of the effect of poly I:C on the expression of TLR3 and cytokines in the HCT 116 cells, and the effects that inhibition of methylation had on this, we wished to examine the effects of TLR3 stimulation in our THP-1 cell line, and whether manipulation with epigenetic modifying drugs would affect it in any way. To examine TLR3 mRNA expression qPCR was performed and relative mRNA expression was quantified using β-Actin as an endogenous control. The fold change relative to the untreated group was calculated using the $2^{-\Delta Ct}$ method. A student's t-test was used to compare the means. A P value of $P < 0.05$ was considered statistically significant.

In figure 5.1A, the result of poly I:C treatment in the THP-1 cells is visible. Interestingly, we noticed that, unlike the HCT 116 cell line, poly I:C treatment does not elicit a response in TLR3, with the untreated (1 ± 0.15) and poly I:C treated groups (1.1 ± 0.1, $P > 0.05$) showing similar expression. Furthermore, the addition of 5-aza-2-deoxycytidine did not decrease TLR3 expression further, but in fact increased it slightly (2.12 ± 0.58) when compared to control. When we examined poly I:C treatment in combination with the 72hr pre-treatment with 5-aza-2-dc there was a further increase in TLR3 expression, such that the difference between the untreated group (1 ± 0.151) and the 5-aza-2-dc + poly I:C group (2.7 ± 0.33, $P < 0.05$) was now significant. However, there was no significant difference between the 5-aza-2-dc only (2.1 ± 0.58) and the 5-aza-2-dc + poly I:C (2.77 ± 0.33, $P > 0.05$) groups, which suggests that the significant

difference between the 5-aza-2-dc + Poly group and the untreated group is a function of the 5-aza-2-dc treatment and not the poly I:C.

When we examined TLR3 protein changes (Fig 5.1B/5.1C) in response to 5-aza-2-dc and poly I:C treatments, we did not observe any significant differences, with values in the untreated (1 ± 0.08), poly I:C treated (1.17 ± 0.06, $P > 0.05$), 5-aza-2-dc treated (1.14 ± 0.07, $P > 0.05$) and 5-aza-2-dc + poly I:C treated (1.08 ± 0.05, $P > 0.05$) being similar across all measurements, with no significant changes seen between any of the groups. This lack of change in protein expression could likely be attributed to the generally low expression of TLR3 in the THP-1 cells. With the mRNA levels being low as they are, and the changes following treatment only being in the region of two fold increases, it is unlikely that these changes in mRNA level would manifest in protein changes

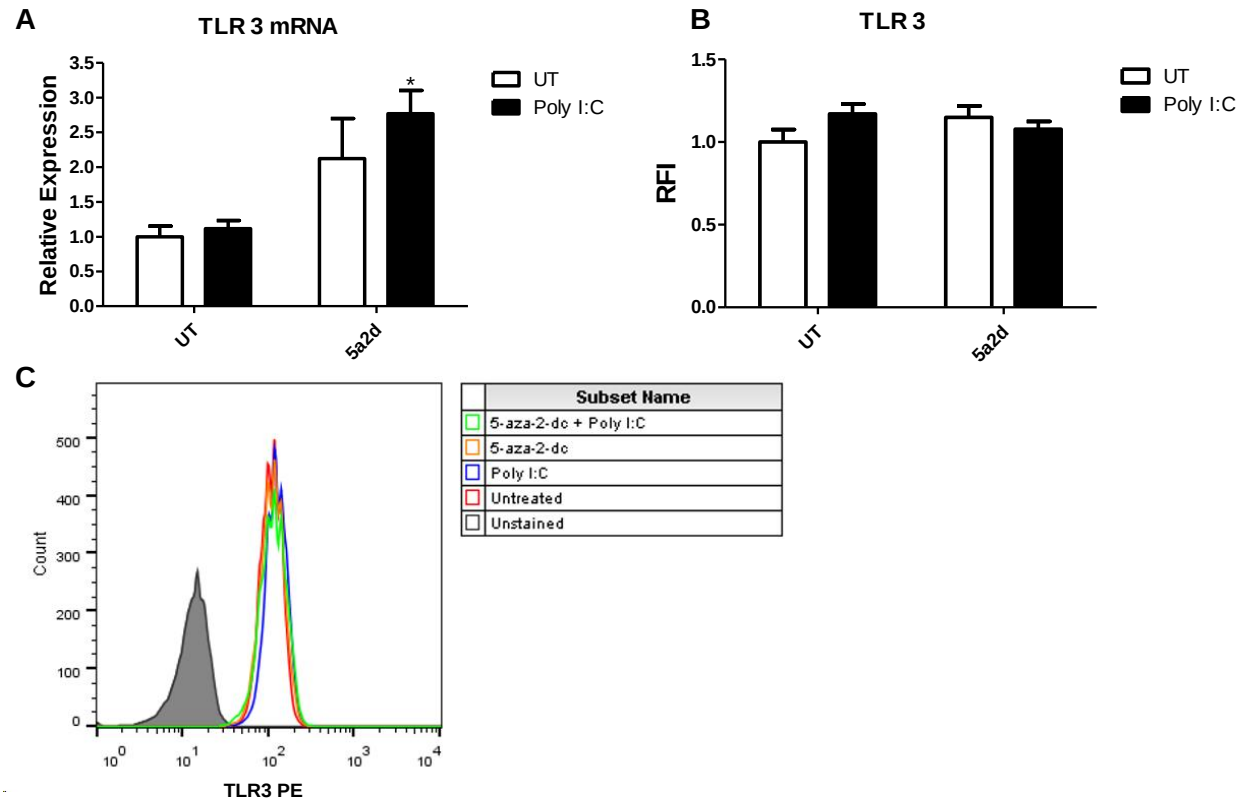


Figure 5.1. Treatment of THP-1 cells with 5-aza-2-deoxycytidine results in slight increases in TLR3 mRNA but not protein expression.

A) Real time PCR analysis was performed to examine the levels of TLR3 mRNA expression in THP-1 cells. mRNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using 2 way ANOVAs. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, * = $P < 0.05$ vs. UT. **B)** Shown is the relative fluorescence intensity (RFI) of the TLR3 PE stain in both THP-1 UT and 5-aza-2-deoxycytidine treated cells. Histograms of raw data are shown in **C).** All fluorescence data was generated using the BD FACS Canto II Flow Cytometer and analysed using FlowJo software.

5.4.2 EFFECT OF POLY I:C ON TLR3 RESPONSES IN THP-1 CELLS TREATED WITH 5-AZA-2-DEOXYCYTIDINE.

Following the investigation of the effects of 5-aza-2-dc and poly I:C treatment on TLR3 expression, we examined the effects these treatments would have on the expression and release of cytokines in this cell line. In figure 5.2 we see the outcome of these studies, with both qPCR and ELISAs being performed to measure changes in cytokine expression and release. To examine cytokine mRNA expression, qPCR was performed and relative mRNA expression was quantified using β-Actin as an endogenous control. The fold change relative to the untreated group was calculated using the $2^{-\Delta Ct}$ method. Cytokine release was measured using ELISAs, with values shown being cytokine release in pg/ml.

When we examined IL6 cytokine mRNA levels, as shown in fig 5.2A, we observed that there was no significant induction of IL6 mRNA following poly I:C stimulation in the THP-1 cells (UT (1 ± 0.2), poly I:C (1.03 ± 0.37, $P > 0.05$). Treatment with 5-aza-2-dc alone (1.4 ± 0.3, $P > 0.05$) or 5-aza-2-dc in combination with poly I:C (1.5 ± 0.5, $P > 0.05$) did not result in significant changes in mRNA expression either. This was in line with the results seen for cytokine release measured using ELISAs (fig 5.2B), with the untreated group (0.46 pg/ml ± 0.4) and the poly I:C treated group (0.1 ± 0.001, $P > 0.05$) not being significantly different. There were visible increases in the 5-aza-2-dc tread group (8.1 pg/ml ± 4.5, $P > 0.05$) and the 5-aza-2-dc + poly I:C group (2.9 pg/ml ± 0.9, $P > 0.05$), but these were modest increases and were not significant changes.

TNFα was the next cytokine examined, and much like IL6, poly I:C alone had no significant effect on mRNA expression of the cytokine (fig 5.2C) (UT (1 ± 0.15), poly I:C (2.4 ± 0.7, $P > 0.05$). However, similar to what was seen with IL6, 5-aza-2-dc treatment did lead to an increase in TNF mRNA (6.9 ± 1.8, $P < 0.01$), and when poly I:C was added there was also a significant increase in mRNA expression observed when compared to the untreated group (9.7 ± 2.8, $P < 0.01$). However, as before, there was no significant difference between the 5-aza-2-dc alone (6.9 ± 1.8) and the 5-aza-2-dc followed by poly I:C groups (9.7 ± 2.8), suggesting that again the increase in TLR3 mRNA was due to the 5-aza- treatment and not a function of the poly I:C.

Upon examination of the ELISA data (fig 5.2D) we noticed that, as with the other cytokines, there was no significant difference between the untreated (4 pg/ml ± 1.3) and the poly I:C treated groups (3.8 pg/ml ± 1.9, $P > 0.05$). There were visible trends in the 5-aza-2-dc (10.8 pg/ml ± 2.1, $P > 0.05$) and the 5-aza-2-dc + poly I:C groups, (13.17 pg/ml ± 3.9, $P > 0.05$) however, in both cases these trends were only towards very minor increases.

Finally, we examined the expression of IFNβ in the THP-1s, and found that the mRNA followed a similar pattern to the other cytokines. In figure 5.2E we see that poly I:C treatment alone has no appreciable effect on expression, with untreated (1 ± 0.3) and poly I:C (0.831 ± 0.2, $P > 0.05$) treated groups showing similar expression. However, 5-aza-2-dc treatment again resulted in a trend towards an increase (2.56 ± 0.64, $P > 0.05$), with the combination of 5-aza-2-dc and poly I:C producing a significant increase in mRNA (2.9 ± 0.5, $P < 0.01$).

In spite of these increases at mRNA level, a resultant change was not observed in the ELISAs (fig 5.2F), with values remaining similar across all groups (UT (4 pg/ml ±

4), poly I:C (2.7 pg/ml ± 1.4, $P > 0.05$), 5-aza-2-dc(2.5 ± 2.4, $P > 0.05$), 5-aza-2-dc + poly I:C (1.6 ± 1.5, $P > 0.05$). Thus, from the results seen we concluded that poly I:C has no impact on cytokine expression or release in THP-1 cells, but rather inhibition of methylation with 5-aza-2-deoxycytidine is the main force behind changes in gene expression. These changes were minor however, and did not result in any significant changes in cytokine release as measured by ELISAs.

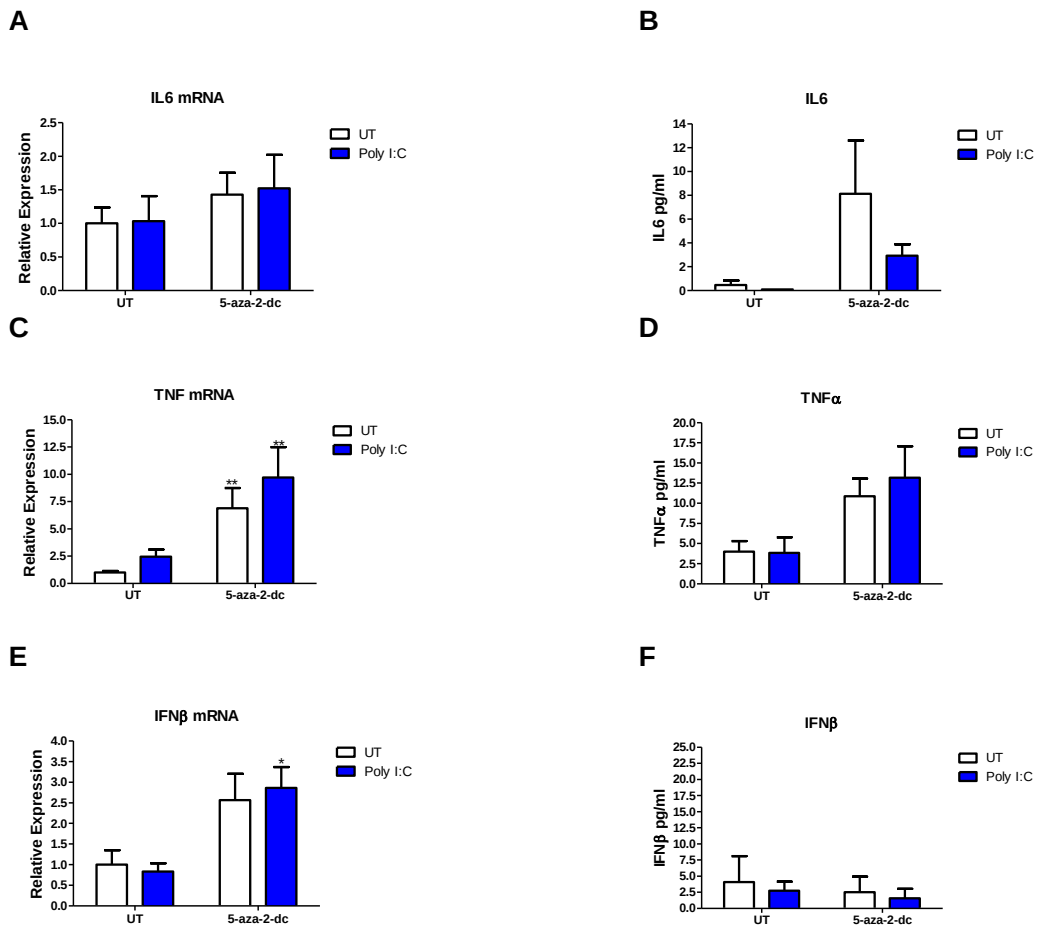


Figure 5.2. Treatment of THP-1 cells with 5-aza-2-dc increases cytokine mRNA expression with no effect on cytokine secretion.

A, C, E) Real time PCR analysis was performed to examine the basal levels of cytokine mRNA expression in both the UT and 5-aza-2-dc treated THP-1 cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3,* = $P < 0.05$ vs. UT, ** = $P < 0.01$ vs. UT, *** = $P < 0.001$ vs. UT. **B, D, F)** IL6, TNF-α & IFNβ protein levels as measured by ELISA. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3.

5.4.3 Effect of poly I:C on TLR3 responses in THP-1 cells treated with SAHA

Following the results obtained for the 5-aza-2-deoxycytidine studies, we wished to examine the effect our other epigenetic drug of choice, SAHA, would have on the expression of TLR3 and cytokines. As before, TLR3 mRNA expression was quantified using qPCR, and changes in expression were calculated using the the $2^{-\Delta Ct}$ method, with the untreated groups being set to one and the fold change being calculated relative to the untreated group. Flow cytometry was performed to measure differences in protein, and again data is presented as relative fluorescence intensity, with values being relative to the untreated control. For both qPCR and flow cytometry, data is expressed as mean ± SEM.

In figure 5.3A we see the effect of SAHA treatment on TLR3 expression in the THP-1 cells. As with the previous data we again observed that poly I:C treatment did not result in an upregulation of TLR3 mRNA, with untreated (1 ± 0.14) and poly I:C treated groups (1.1 ± 0.14, $P > 0.05$) groups showing similar expression of TLR3. Treatment with SAHA however did result in a significant increase in expression (3.1 ± 0.5, $P > 0.01$) when compared to the control group. When SAHA was paired with poly I:C treatment there was also a significant increase in expression when compared to control (3.2 ± 0.5, $P > 0.01$), however there was no discernible difference between the SAHA group and the SAHA + poly I:C group. This again suggests that the increase in mRNA expression is solely the function of the HDAC inhibition, with poly I:C having no effect.

When we analysed the flow cytometry data (Fig 5.3B/5.3C) for changes in TLR3 protein, as with the 5-aza-2-dc treatments, there was no significant difference between

the untreated (1 ± 0.08) and the SAHA treated (1.17 ± 0.06, $P > 0.05$) groups, implying again that the changes in mRNA were not resulting in changes at the protein level. Again this is likely due to the low basal level of TLR3 expression being unlikely to be affected by such a minor increase in mRNA expression.

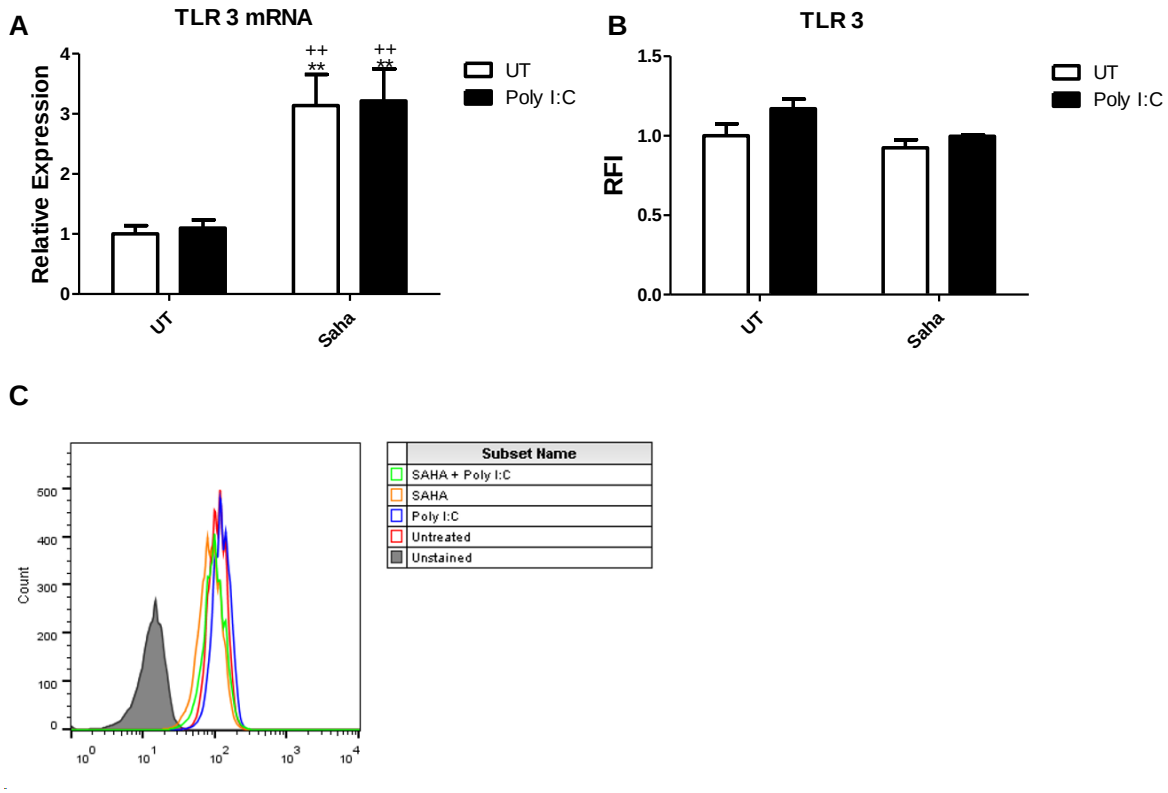


Figure 5.3. Treatment of THP-1 cells with SAHA increases TLR3 mRNA expression with no change in protein level.

A) Real time PCR analysis was performed to examine the levels of TLR3 mRNA expression in THP-1 cells. mRNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a students t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, ** = $P < 0.01$ vs UT, ++ = $P < 0.01$ vs poly I:C. **B)** Shown is the relative fluorescence intensity (RFI) of the TLR3 PE stain in both THP-1 UT and SAHA treated cells. All fluorescence data was generated using the BD FACS Canto II Flow Cytometer and analysed using FlowJo software.

5.4.4 Effect of poly I:C on TLR3 responses in THP-1 cells treated with SAHA.

Following the investigation of the effects of SAHA and poly I:C treatment on TLR3 expression, we examined the effects these treatments would have on the expression and release of cytokines in this cell line. In figure 5.4 we see the outcome of these studies, with both qPCR and ELISAs being performed to measure changes in cytokine expression and release. To examine cytokine mRNA expression qPCR was performed and relative mRNA expression was quantified using β-Actin as an endogenous control. The fold change relative to the untreated group was calculated using the $2^{-\Delta Ct}$ method. Cytokine release was measured using ELISAs with values shown being cytokine release in pg/ml. All data shown is mean ± SEM.

In figure 5.4A we see the effect of SAHA treatment on IL6 mRNA expression in the THP-1 cell line. As seen previously, poly I:C treatment by itself is not sufficient to increase expression, with the untreated group (1 ± 0.2) and the poly I:C group (1.4 ± 0.1, $P > 0.05$) showing no significant difference in expression. However, similar with what was seen in the 5-aza-2-dc treated cells, treatment with SAHA increases mRNA expression (6.1 ± 0.9, $P < 0.001$) when compared to the untreated group. The SAHA + poly I:C group also saw a significant increase in expression (4.8 ± 0.8, $P < 0.001$) when compared to the untreated group, but there was no significant difference between the SAHA only group and the SAHA + poly I:C group. This again infers that the increase in IL6 mRNA is a result of the SAHA treatment and poly I:C has no bearing on the upregulation seen.

When we look at actual cytokine release as measured by ELISA, there is very little difference between the untreated group (0.46 pg/ml ± 0.36) and the poly I:C treated group (0.1 pg/ml ± 0.001, $P > 0.05$) . Despite the change in mRNA expression, treatment with SAHA alone (2.1 pg/ml ± 0.2, $P > 0.05$) or SAHA + poly I:C (0.8 pg/ml ± 0.8, $P > 0.05$) had no effect on IL6 release, suggesting that changes in mRNA level elicited by SAHA treatment did not result in altered cytokine release.

The effect on TNFα was the next experiment performed, and we again observed that treatment with poly I:C alone did not result in a significant change in expression (UT (1 ± 0.05), poly I:C (1.75 ± 0.2, $P > 0.05$). The trend of SAHA altering mRNA expression continued however, with SAHA treatment resulting in a significant increase in TNFα mRNA expression (2.1 ± 0.15, $P < 0.001$) when compared to the untreated control. When poly I:C treatment was preceded by SAHA there was also a significant increase in mRNA expression (3.2 ± 0.3, $P < 0.001$) when compared to the untreated group. Contrary to what was seen with IL6 however, there was a significant difference between SAHA (2.09 ± 0.15) and SAHA + poly I:C (3.2 ± 0.3, $P < 0.001$ (vs SAHA only)). There were no differences observed at the protein level (fig 5.4D), with TNFα release being similar across all groups (UT (4 pg/ml ± 1.3), poly I:C (3.8 pg/ml ± 1.9), SAHA (6.4 pg/ml ± 0.98), SAHA + poly I:C (3.4 pg/ml ± 0.65), $P > 0.05$ for all groups).

IFNβ was the last cytokine examined, and the effect of SAHA on IFNβ mRNA expression can be seen in figure 5.4E. As with the other cytokines, poly I:C alone does not elicit a response, with levels of mRNA being similar between the untreated (1 ± 0.08) and the poly I:C treated cells (1.2 ± 0.15, $P > 0.05$). Once again however, SAHA treatment results in an increase in IFNβ mRNA expression, with a fivefold increase seen in the SAHA group (5.05 ± 0.7, $P < 0.001$) when compared to the untreated group.

Interestingly however the combination of SAHA and poly I:C did not result in a change when compared to the untreated group, with no significant difference between both (UT (1 ± 0.08), SAHA + poly I:C (1.5 ± 0.2, P > 0..05). Upon examination of the ELISA data for IFNβ, as seen in figure 5.4F, we noted that there was no discernible differences across any of the treatment groups, with all of the values being close to the limit of detection (UT (4.1 pg/ml ± 4), poly I:C (2.6 pg/ml ± 1.4, P > 0.05), SAHA (4.8 pg/ml ± 1.9, P > 0.05), SAHA + poly I:C (5.7 pg/ml ± 2.7, P > 0.05)). The data collected for SAHA bears a resemblance to that from the 5-aza-2-deoxycitidine studies, in that treatment with SAHA can result in an upregulation of cytokine expression at the mRNA level, but these changes do not manifest themselves as meaningful changes in cytokine release. The exact reason for this is unclear but if one was to speculate, it could be that actual levels of TLR3 are still too low to induce actual cytokine release.

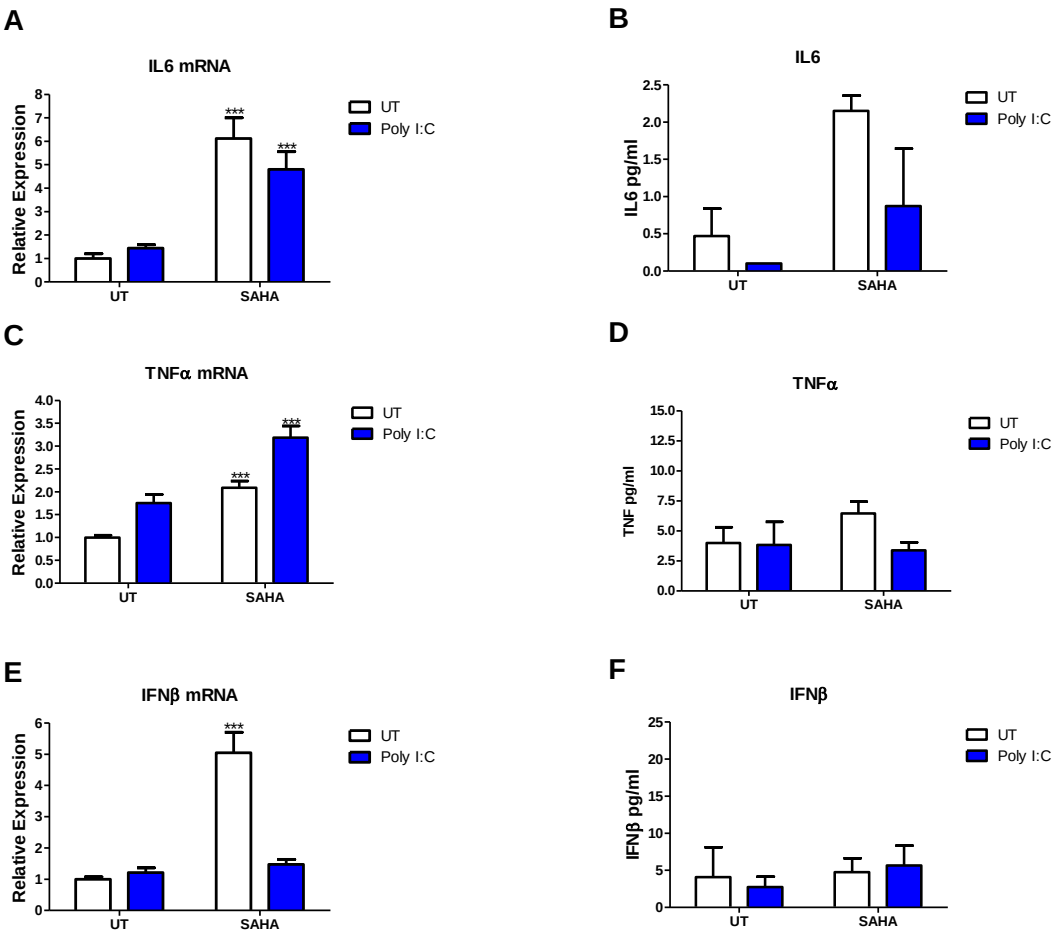


Figure 5.4. Treatment of THP-1 cells with SAHA resulting in increased cytokine mRNA expression with no change in cytokine release observed.

A, C, E) Real time PCR analysis was performed to examine the levels of TLR3 mRNA expression in both the UT and SAHA treated THP-1 cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. WT. **B, D, F)** IL6, TNF-α & IFNβ protein levels as measured by ELISA. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. untreated.

5.4.5 EFFECT OF POLY I:C AND EPIGENETIC DRUG TREATMENTS ON PHOSPHORYLATION OF SIGNALLING PROTEINS IN THP-1 CELLS.

In light of the results obtained regarding TLR3 expression and activation in the THP-1 cells, and the seemingly absent response of the cell line to poly I:C induced TLR3 activation and cytokine release, we sought to examine the signalling pathway involved in transducing the signals of activated TLR3. To this end, we performed western blots on cell lysates, with images being generated using the Licor Odyssey western blot scanner and analysed using Image Studio Lite imaging software.

Figure 5.5A shows representative blots for each of the phosphorylated signalling proteins of interest in the pathway. In row 1 of fig 5.5A and 5.5B we see the effect of our treatments of P-ERK levels, with no increase in P-ERK seen between the untreated (1 ± .9) group and the poly I:C (0.66 ± 0.76, $P > 0.05$) treated group. Treatment with both 5-aza-2-dc (.7 ± 1, $P > 0.05$) and SAHA (1.6 ± 1.9, $P > 0.05$) does not result in significant changes in the levels of P-ERK, with the combinations of 5-aza-2-dc and poly I:C (1.16 ± 1.8, $P > 0.05$) and SAHA and poly I:C (0.88 ± 1.15) showing similar results.

P-SAPK/JNK (5.5A, 5.5C) did not show any significant change across all the groups, with similar, albeit very low, levels of the phosphorylated protein seen with all treatments (UT (1 ± .15), poly I:C (1 ± 0.09), 5-aza-2-dc (1 ± 0.03), SAHA (1.1 ± 0.14), 5-aza-2-dc + poly I:C (1.1 ± .12), SAHA + poly I:C (1.1 ± 0.15), $P > 0.05$ vs. UT for all groups). The last protein examined in the MAPK signalling pathway was P-P38 (5.5A row 5, 5.5D), for which there was no difference observed between the untreated (1±

0.09) and the poly I:C treated group (0.7 ± .2, P > 0.05). There was a no increase seen in the 5-aza-2-dc group alone (0.951 ± 0.001), or with poly I:C treatment (0.9 ± 0.03), when compared to the UT (P > 0.05 for both). There was however a significant increase in P-P38 seen in the SAHA group (1.969 ± 0.018, P < 0.05) however this was absent in the SAHA + poly I:C group (1.2 ± 0.25, P > 0.05).

In 5.5A (row 7) and 5.5E, the results observed for P-IκBα are shown, and as before, poly I:C treatment (0.696 ± 0.05, P > 0.05) does not result in an increase in levels of the phospho protein. Treatment with 5-aza-2-dc alone (0.664 ± 0.07) or with poly I:C (0.5 ± 0.05, P > 0.05) did not alter P-IκB significantly. Similar results were seen with SAHA treatment, with SAHA alone (0.9 ± 0.35, P > 0.05) and SAHA + poly I:C (0.7 ± 0.2, P . 0.05) not producing any significant alterations in protein phosphorylation.

Upon examination of P-NF-κB (5.5A row 9, 5.5F), we did not observe any significant change across any of the groups. The UT (1 ± 0.04) and poly I:C (.94 ± 0.2, P > 0.05) groups all showed similar levels, indicating that poly I:C was having no effect. Furthermore, treatment with 5-aza-2-dc (5-aza-2-dc (0.8 ± 0.02, P > 0.05), 5-aza-2dc + poly I:C (0.8 ± 0.09, P > 0.05), SAHA (SAHA (0.99 ± 0.14, P > 0.05), or SAHA + poly I:C (0.83 ± 0.03, P > 0.05) did not result in any discernible changes in NF-κB phosphorylation. Finally, we examined P-IRF3 (5.5A row 10, 5.5G), and upon examination, we found that levels were similar between the UT (1 ± 0.017) and the poly I:C treated group (1 ± 0.05, P > 0.05). In 5-aza-2-dc (5-aza-2-dc (.73 ± 0.167, P > 0.05), 5-aza-2-dc + poly I:C (0.59 ± 0.01, P > 0.05) and SAHA (SAHA (0.6 ± 0.01, P > 0.05), SAHA + poly I:C (0.66 ± 0.24, P > 0.05)) treated groups there were no significant

changes, although there were slight decreases in expression which did not reach significance.

Thus, from our examination of the signalling proteins of interest in the THP-1 cells, we determined that poly I:C treatment does not result in any increase in the levels of phospho proteins when compared to our controls, supplementing the data shown in chapter 3, which showed that in the time response study in THP-1s, no discernible response was seen at any time point. Any changes seen in the levels of phospho protein are in the 5-aza-2-dc/SAHA treatment groups, which complements the previous data showing upregulation of TLR3 and cytokine mRNA expression in the groups treated with epigenetic modifying drugs

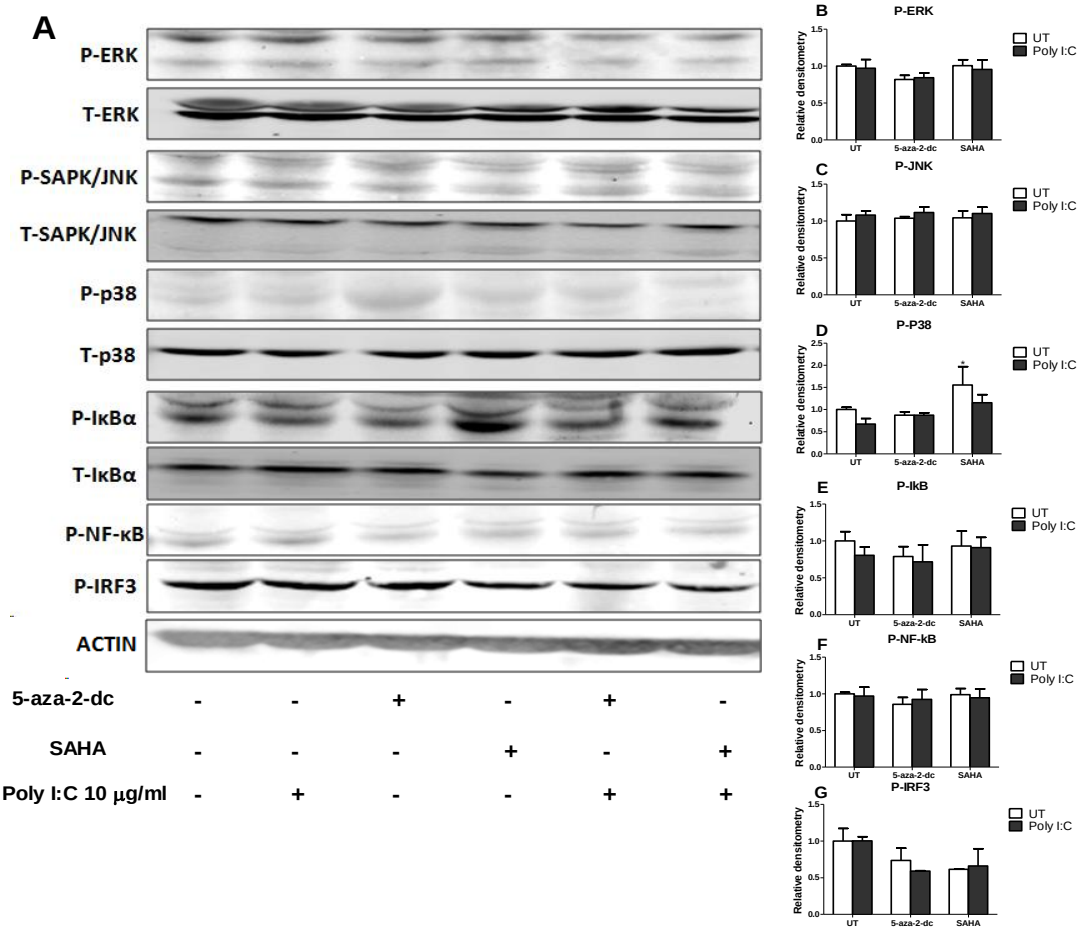


Figure 5.5. Stimulation of THP-1 cells with poly I:C alone, or in the presence of 5-aza-2-dc or SAHA, does not result in signalling protein phosphorylation

A) Cell lysates were analysed using western blot analysis using phospho-specific antibodies. Images were acquired using the Licor Odyssey with images generated using Image studio lite. Blots shown are representative images. N=3 for all groups. **B-G)** Using analysis software in image studio lite, blots were analysed for densitometry, with each band being set relative to β-Actin loading control. Values were then set relative to the UT group such that data is expressed as mean relative densitometry ± SEM. Data was analysed using a 2 way ANOVA, with Bonferroni pots hoc test being used. $P < 0.05$ was considered significant.

5.4.6 DIFFERENTIATION OF THP-1 CELLS INTO MACROPHAGES ALTERS TLR3 EXPRESSION AND FUNCTION.

Given that macrophages become active in the presence of an immune challenge, and have an important role in the activation of the innate immune response due to their role in antigen presentation, we sought to determine whether differentiation of THP-1 cells into macrophages would alter the expression and function of TLR3. In figure 5.6 we see the outcome of these studies, with qPCR, flow cytometry and ELISAs being performed to measure changes in TLR3 expression, and cytokine expression and release. To examine mRNA expression qPCR was performed and relative mRNA expression was quantified using β-Actin as an endogenous control. The fold change relative to the untreated group was calculated using the $2^{-\Delta Ct}$ method. Cytokine release was measured using ELISAs with values shown being cytokine release in pg/ml and flow cytometry was performed with the changes being calculated relative to the untreated group. All data cited is mean ± SEM.

Figure 5.6A shows the effect of poly I:C stimulation on the expression of TLR3, with stimulation resulting in an increase in TLR3 mRNA level relative to the untreated group (UT (1 ± 0.18), poly I:C (2.9 ± 0.4, $P < 0.001$). Thus at the mRNA level at least, there is a visible response to poly I:C, which is not present in the THP-1 cells. At the protein level, although there was a trend towards an increase in TLR3 in the poly I:C treated macrophages (UT (1 ± 0.06), poly I:C (1.3 ± 0.1, $P > 0.05$), this did not reach significance.

When we examined the effect of poly I:C treatment on cytokine expression and release in the macrophages, we noticed that, where the THP-1s were non responsive to poly I:C, poly I:C stimulation in the macrophages seems to result in some upregulation and release of cytokines. In fig 5.6C we see the effect of poly I:C on IL6 mRNA level. There was no significant increase seen, although there was a visible trend present when the untreated (1 ± 0.3) and poly I:C treated (2.1 ± 0.5, $P > 0.05$) groups were compared. However when we examined cytokine release using ELISA (fig 5.6D) we noticed that poly I:C treatment lead to a small but significant increase in cytokine release when compare to the untreated group (UT (2.8 pg/ml ± 0.2), poly I:C (12.5 pg/ml ± 1.9, $P < 0.01$)).

In fig 5.6E we see that poly I:C treatment did not seem to increase TNFα mRNA in the macrophages, with similar expression seen between both groups (UT (1 ± 0.1), poly I:C (0.8 ± 0.2, $P > 0.05$). However, despite the lack of response detected at the mRNA level, we did notice a response to poly I:C stimulation when we performed ELISAs (fig 5.6F), with the stimulated group (39 pg/ml ± 3, $P < 0.001$) showing significantly higher release of TNFα when compared to the untreated group (5 pg/ml ± 1.2).

Finally we examined the effect of poly I:C in macrophages on IFNβ expression and release. We observed that poly I:C treatment showed a trend towards a non significant increase in IFNβ mRNA, with slightly higher expression seen in the poly I:C treated group (2.1 ± 0.2, $P > 0.05$) relative to the untreated (1 ± 0.2). Once again, this modest increase in mRNA expression was followed by a statistically significant increase in IFNβ secretion, as measured by ELISA, with the poly I:C treated group showing a

small but significant increase in release (14 pg/ml ± 12, $P < 0.001$) when compared to the untreated group (1 pg/ml ± 0.5). Thus, from the data discussed here, it seems evident that differentiation of THP-1 cells into macrophages results in an increase in responsiveness to TLR3. Following this, we examined whether this perceived increase led to alterations in the signalling protein profile.

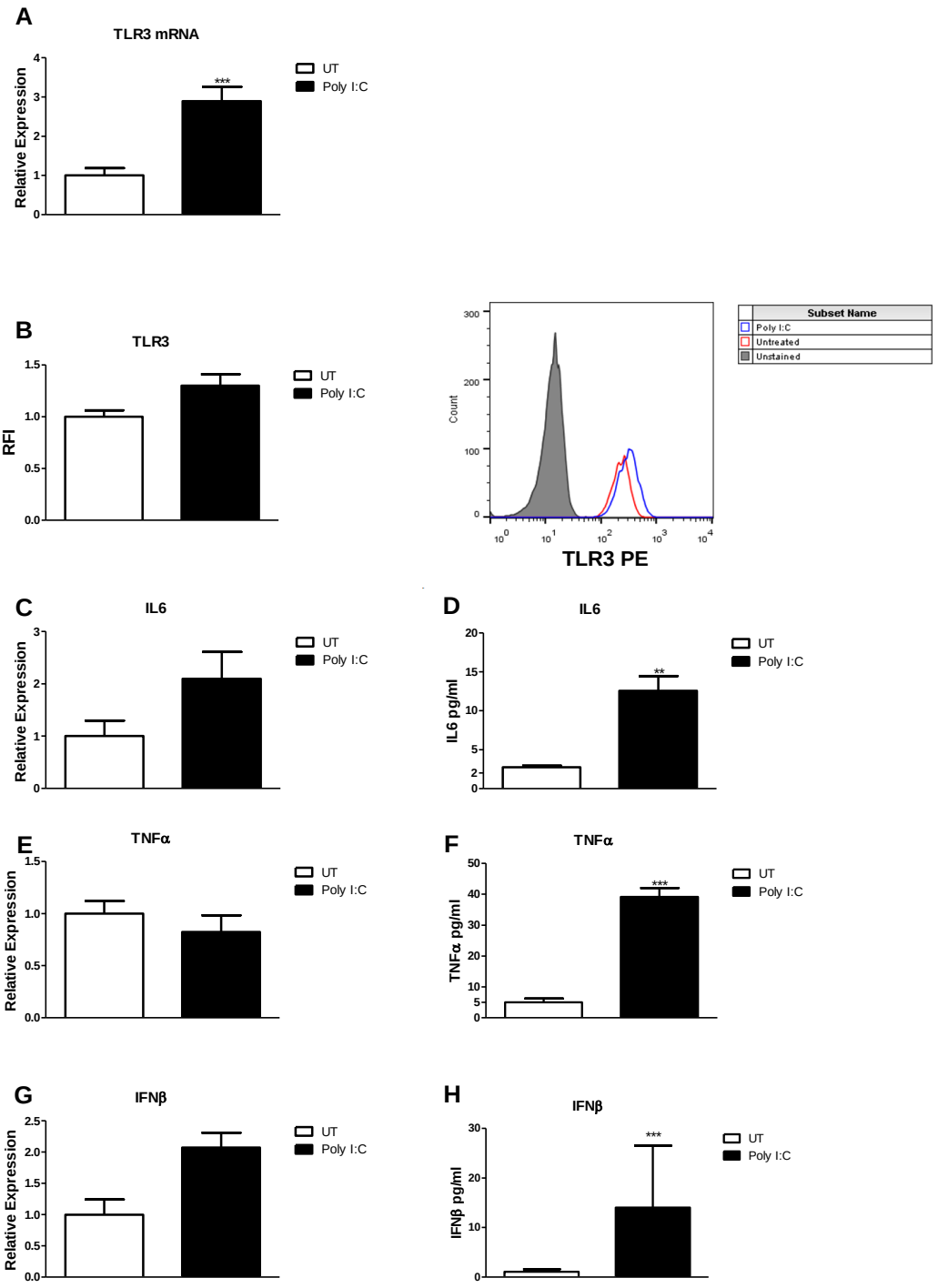


Figure 5.6. Stimulation of THP-1 derived macrophages with poly I:C results in increased TLR3 expression and cytokine release.

A, C, E, G) qPCR was performed to measure changes in TLR3 and cytokine expression in the monocyte derived macrophages following poly I:C stimulation. RNA expression

was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs. UT. **B)** Intracellular flow cytometry was performed to measure TLR3. Shown on left is the relative fluorescence intensity (RFI) of the TLR3 PE stain in both THP-1 UT and SAHA treated cells. Histograms of TLR3 PE is shown on the right. All fluorescence data was generated using the BD Facs Canto II Flow Cytometer and analysed using FlowJo software. **D, F, H)** IL6, TNFα & IFNβ protein levels as measured by ELISA. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, ** = $P < 0.01$ vs. UT, *** = $P < 0.001$ vs. UT**.**

5.4.7 EFFECT OF POLY I:C AND EPIGENETIC DRUG TREATMENTS ON PHOSPHORYLATION OF SIGNALLING PROTEINS IN MACROPHAGES.

In light of the results obtained regarding TLR3 expression and activation in the macrophages, and the development of a response to poly I:C following differentiation, we sought to examine the signalling pathway involved in transducing the signals of activated TLR3. To this end we performed western blots on cell lysates, with images being generated using the Licor Odyssey western blot scanner and analysed using Image Studio Lite imaging software. Figure 5.7A shows representative blots for each of the phosphorylated signalling proteins of interest in the pathway.

In row 1 of fig 5.7A, and 5.7B, we see the effect of our treatments of P-ERK levels, with no increase in P-ERK seen between the untreated (1 ± .0.08) group and the poly I:C (0.713 ± 0.05, $P > 0.05$) treated group. Treatment with both 5-aza-2-dc (.857 ± 0.17, $P > 0.05$) and SAHA (1.8 ± 0.15, $P > 0.05$) does not result in significant changes in the levels of P-ERK, with the combinations of 5-aza-2-dc and poly I:C (0.857± .17, $P > 0.05$) and SAHA and poly I:C (1.493 ± .13, $P > 0.05$) showing similar results. P-SAPK/JNK (5.7A, 5.7C) did not show any significant change across all the groups, with similar levels of the phosphorylated protein seen with all treatments (UT (1 ± .015), poly I:C (1 ± 0.019), 5-aza-2-dc (1.15 ± 0.23), SAHA (1.1 ± 0.084), 5-aza-2-dc + poly I:C (1.24 ± .12), SAHA + poly I:C (1.021 ± 0.08), $P > 0.05$ vs UT for all groups). The last protein examined in the MAPK signalling pathway was P-P38 (5.7A row 5, 5.7D), for

which there was no difference observed between the untreated (1± 0.04) and the poly I:C treated group (0.993 ± .016, P > 0.05). There was a no increase seen in the 5-aza-2-dc group alone (0.842 ± 0.226), or with poly I:C treatment (0.756 ± 0.15) when compared to the UT (P > 0.05 for both). SAHA treatment also did not show significant changes in levels of P-P38 (SAHA (1.2 ± .3, P > 0.05), SAHA + poly I:C (0.85 ± 0.2, P > 0.05).

In 5.7A (row 7) and 5.7E, the results observed for P-IκBα are shown, poly I:C treatment (1.689 ± 0.07, P > 0.05) result in a slight increase in levels of the phospho protein, however this did not reach significance. Treatment with 5-aza-2-dc alone (1.04 ± 0.05, P > 0.05) or with poly I:C (1.223 ± 0.14, P > 0.05) did not alter P-IκB significantly. Similar results were seen with SAHA treatment, with SAHA alone (1.218 ± 0.045, P > 0.05) and SAHA + poly I:C (0.78 ± 0.2, P . 0.05) not producing any significant alterations in protein phosphorylation. Upon examination of P-NF-κB (5.7A row 9, 5.7F), we observed a significant increase in levels of the phospho-protein following poly I:C treatment (UT (1 ± 0.130), poly I:C (8.9 ± 0.720, P < 0.05). There was no significant difference seen with the other groups, with treatment with 5-aza-2-dc (5-aza-2-dc (1.67 ± 0.1, P > 0.05), 5-aza-2dc + poly I:C (1.08 ± 0.126, P > 0.05) or SAHA (SAHA (0.99 ± 0.14, P > 0.05), SAHA + poly I:C (1.92 ± 0.03, P > 0.05) not producing any discernible changes in NF-κB phosphorylation.

Finally we examined P-IRF3 (5.7A row 10, 5.7G), and upon examination, we found that there were non significant increases in every treatment group except SAHA (UT (1 ± 0.154), poly I:C (2.4 ± 0.17, P > 0.05), 5-aza-2-dc (2.3 ± 0.16, P > 0.05), SAHA (0.3 ± 0.2, P > 0.05), 5-aza-2-dc + poly I:C (1.95 ± 0.323, P > 0.05), SAHA +

poly I:C (1.937 ± 0.187, $P > 0.05$). Thus, from our examination of the signalling proteins of interest in the macrophages, it is apparent that there is a certain level of protein phosphorylation occurring, with NF-κB activation being the most notable occurrence that is absent in the THP-1 cells. It seems that differentiation of THP-1 cells into macrophages allows the activation of certain components of the TLR3 signalling pathway.

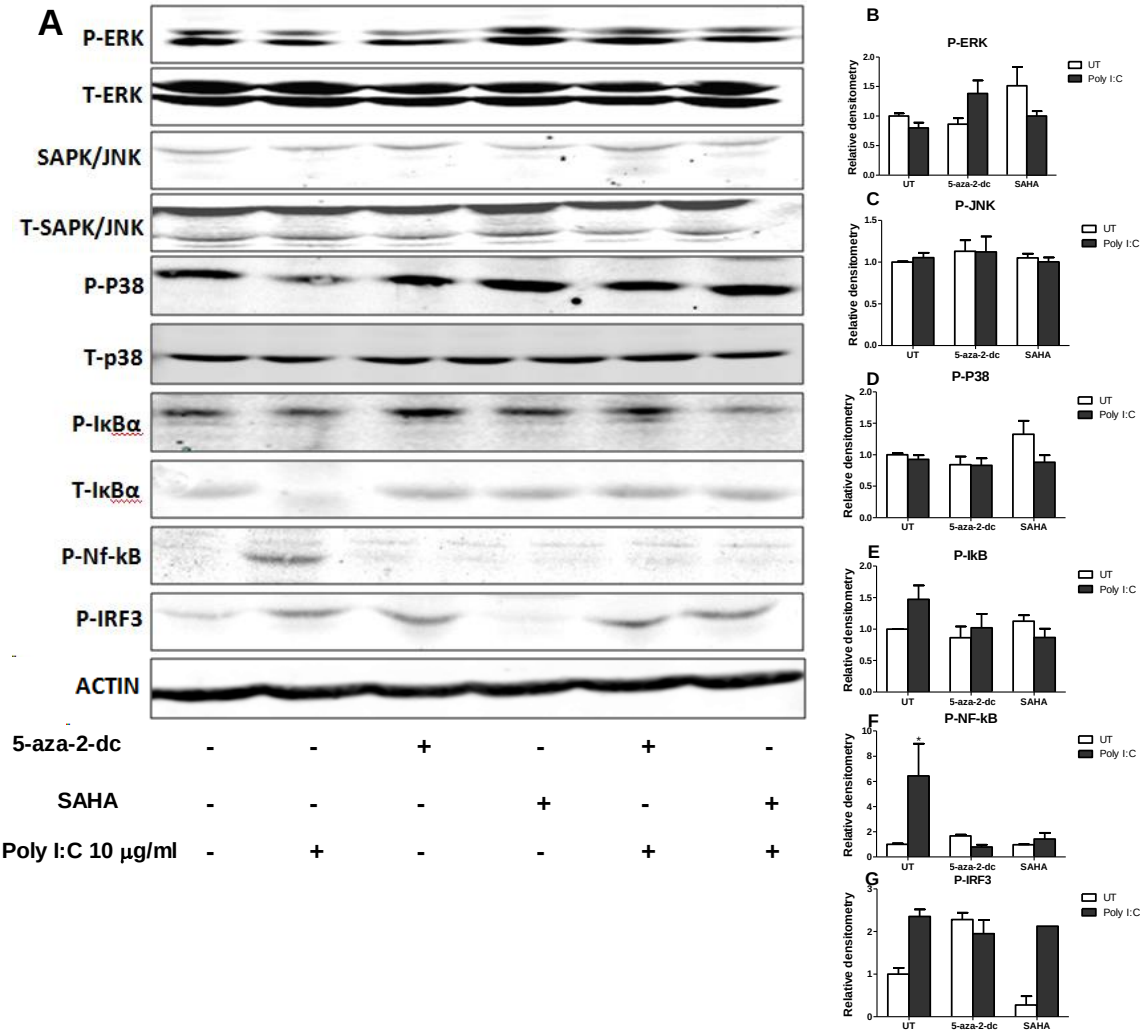


Figure 5.7. Stimulation of THP-1 derived macrophages with poly I:C results in signalling protein phosphorylation, which is subsequently inhibited by treatment with 5-aza-2-dc or SAHA.

A) Cell lysates were analysed using western blot analysis using phospho specific antibodies. Images were acquired using the Licor Odyssey with images generated using Image studio lite. Blots shown are representative images. N=3 for all groups. **B-G)** Using analysis software in image studio lite, blots were analysed for densitometry, with each band being set relative to β-Actin loading control. Values were then set relative to the UT group such that data is expressed as mean relative densitometry ± SEM. Data

was analysed using a 2 way ANOVA, with Bonferroni pots hoc test being used. $P < 0.05$ was considered significant. *** = $P < 0.001$ vs. UT.

5.5 DISCUSSION

In this chapter TLR3 expression and function was examined in the monocytic cell line, and then differentiated macrophages. These cells provided an interesting platform to examine the TLR3 expression given that the expression is almost non-existent in the non-differentiated monocytes, and the expression increases following differentiation into macrophages, as we had shown in the initial results chapter (FIG 3.19).

Our initial studies were focused on the TLR3 responses, or the lack thereof, in THP-1 cells. There is evidence that TLR3 responses are lacking in THP-1 cells due to the absence of any measurable expression of TLR3 (Carpenter et al., 2011). There are several other studies which also examined TLR expression in human monocytes, all of which determined that TLR3 expression is nonexistent in these cells (Heinz et al., 2003; Visintin et al., 2001; Zarember and Godowski, 2002). Our findings echoed those of Carpenter *et al*, in that there was no measurable response to poly I:C seen with any of the analysis methods used. There was no change in mRNA or protein expression of TLR3 following stimulation with poly I:C, and stimulation produced no induction of signaling protein phosphorylation or cytokine release. However, once we examined the effects of epigenetic modifying drugs on the THP-1 cells, we began to see changes in mRNA expression of some of our genes of interest. Treatments with 5-aza-2-dc and SAHA increased the expression of TLR3 and certain cytokines at the mRNA level, which was the opposite effect to what we observed in the HCT 116 cells; however, this did not carry over to the protein level. In spite of this, however, these changes were interesting, with modulation of the epigenome in these cells seemingly able to increase the expression of a previously absent receptor. Although the changes observed at the

mRNA level were not sufficient to induce any measurable changes in protein expression or cytokine secretion.

Upon differentiation into macrophages however, the TLR3 expression profile changes dramatically. The cells have a much higher expression of the receptor, and become responsive to stimulation of the receptor with poly I:C. This finding is supported by another study which examined poly I:C responses in macrophages, showing that the cells are indeed responsive to stimulations with the TLR3 ligand (W.-Y. Cui et al., 2013). Clearly there is a dramatic shift in the machinery controlling regulation of TLR3 upon differentiation, with the cells now responding to poly I:C and transducing the TLR3 activation signals. Furthermore, the effects of epigenetic drug treatments now mirror those seen in the HCT 116 cells, with 5-aza-2-dc and SAHA preventing the poly I:C induced upregulation of TLR3, and the activation of the signaling cascade. The drug treatments are also preventing the ability of the differentiated cells to produce cytokines in response to poly I:C treatment, another result that echoes what we observed in the HCT 116 cells.

Thus, the evidence would suggest that the act of differentiation, as well as treatments with epigenetic modifying drugs, is having dramatic effects on TLR3 and its associated signaling. There is a paucity of information on the epigenetic regulation of inflammatory processes in these cell lines, with only one study showing a direct effect of HDAC inhibitors on LPS induced cytokine release in THP-1 cells. Treatment with the HDAC inhibitor LAQ824 was shown to prevent LPS induced cytokine release from THP-1 cells (Brogdon et al., 2007). Another study illustrated the potential anti inflammatory effects of SAHA *in* vivo, with treatment with the drug preventing LPS induced cytokine release in mice, as well as reducing hepatic injury (Leoni et al., 2002).

In the broader context of inflammation, the inhibition of cytokine release and signaling seen in the macrophages following treatment with the DNMT and HDAC inhibitors indicates that the inflammatory actions of macrophages may be susceptible to epigenetic drugs. This could be beneficial in the event of an unwanted or prolonged anti-viral inflammatory response, in which the epigenetic modifying drugs could modulate macrophage activation and function.

From here we set out to try to identify a potential mechanism that ties together the responses observed both here and in the previous chapter, some uniting factor that could potentially be behind our observations to date. Literature searches led us towards IRF8, which highlighted that IRF8 has an essential role in TLR3 regulation, acting as a negative regulator of TLR3 by inhibiting transcription factor binding at the TLR3 promoter region (Fragale et al., 2011). Furthermore, there was evidence which showed that IRF8 expression in THP-1 cells was markedly higher than in other cell lines (Kubosaki et al., 2010). Based on this, we theorized that the differences observed in TLR3 expression might be due to differences in IRF8 expression. The role of IRF8 in the regulation of TLR3 is the focus of the final chapter.

5.6 CONCLUSION

The results obtained here highlighted the different functional responses of TLR3 in monocytes and macrophages. The differences potentially reflect the different physiological roles of the two distinct cell types. While macrophages have a need to recognize PAMPs such as TLR3 in order to effectively phagocytose pathogens, precursor cells such as monocytes do not require the same pattern recognition. The changes in TLR3 function seen with differentiation led to speculation that changes in

expression of a potential regulator of TLR3 may be behind the differential expression observed. This search for a common negative regulator will form the focus of the final chapter,

Chapter 6: Investigating the mechanisms of TLR3 regulation by epigenetic modifications.

6.1 INTRODUCTION

Following the outcomes of the previous results chapters, we believed there may exist a uniting factor behind the differential expression of TLR3 across our different cell types. Literature searches of TLR regulating proteins led us to IRF8, a protein which has an essential role in the regulation of TLR3 expression (Fragale et al., 2011). Evidence shows that IRF8 has the ability to bind to the TLR3 promoter competitively, thus preventing the binding of pro-TLR3 transcription factors such as IRF1 and IRF2. Our interest in this particular protein was heightened by the knowledge that THP-1 cells highly express IRF8, with its function being an essential component in the differentiation of monocytes into macrophages (Kurotaki et al., 2013).

To this end we sought to compare IRF8 expression across our different cell types, and to invesigate how its expression responded to treatment with epigenetic modifying drugs. The relationship between IRF8 and TLR3 expression in each cell type led us to believe that IRF8 was regulating TLR3 expression, and that, rather than TLR3 being directly affected by the epigenetic modifying drugs, that IRF8 expression was instead modulated by these epigenetic changes. To investigate this we transfected our HCT 116 cell line (WT and DKO) with *hTLR3*, to determine if addition of a plasmid could restore the TLR function in the DKO cells, and whether it would alter expression in the WT cells as well. Following this we also transfected our cells with *hIRF8*, in order to determine if expressing this gene in the HCT 116 cells, which have low basal IRF8 expression, would alter TLR3 expression and function. We also wished to overexpress

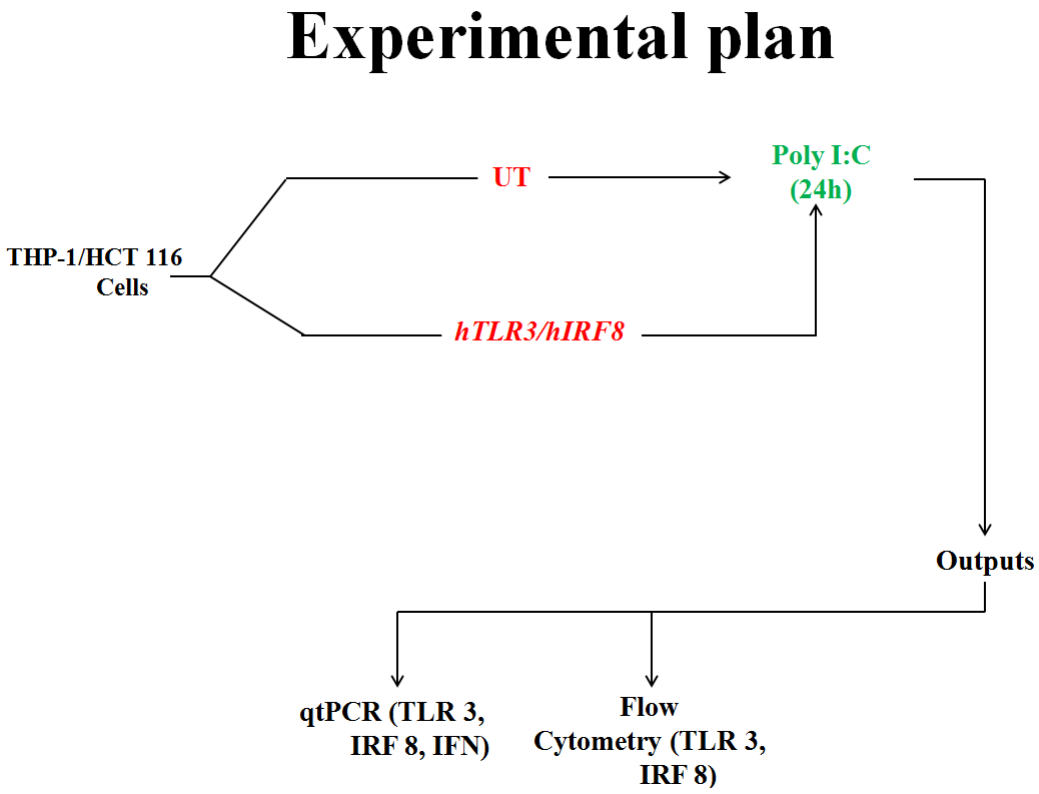
hTLR3 in the THP-1 cells, in order to examine the effects of overexpressing our gene of interest in a cell line abundant in the protein, which we believed to be controlling its expression. We also wished to confirm our belief that the effect of the epigenetic modifying drugs on TLR3 expression was not due to direct action at the TLR3 promoter. To this end we sought to perform methylation sequencing analysis, however, for reasons outlined later in this chapter, this process was unsuccessful

Thus, the aim of this chapter was to help discern the mechanism behind the epigenetic induced changes in TLR3 expression and signaling. To this end, we overexpressed both TLR3 and IRF8 in HCT 116 cells and THP-1 cells and measured the responses of the transfected cells to poly I:C treatment. We hypothesized that there would be significant differences in IRF8 expression between our different cell lines. Furthermore, the changes seen in TLR3 expression following epigenetic modifications would correspond with changes in IRF8 expression. Following that, we hypothesized that overexpression of TLR3 in the DKO HCT 116 cells and THP-1 cells would provide a rescue effect, and that overexpression of IRF8 in the WT HCT 116 cells would inhibit TLR3 activation and function.

6.2 METHODS

The methods used in this chapter did not differ from those outlined in chapter 2.

6.3 EXPERIMENTAL DESIGN



The experimental design described in the graphic above was followed. HCT 116 cells were transfected with *hTLR*/*hIRF8*and THP-1 cells were transfected with *hTLR3*. Cells were then treated with poly I:C and analysed for changes in TLR3/IRF8 and IFN expression by PCR and flow cytometry. Treatments were performed for n=3 for each group and $P < 0.05$ was considered significant.

6.4 RESULTS

6.4.1 DIFFERENCES IN GENE EXPRESSION ACROSS CELL LINES

In the previous two results chapters, we saw the markedly different responses in the HCT 116 and the THP-1 cells, and also how the THP-1 cell responses differed once they underwent differentiation into macrophages. In light of these results, we sought to examine whether there were differences in the expression of both TLR3 and IRF8 between the different cell lines. To examine this we performed qPCR, and relative mRNA expression was quantified using β-Actin as an endogenous control. The fold change relative to the THP-1 cell line was calculated using the $2^{-\Delta Ct}$ method, with the data expressed as mean fold change ± SEM. Differences were analysed using a one way ANOVA and $P < 0.05$ was considered significant.

In figure 6.1A we see the difference in TLR3 mRNA expression across the three cell lines, with the macrophages having a significantly higher mRNA expression of TLR3 when compared to the THP-1 Cells (THP-1 (1 ± 0.19), Macrophages (8.84 ± 1.65, $P < 0.001$)). The difference between the THP-1 and the HCT 116 cells was even greater still, with a large difference in mRNA expression seen between the two cell lines (THP-1 (1 ± 0.19), HCT 116 (63.5 ± 6.3, $P < 0.001$)). Thus, there are major differences in TLR3 expression between our three cell types, and interestingly, there is also large differences seen in IRF8 expression (Fig 6.1B). While the THP-1 cells have to lowest TLR3 expression of the three cell types, with its expression being effectively nonexistent, the monocytes have the highest IRF8 expression of the three cell lines, with the macrophages having approximately 10 times lower expression (0.098 ± 0.013

(relative to THP-1), $P < 0.001$) and the HCT 116 cells having over 200 times lower expression (0.0003 ± 0.0001 (relative to THP-1), $P < 0.001$). Given the evidence from previous studies that IRF8 acts as a negative regulator of TLR3, this suggests that the differences observed between the cell lines could be related to the hugely differing IRF8 expression.

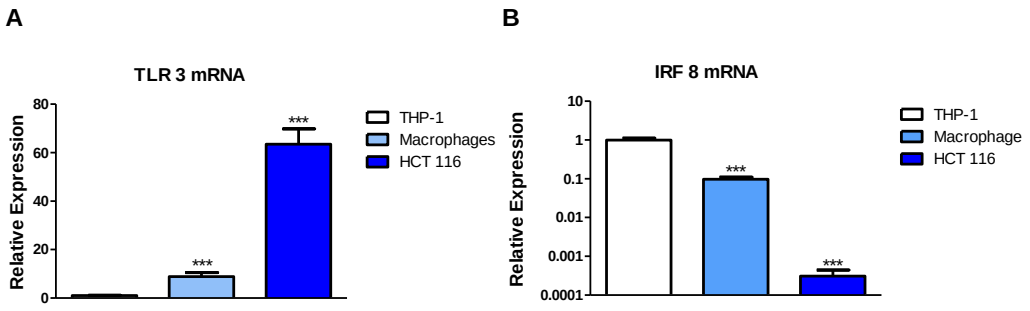


Figure 6.1. Differences in mRNA expression of TLR3 and IRF8 were observed across the different cell lines.

A-B) Real time PCR analysis was performed to examine the levels of IRF8 and TLR3 mRNA expression in THP-1, macrophage and HCT 116 cells. mRNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using 1 way ANOVA. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, *** = $P < 0.001$ vs THP-1.

6.4.2 EPIGENETIC MODIFICATIONS IN HCT 116 CELLS ALTER IRF8 MRNA, BUT NOT IRF8 PROTEIN LEVEL

Based on our observations that IRF8 levels were drastically different across our different cell lines, we posited that the large decrease in TLR3 expression, and the subsequent prevention of TLR3 responses, as outlined in chapter 4, could possibly be related to changes in IRF8 expression in the HCT 116 cells. To analyse the changes, we performed qPCR to measure mRNA expression and flow cytometry to measure changes in the protein level. Data was calculated as fold change relative to the untreated control, and is displayed as mean ± SEM. T-tests were used to examine whether there were significant changes between the groups and $p < 0.05$ was considered significant.

In fig 6.2A we see the difference in IRF8 mRNA expression on the WT vs DKO HCT 116 cells. There was a significantly higher IRF8 expression seen in the DKO cells when compared to the WT cells (WT (1 ± 0.44), DKO (17.3 ± 3, $P < 0.001$). A similar outcome was observed when the HCT 116 cells were treated with 5-aza-2-dc (Fig 6.2B), with the drug treatment resulting in a large increase in IRF8 mRNA expression (UT (1 ± 0.4), 5-aza-2-dc (24 ± 2.88, $P < 0.001$). Lastly, we examined whether inhibition of HDACs with SAHA would produce similar results to the inhibition/knockout of the DNMTs. In figure 6.2C we observed that treatment with SAHA resulted in a significant increase in IRF8 mRNA (UT (1 ± 0.44), SAHA (24.3 ± 4.46, $P < 0.001$). As such, it is evident that the epigenetic modifying drugs we have examined across have a large effect on the expression of IRF8 mRNA expression in HCT 116 cells. These results highlight a potential role for IRF8 in the epigenetic induced changes seen in TLR3 expression.

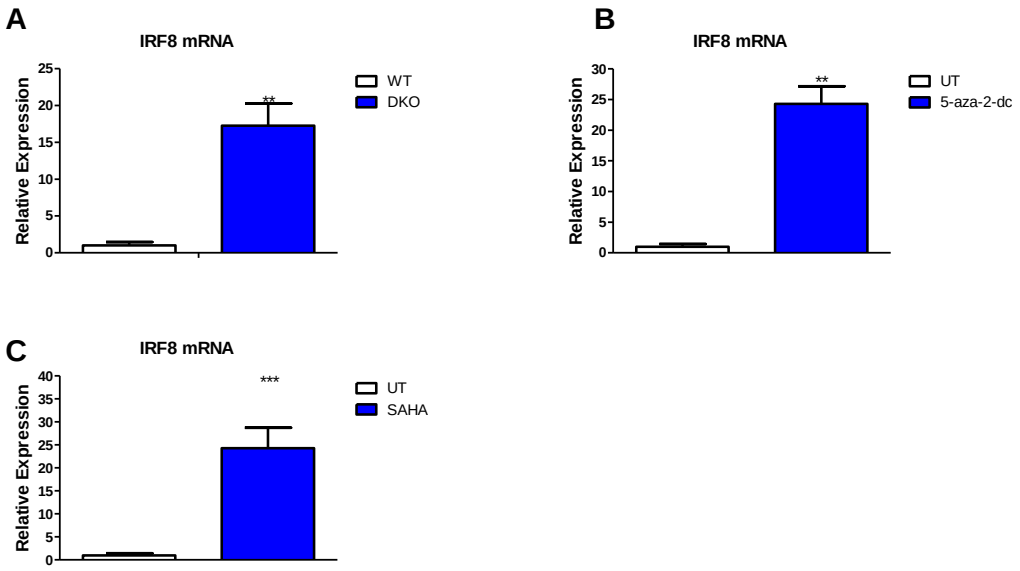


Figure 6.2. Inhibition of DNMTs or HDACs results in increased IRF8 mRNA expression in HCT 116 cells

A-C) Real time PCR analysis was performed to examine the levels of IRF8 mRNA expression in the WT, DKO, 5-aza-2-dc and SAHA treated cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, ** = $P < 0.01$ vs UT, *** = $P < 0.001$ vs UT.

6.4.3 EPIGENETIC MODIFYING DRUGS ALTER IRF8 MRNA EXPRESSION, BUT NOT IRF8 PROTEIN EXPRESSION, IN THP-1 CELLS.

Based on the effect of the epigenetic modifying drugs in the HCT 116 cells, we sought to examine whether these drug treatments were altering IRF8 expression in THP-1 cells in any way. To analyse the changes we performed qPCR to measure mRNA expression and flow cytometry to measure changes in the protein level. Data was calculated as fold change relative to the untreated control, and is displayed as mean ± SEM. T-tests were used to examine whether there were significant changes between the groups and $P < 0.05$ was considered significant.

In figure 6.3A, we see the effect of 5-aza-2-dc treatment on IRF8 mRNA expression in the THP-1 cells, with the drug treatment resulting in over a 5 fold decrease in mRNA expression (UT ($1 \pm .304$), 5-aza-2-dc (0.18 ± 0.07, $P < 0.05$). Similar results were seen with the SAHA treated THP-1 cells (fig 6.3B), with a large decrease in mRNA seen following SAHA treatment (UT (1 ± 0.3), SAHA (0.07 ± 0.05, $P < 0.05$). Following the observations in the HCT-116 cells, this supports the theory that IRF* expression can be altered by epigenetic modifications. It also supports the hypothesis that TLR3 expression changes observed as a result of epigenetic modifications may be related to changes in IRF8 expression.

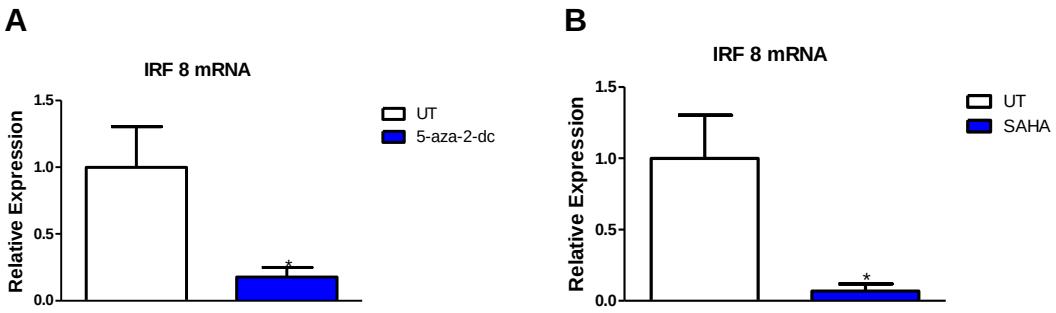


Figure 6.3. Inhibition of DNMTs or HDACs results in decreased IRF8 expression in THP-1 cells

A-B) Real time PCR analysis was performed to examine the levels of IRF8 mRNA expression in both the WT UT and WT 5-azz-2-dc/SAHA treated cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a student's t-test. $P < 0.05$ was considered significant. Data shown is mean ± SEM, N = 3, * = $P < 0.05$ vs. UT.

6.4.4 Effect of TLR3 Overexpression in HCT 116 and THP-1 Cells

In order to further investigate the relationship between TLR3 and IRF8, and how these affect each other in the different cell lines, we embarked on a series of overexpression studies in which we transfected our cell lines with plasmids to overexpress the genes of interest. In this section, we examine the effects of overexpression of TLR3 in the HCT 116 and THP-1 cells. The cells were transfected as described in the methods section and underwent stimulations with poly I:C as previously described. To measure changes induced by transfections, we used qPCR to measure changes in mRNA expression and flow cytometry to measure changes in protein levels. Data was calculated as fold change relative to the untreated control, and is displayed as mean ± SEM. Two way ANOVAs were used to determine whether there were significant differences between the groups and Bonferronis post hoc test was used. $P < 0.05$ was considered significant.

The results for the non-transfected HCT 116 cells (Fig 6.4A) were the same as those seen in chapter 4, with a significant increase in TLR3 mRNA expression in the WT cells following stimulation (WT (1 ± 0.2), Wt + poly I:C (6.2 ± 1.016, $P < 0.001$), and significantly lower basal expression in the DKO cells that did not change following stimulation (DKO (0.13 ± 0.025), DKO + poly I:C (0.06 ± 0.01, $P > 0.05$). Following transfection with *hTLR3* there is an increase seen in both the WT cells (WT (22.47 ± 8), WT + poly I:C (19.9 ± 5.25), and in the DKO cells (DKO (7.1 ± 2.3), DKO + poly I:C (6.9 ± 1.1, $P > 0.05$). When we examined the changes in TLR3 protein level following transfection (Fig 6.4B), we observed that, in the non-transfcted groups, there was a significant increase in TLR3 expression following poly I:C stimulation (WT UT (1 ± 0.02), WT + poly I:C (1.5 ± 0.04, $P < 0.001$), with no changes seen in the DKO cells (DKO UT (0.9 ± 0.04) DKO + poly I:C (1 ± 0.012, $P > 0.05$). Following transfection,

there was an increase in TLR3 protein levels across all groups, (WT + *hTRL3* (1.69 ± 0.057, P < 0.001 vs WT UT),WT + *hTRL3*+ poly I:C (1.982 ± 0.01, P < 0.001 vs WT UT, P < 0.001 vs WT + *hTRL3*), DKO + *hTRL3* (1.7 ± 0.09, P < 0.001 vs WT UT) DKO + *hTRL3* + poly I:C (2.09 ± 0.03, P < 0.001 vs WT UT, P < 0.01 vs WT + *hTRL3*). As is evident, while the significant increase in TLR3 expression was maintained in the WT + *hTRL3* following transfection (WT + *hTRL3*(1.69 ± 0.05) WT + *hTRL3* + poly I:C (1.982 ± 0.09, P < 0.001), we also observed a significant increase in the DKO + *hTRL3* cells following poly I:C stimulation (2.093 ± 0.03, P < 0.001) When compared to the DKO + *hTRL3* cells alone (1.723 ± 0.092). This suggests that the transfection is having a rescue effect on the DKO cells, and that the overexpression of *hTRL3* allows them to become responsive to poly I:C stimulation.

In figure 6.4C the effects of *hTRL3* transfection on IRF8 mRNA are shown, with no significant difference between the WT and WT + poly I:C group (WT UT (1 ± 0.4) WT + poly IC: (0.23 ± 0.06, P > 0.05), however both the DKO (20.9 ± 3.905, P < 0.001) and the DKO + poly I:C (27.6 ± 3.2, P < 0.001), had significantly higher expression of IRF8, in line with our findings in chapter 4. Transfection with *hTLR3* did not affect the expression of IRF8 significantly, with the expression profile in the transfected cells similar to that seen in the control group (WT + *hTLR3* (0.48 ± 0.093), WT *hTLR3* + poly I:C (0.6 ± 0.19, P > 0.05), DKO + *hTLR3* (44.9 ± 9.8, P < 0.001 vs both WT and WT *hTLR3*), DKO + *hTLR3* + poly I:C (27.6 ± 3.2, P < 0.001 vs WT UT and WT *hTLR3*). At the protein level (Fig 6.4D) however, we observed no major effect of *hTLR3* transfection on IRF8 expression, with similar expression seen across all plasmid treated groups.

In figure 6.4E, we examined the effect of *hTLR3* transfection on IFNβ expression. As shown previously, treatment of WT HCT 116 cells with poly IC: results in an increase in IFN expression (WT UT (1 ± 0.15), WT + poly I:C (4.7 ± 0.9, $P < 0.001$), with no significant response seen in the DKO cells. However, upon transfection with the *hTLR3*, the increase in IFNβ mRNA expression following poly I:C treatment is seemingly absent (WT + *hTLR3* (1 ± 0.13), WT + *hTLR3* + poly I:C (1.4 ± 0.1, $P > 0.05$). Once again, no change was observed in the DKO cells. Following the examination of the effect of transfection in the HCT 116 cells, we examined whether transfection with *hTLR3* could make the normally non responsive THP-1 cells, respond to poly I:C. In figure 6.4F, we see the effect of transfection on TLR3 mRNA expression. Poly I:C treatment of the non transfected THP-1 did not result in and change in TLR3 expression (UT (1 ± 0.5), poly I:C (0.56 ± 0.17, $P > 0.05$). However, upon transfection with *hTLR3*, there is a huge increase in TLR3 expression seen in the THP-1 cells (THP-1 (1 ± 0.46), THP-1 ± *hTLR3* (1082 ± 300.8, $P < 0.001$). Furthermore, following poly I:C stimulation in the transfected THP-1 cells, there is a large increase in expression of TLR3 (8608 ± 1250, $P > 0.05$), however this did not reach significance when compared to the transfected but unstimulated THP-1 cells.

When TLR3 protein levels were examined in the THP-1 cells (fig 6.4G) following transfection, we found similar results to what was seen at the mRNA level. There was no discernible difference between the THP-1 UT ant the poly I:C treated groups (UT (1 ± 0.03), UT + poly I:C (1.12 ± 0.04, $P > 0.05$). Transfection of the THP-1 cells with *hTLR3* resulted in a significant increase in both the *hTLR3* alone (1.318 ± 0.050, $P < 0.05$) and *hTLR3* followed by poly I:C (1.4 ± 0.07, $P < 0.05$) groups. Thus, the transfection was increasing TLR3 expression, albeit very slightly at the protein level,

and, unlike what we observed with the mRNA data, the transfection did not make the cells responsive to poly I:C, as there was no difference in expression between the UT or poly I:C treated transfected cells.

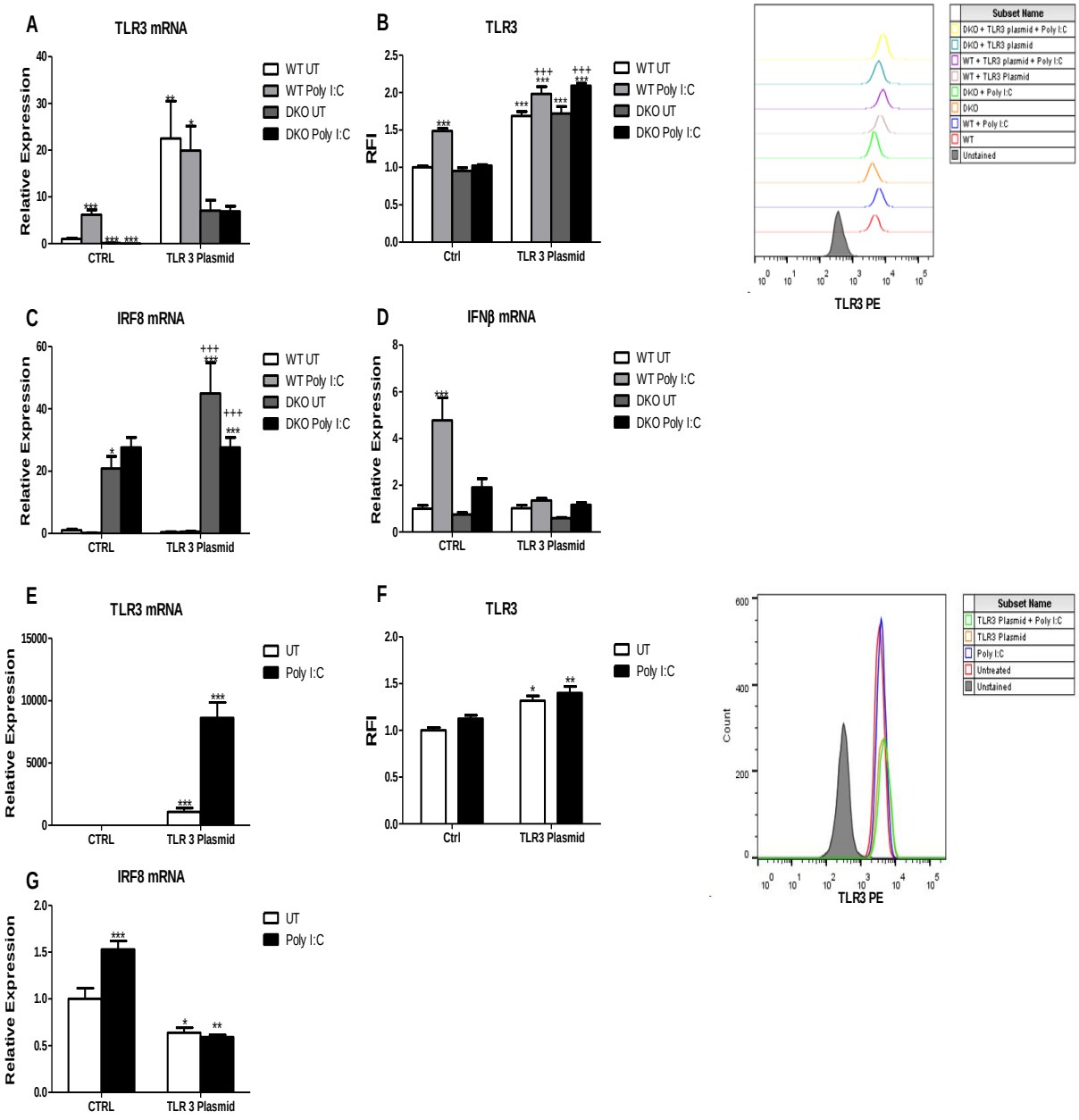


Figure 6.4 Effects of *hTLR3* overexpression in HCT 116 and THP-1 Cells

A, C, D, E, G) Real time PCR analysis was performed to examine the levels of IRF8, TLR3 and IFNβ mRNA expression in both the HCT 116 and THP-1 cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using a 2 way ANOVA. $P < 0.05$ was considered

significant. Data shown is mean ± SEM, N = 3, * = P < 0.05 vs WT UT, ** = P < 0.01 vs WT UT, *** = P < 0.05 vs WT UT, + = P < 0.05 vs WT Plasmid UT, ++ = P < 0.01 vs WT plasmid UT, +++ = P < 0.05 vs WT plasmid UT. **B, F)** Shown is the relative fluorescence intensity (RFI) of the TLR3 PE stain in HCT 116 and THP-1 cells, with or without TLR3 plasmid, SAHA, 5-aza-2-dc and poly I:C. RFI is calculate relative to the control group. Histograms of raw flouresence is shown on the right. P < 0.05 was considered significant. * = P < 0.05 vs WT UT, ** = P < 0.01 vs WT UT, *** = P < 0.05 vs WT UT, + = P < 0.05 vs WT Plasmid UT, ++ = P < 0.01 vs WT plasmid UT, +++ = P < 0.05 vs WT plasmid UT.

6.4.5 EFFECT OF IRF8 TRANSFECTION ON GENE EXPRESSION IN HCT 116 CELLS.

Following on from the results seen with the TLR3 transfection studies, we sought to determine what effect IRF8 transfection would have on the expression of TLR3 in the HCT 116 cells. Given that these cells have very low IRF8 expression, and very high TLR3 expression, we posited that IRF8 over expression might alter the TLR3 expression profile. The cells were transfected as described in the methods section and underwent stimulations with poly I:C as previously described. To measure changes induced by transfections, we used qPCR to measure changes in mRNA expression and flow cytometry to measure changes in protein levels. Data was calculated as fold change relative to the untreated control, and is displayed as mean ± SEM. 2 way ANOVAs were used to determine whether there were significant differences between the groups and Bonferronis post hoc test was used. $P < 0.05$ was considered significant.

In figure 6.5A we that, as seen previously, stimulation with poly I:C increases TLR3 expression significantly (WT UT (1 ± 0.2), WT poly I:C (6.2 ± 1.016, $P < 0.001$), and that expression is greatly reduced in the DKO cells, and they do not respond to the poly I:C stimulation. However, upon transfection of WT HCT 116 cells with *hIRF8*, we found that overall TLR3 expression decreases, with the cells no longer being responsive to poly I:C stimulation (WT *hIRF8* (0.187 ± 0.097), (WT *hIRF8* + poly I:C (0.173 ± 0.04, $P > 0.05$). However, when we examined TLR3 protein expression, we found that the IRF8 plasmid did not have the same effect at the protein level. There was a significant increase in TLR3 expression seen in the WT cells following poly I:C

treatment (1.489 ± 0.04, P < 0.001) when compared to the WT UT cells (1 ± 0.020), and this response to poly I:C was still present after transfection with *hIRF8* (WT *hIRF8* (1.17 ± 0.04), WT *hIRF8* + poly I:C (1.5 ± 0.01, P < 0.001). There were no changes in TLR3 expression in the DKO cells across any of the groups.

In figure 6.5C we measured the IRF8 mRNA level in response to the transfection and its evident that there was a large increase in mRNA expression. The IRF8 expression in the untransfected cells was similar to what we observed previously, with higher expression of IRF8 in the DKO cells lines when compared to the WT cells (WT UT (1 ± 0.44), WT poly I:C (0.2 ± 0.06, P > 0.05), DKO UT (20.9 ± 3.9, P < 0.001 vs WT UT), DKO + poly I:C (27.6 ± 3.2, P < 0.001 vs WT UT). Following transfection however there was a colossal increase in IRF8 mRNA level (WT *hIRF8* (489987 ± 48692, P < 0.001). WT *hIRF8* + poly I:C (593150 ± 42738, P < 0.001), DKO *hIRF8* (1430337 ± 125661, P < 0.001), DKO *hIRF8* + poly I:C (1265411 ± 253372, P < 0.001). These changes were also reflected at the protein level (6.5D), with huge increases in IRF8 seen post transfection level (WT *hIRF8* (15.5 ± .885, P < 0.001). WT *hIRF8* + poly I:C (17.3 ± 1.57, P < 0.001), DKO *hIRF8* (14.53 ± .3, P < 0.001), DKO *hIRF8* + poly I:C (13.49 ± .597, P < 0.001).

Finally, in figure 6.5E, we see the effect of IRF8 transfection of IFNβ, mRNA expression. We observed that, as seen previously there was a significant increase in IFN expression following poly I:C treatment (WT UT (1 ± 0.152), WT poly I:C (4.78 ± 0.96, P < 0.001). However, following transfection with IRF8, the poly I:C induced increase in IFNβ is no longer present level (WT *hIRF8* (1.85 ± .167, P < 0.001). WT *hIRF8* + poly I:C (1.639 ± .309, P > 0.05 vs WT *hIRF8*)). Thus, this data would suggest that the

overexpression with IRF8 has the ability to effect change at the mRNA level, but has seemingly no effect on the protein expression of TLR3.

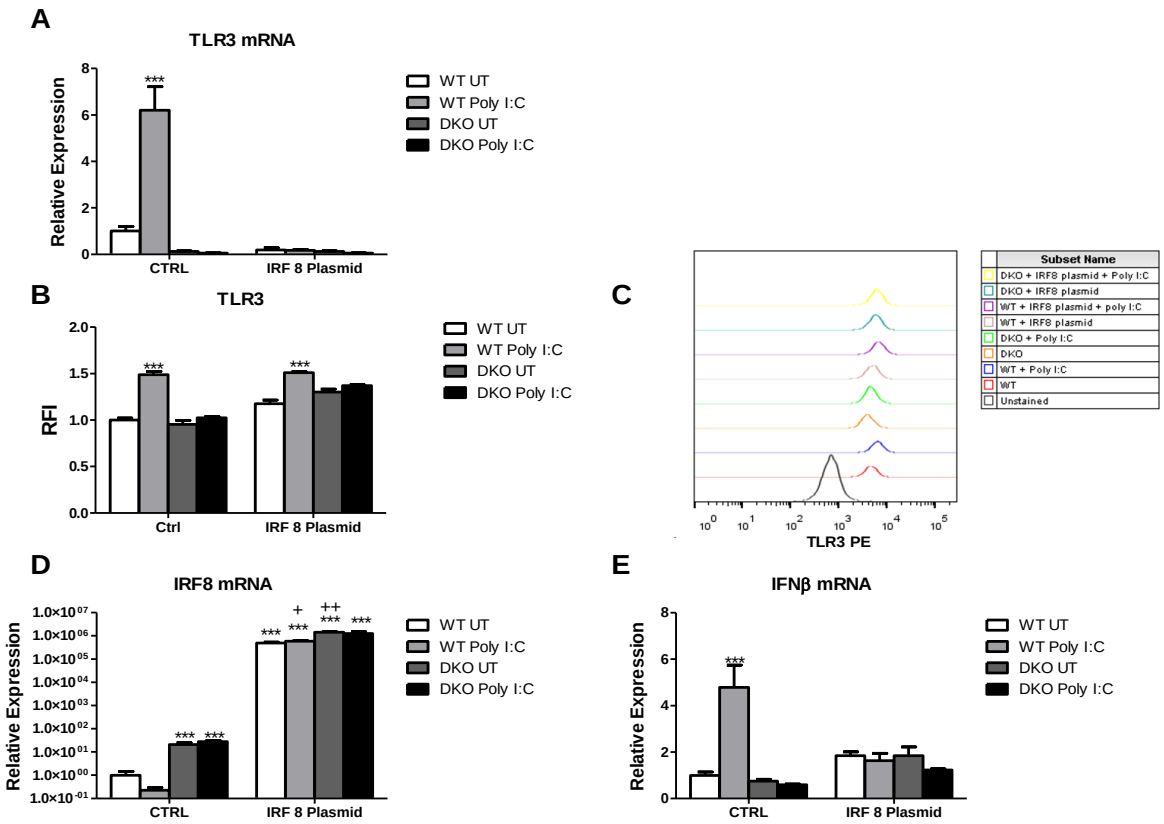


Figure 6.5. Effects of *hIRF8* overexpression in HCT 116 cells

A, D, E) Real time PCR analysis was performed to examine the levels of IRF8, TLR3 and IFNβ mRNA expression in both the HCT 116 cells. RNA expression was quantified using the Roche LightCycler 480 and the relative fold change was calculated. Data was analysed using 2 way ANOVAs. P < 0.05 was considered significant. Data shown is mean ± SEM, N = 3, * = P < 0.05 vs WT UT, ** = P < 0.01 vs WT UT, *** = P < 0.05 vs WT UT, + = P < 0.05 vs WT Plasmid UT, ++ = P < 0.01 vs WT plasmid UT, +++ = P < 0.05 vs WT plasmid UT. **B)** Shown is the relative fluorescence intensity (RFI) of the IRF8 APC stain in both HCT 116 UT and 5-aza-2-dc/SAHA treated cells. RFI is calculated relative to the control group.. P < 0.05 was considered significant. * = P < 0.05 vs WT UT, ** = P < 0.01 vs WT UT, *** = P < 0.05 vs WT UT, + = P < 0.05 vs

WT Plasmid UT, ++ = $P < 0.01$ vs WT plasmid UT, +++ = $P < 0.05$ vs WT plasmid UT.

Histograms of raw flouresence data is shown in **C)**

6.4.6 PRIMER DESIGN FOR BISULFITE SEQUENCING

In order to perform bisulfite sequencing of DNA, primers must be designed specifically for the bisulfite converted DNA, which is significantly different from the non-converted sequence it originates from. We converted genomic HCT 116 WT DNA as described in the methods and we wished to sequence the bisulfite-converted products to determine the extent of methylation in the promoter region of TLR3. In figure 6.6 we show a diagram of the TLR3, gene, with the exons numbered 1-5. The promoter region, which is shown to be on the first exon, is expanded to show the 900 base pair sequence that makes up the region. In grey, beginning at base pair 806, is the predicted region of IRF-E (interferon elements) binding. This is significant as it's the region which IRF1/2, which are known to initiate TLR3 transcription, bind. It is also the region at which IRF8 is purported to competitively bind to, preventing IRF1/2 induced transcription initiation.

In figure 6.7A we see the sequence for the TLR3 promoter region, which we obtained from the Genecards database, with the CG pairs highlighted in grey. As is evident from the image, there is very little overall CG content, suggesting that the potential for regulation of this region by methylation is relatively low. Furthermore, there is only one CpG island, which consists of CGCG from bp 325-329. In figure 6.7B, we see the bisulfite converted promoter sequence, and we can see that the software used to predict the conversion shows the conversion of almost all of the cytosines. The only non-converted regions are the CGs, which are again highlighted in grey.

Figure 6.7C shows the sequence alignment of the first primer pair used. The amplicon length of this primer pair was 988 bases, and we designed it to encompass the

whole TLR3 promoter region. Unfortunately, as is seen in figure 6.7A-C (primer pair 1), these primers were unsuccessful in amplifying the target region. There is a number of possible reasons for this, however the most likely is that the amplicon size was too large, seen as smaller amplicon sizes are recommended for bisulfite converted PCR. With this in mind, the second and third set of primers we designed (figure 6.7D-E) were designed to have shorter amplicons (395 and 397 bp respectively). However, once these primers were designed, we discovered that primer pair 2 and primer pair 3 (table 2.5, chapter 2: methods) had almost the exact same start and end points (pair 1 (start 42, stop 436), pair 2 (start 40, stop 436)). Thus, we only utilized one of the sets in PCR reactions as they were essentially the same primers. Similar to what we observed with the first primer set, there was no amplification seen in any of our PCR reactions (figure 6.8A-C, primer pair 2), suggesting that the primers were ineffective.

By using positive controls, which amplified non converted HCT 116 WT DNA with primers for the non converted sequence, to demonstrate that the PCR reaction itself, as well as the DNA isolation, was not at fault, we determined that the issue was solely with the primers themselves. As a last effort, we sought to combine the forward primer from one set, with the reverse from another, and vice versa. The hope was that they might combine to potentially amplify a section of the promoter region, however as before, no amplification occurred with either pair (figure 6.8D). Following the failure of any of our primers to amplify our region of interest, we attempted to redesign more primers with different amplicon lengths and binding sites. However, following further analysis of the bisulfite converted region, it was determined that we could not design any further primer pairs which would encompass our region of interest.

Following the bisulfite conversion, as is seen in figure 6.7B, the region contains a large amount of thymine and adenine, which recur in long runs of Ts and As. This makes it next to impossible to design primers for these regions, as they would be incredibly non-specific. Hence, we had to abandon our hopes of sequencing the bisulfite converted TLR3 promoter region, although given the lack of CG content in the promoter region itself, one could speculate that the gene expression is unlikely to be directly regulated by methylation.

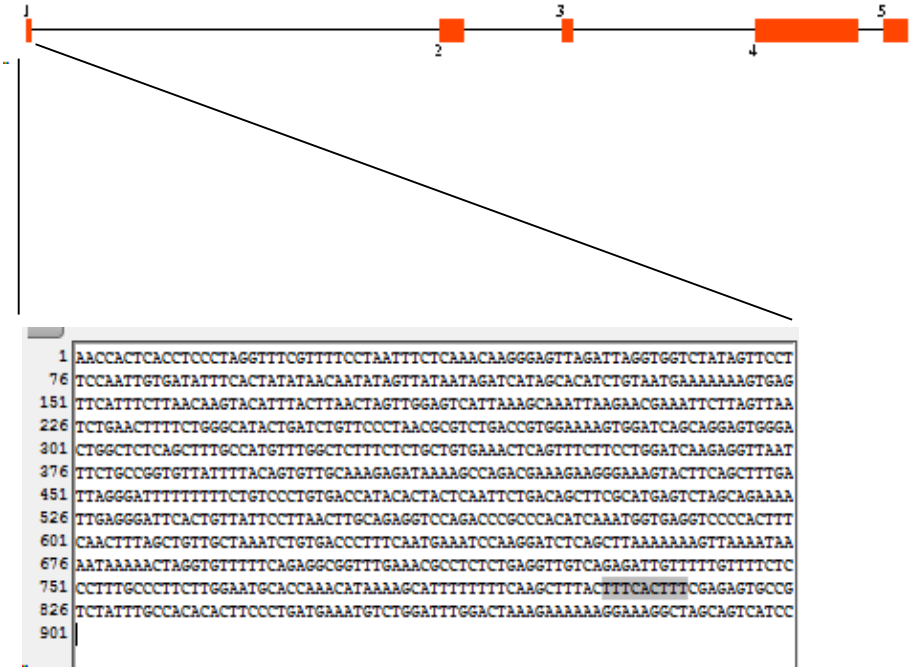
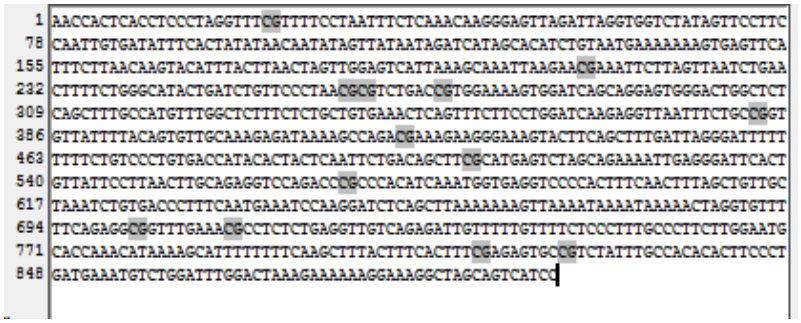


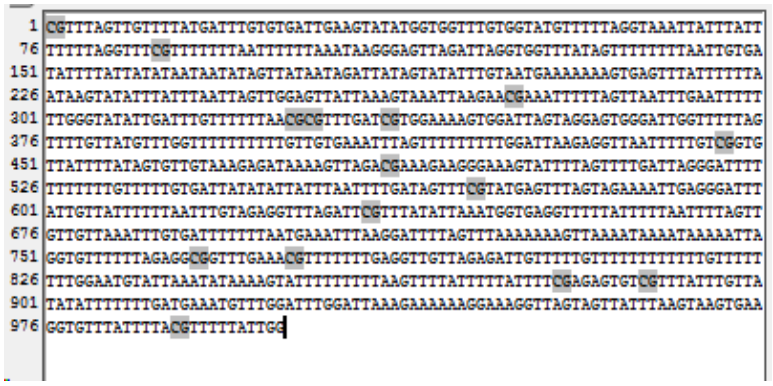
Figure 6.6. TLR3 gene structure, promoter sequence, and predicted IRF8 binding location.

THE TLR3 gene structure is outlined, with the five exons shown. The TLR3 promoter region is believed to be on exon 1, as indicated by the diagram. The promoter region is a 900 base pair (BP) region upstream of the transcription start site. The sequence highlighted in grey is the alleged binding position of IRF elements in the TLR3 promoter region.

A



B



C

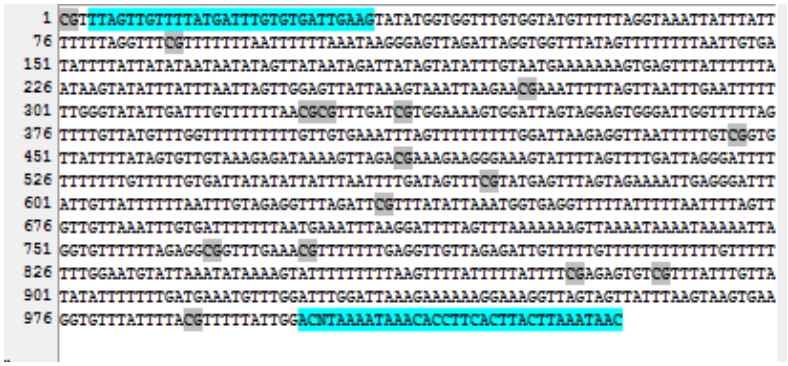


Figure 6.7 (1 of 2). Predicted bisulfite converted TLR3 promoter sequence and primer alignments.

A) TLR3 promoter sequence, with 50 bases added either side to allow design of primers, which would encompass the whole region. CG content is highlighted in grey **B)** Predicted bisulfite converted TLR3 promoter region sequence. Using Zymo's bisulfite primer seeker we were able to predict the sequence of the bisulfite converted TLR3 promoter. **C)** The sequence alignments of forward and reverse primer pair 1. Binding sites are shown in blue.

D

E

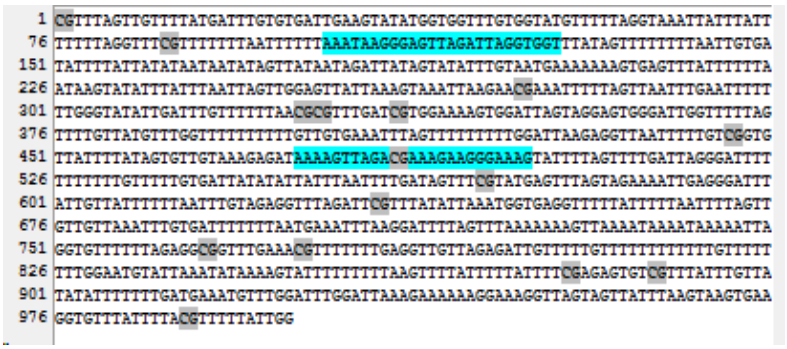


Figure 6.7 (2 of 2). Predicted bisulfite converted TLR3 promoter sequence and primer alignments.

A) TLR3 promoter sequence, with 50 bases added either side to allow design of primers, which would encompass the whole region. CG content is highlighted in grey **B)** Predicted bisulfite converted TLR3 promoter region sequence. Using Zymo's bisulfite primer seeker we were able to predict the sequence of the bisulfite converted TLR3 promoter. **C)** The sequence alignments of forward and reverse primer pair 1. Binding sites are shown in blue. **D)** The sequence alignments of forward and reverse primer pair 2. Binding sites are shown in blue. **E)** The sequence alignments of forward and reverse primer pair 3. Binding sites are shown in blue

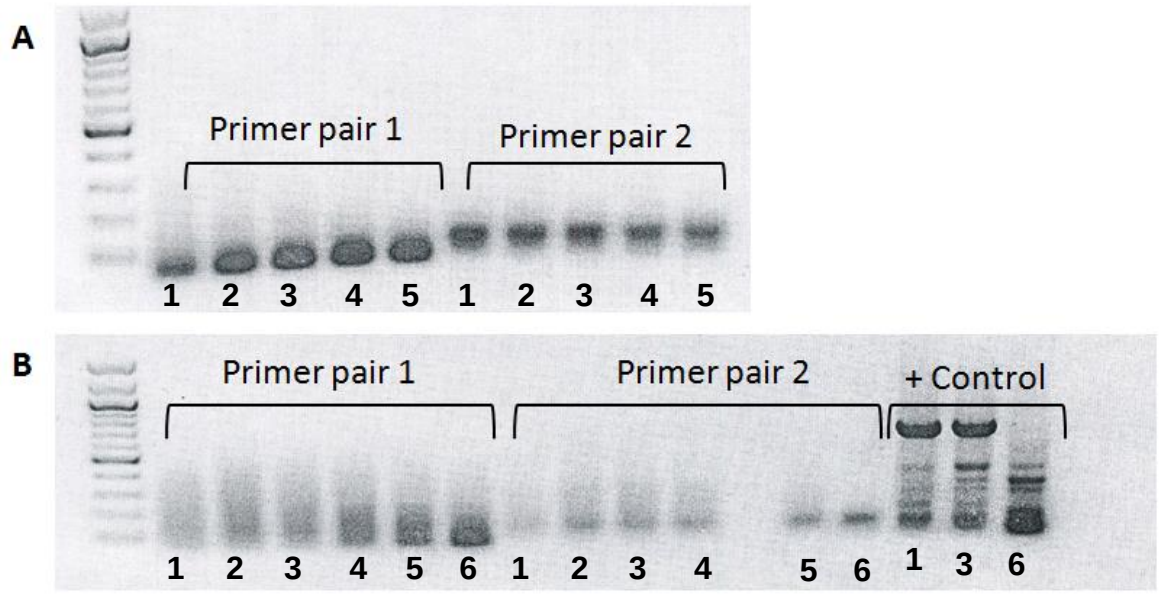


Figure 6.8 (1 of 2). Results of bisulfite converted PCR with primers for TLR3 promoter region.

A-B) Outlined here are the results of PCR reactions involving bisulfite converted DNA and the primers designed to amplify the TLR3 promoter region. All samples were WT HCT 116 genomic DNA. Each lane is the same DNA sample at a different annealing temperature. Temps used were as follows **A)**1 (53.1°C),2 (56.1°C), 3 (60.6°C), 4 (63.5 °C), 5 (64.5 °C).**B)** 1 (42.1°C),2 (43.5°C), 3 (46.9°C), 4 (51.8 °C), 5 (55.1 °C), 6 (56.5°C). PCR products were run on 1% agarose gels with ethidium bromide. The positive control used here is non bisulfite converted DNA which is amplified with TLR3 primers designed for the non-converted TLR3 promoter region. The three lanes correspond to three positive controls with the same DNA/primer mix, at the temperatures indicated.

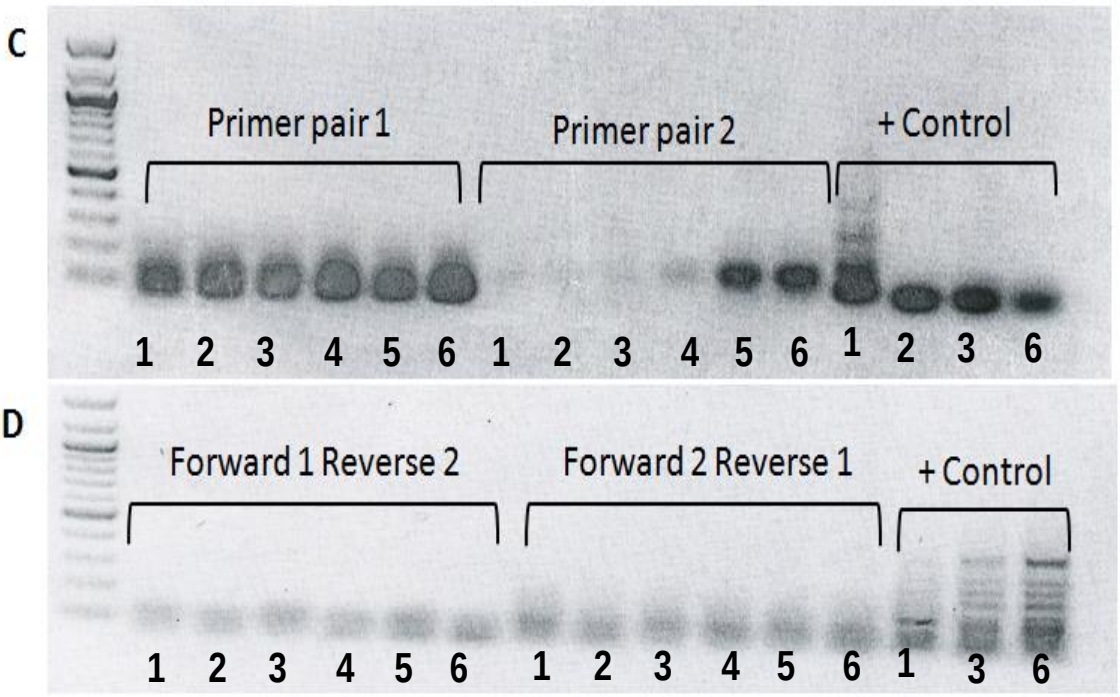


Figure 6.8 (1 of 2). Results of bisulfite converted PCR with primers for TLR3 promoter region.

C-D) Outlined here are the results of PCR reactions involving bisulfite converted DNA and the primers designed to amplify the TLR3 promoter region. All samples were WT HCT 116 genomic DNA. Each lane is the same DNA sample at a different annealing temperature. Temps used were as follows **C-D)** 1 (42.1°C),2 (43.5°C), 3 (46.9°C), 4 (51.8 °C), 5 (55.1 °C), 6 (56.5°C). PCR products were run on 1% agarose gels with ethidium bromide. The positive control used here is non bisulfite converted DNA which is amplified with TLR3 primers designed for the non-converted TLR3 promoter region. The lanes correspond to positive controls with the same DNA/primer mix, at the temperatures indicated.

6.5 DISCUSSION

The goal of this chapter was to examine the potential factors regulating TLR3 expression, and we hoped to confirm whether TLR3 was being regulated by an external factor such as a negative regulator, or whether the effects we have observed throughout this thesis were due to the direct actions of epigenetic modifications on the TLR3 promoter region.

The initial studies involved examining the differences in IRF8 expression across our different cell lines. We were aware from previous studies that IRF8 has an essential role in TLR3 regulation, acting as a negative regulator of TLR3 by inhibiting transcription factor binding at the TLR3 promoter region (Fragale et al., 2011). Furthermore, there was evidence which showed that IRF8 expression in THP-1 cells was markedly higher than in other cell lines (Kubosaki et al., 2010). Based on this, we theorized that the differences observed in TLR3 expression might be due to differences in IRF8 expression.

To investigate this, we examined TLR3 and IRF8 expression in our three cell lines. THP-1 cells had the highest expression of IRF8 and the lowest TLR3 expression, with the HCT 116 cells showing the opposite results. Furthermore, differentiation of the THP-1 cells into macrophages resulted in a downregulation of IRF8 and an upregulation of TLR3. These expression patterns led to further interest in IRF8, and we discovered that, in any instance in which we observed decreased TLR3 expression in our HCT 116 cells, we observed a corresponding increase in IRF8 expression.

Inhibition of the DNMTs and HDACs in HCT 116 cells produced large decreases in TLR3 expression and large increases in IRF8 expression. We then wished to examine what effect overexpression of TLR3 in our non responsive cell lines would have, as well as the effect of overexpression of IRF8 in the WT HCT 116 cells. We hoped this would help confirm that IRF8 could be regulating TLR3 expression, and that inhibition of methylation and acetylation in the DKO/DNMT inhibitor and HDAC inhibitor treated cells was upregulating IRF8, causing the inhibition of the TLR3 response.

Transfection of THP-1 cells and DKO HCT 116 cells with *hTLR3* produced a rescue effect, with both cell lines showing increased expression of TLR3 at the mRNA and protein level, and both becoming responsive to stimulation with poly I:C. Transfection with *hIRF8* yielded some interesting results, with overexpression of IRF8 in the WT HCT 116 cells preventing poly I:C induced increases in TLR3 mRNA expression and IFNβ expression. However, no effect of IRF8 overexpression was observed at the protein level, with cells seemingly still responsive to poly I:C stimulation despite the overexpression. This was a somewhat frustrating result as it did not provide a definitive answer either way as to whether IRF8 was behind the changes in TLR3 expression, although an argument could still be made that, based on the mRNA data, IRF8 overexpression did prevent TLR3 function. If we are to believe the protein results, it would seem that the overexpression of IRF8 was not affecting TLR3 in the WT HCT 116 cells. If, however, the protein data is unreliable, the overexpression of IRF8, which is inhibiting poly I:C induced TLR3 and IFNβ upregulation, could be negatively regulating TLR3. If this is the case, then IRF8 is a key component in viral immunity, suggesting that its level of expression is closely related to TLR3 function. In

the context of viral infection, upregulation of IRF8 could be a mechanism by which the anti-viral response is 'switched off' once the process of pathogen elimination is complete. Furthermore, absence, or downregulation of, IRF8 could lead to an excessive viral response, and could potentially be a factor in viral induced auto-immunity or chronic inflammation. There are studies which investigate the role of viral infections in the development of autoimmunity (Ercolini and Miller, 2009; Gilliet et al., 2008), perhaps IRF8 and its regulation of TLR3 could be another mechanism of viral induced autoimmunity.

The last set of studies outlined in this chapter sought to determine whether the TLR3 promoter region was directly affected by changes in the epigenetic state. Bioinformatic analysis showed that there was very little CG content in the TLR3 promoter region, which suggests that it is unlikely to be directly regulated by changes in methylation or acetylation state, as direct epigenetic control of gene regulation is correlated with CG density (Deaton and Bird, 2011). We wished to confirm this however, so we bisulfite converted DNA from our different treatments in order to perform sequencing of the bisulfite treated DNA. This would have allowed us to visualize any changes in methylation state of the promoter region with a single base resolution. Unfortunately, the sequencing of bisulfite treated DNA is an extremely complex process, and often involves an element of luck. We designed several primer pairs in an attempt to amplify our region of interest, but we were unsuccessful. This could be attributed to a number of factors, most notably, the difficulty in designing effective primers for bisulfite DNA. Once DNA is converted using bisulfite treatment it becomes extremely fragmented, and it is no longer double stranded. This means primers must be large in size (36-34 base pairs) and have a relatively short amplicon length

(150-300 base pairs). This means that there are very limited potential binding sites for primers, and once you have exhausted your options, you will not be able to amplify your region of interest. Unfortunately, many of the methods of determining methylation of specific regions require primer design, and as such most would prove difficult for the reasons outline above. There are other methods of examining methylation levels, however many of these, such as methylation specific western blotting or ELISAs, only show total levels of methylation in a sample, and not what specific genetic regions are methylated.

6.6 CONCLUSION

The disparity between the effect of IRF8 overexpression on TLR3 and IFNβ mRNA expression, and TLR3 protein expression, has left us with uncertainty regarding its role in regulating TLR3s function. While there is a strong case for IRF8 as a negative regulator of TLR3, we failed to provide a definitive result which would convince us that this was the case. We also encountered the difficulty associated with designing primers for bisulfite converted DNA, and our attempts to determine the methylation status of the TLR3 promoter region were wholly unsuccessful.

Chapter 7: General discussion

In the wake of the human genome project the assumptions that a large part of the human genome was just 'junk DNA' with no discernible purpose was disproven, and the importance of these once overlooked elements of the human genome became apparent (Ko and Susztak, 2013). The projects that followed the human genome project opened the door to a whole new area of human biology, one that was previously poorly understood. The ENCODE project led to the reevaluation of the term epigenetics, a term once loosely defined as 'the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence' (Felsenfeld, 2014). The term has now come to encompass a form of non-traditional genetics, in which gene expression is altered without changing the nucleotide sequence. The possibilities for research involving epigenetics are endless, and in our role as immune researchers, we sought to examine whether this new field in molecular biology had any bearing on regulation of the Toll-like Receptors.

This area of research was an expanding field of interest, and there was ample evidence to suggest that epigenetics played a major role in the development of many inflammatory conditions. Hypomethylation of the TR2 promoter had been shown to be associated with increased inflammatory responses in cystic fibrosis (Shuto et al., 2006). TLR 4 was shown to be regulated by both DNA methylation and histone acetylation in intestinal epithelial cells (Takahashi et al., 2009), and multiple other examples setting the precedent that epigenetics have a role to play in the regulation of TLR expression and signalling. Our initial studies focused on the difference in TLR gene expression in the WT and DKO HCT 116 cell line. We hoped to determine whether knockout of the methylation machinery would alter the expression of members of the TLR family, and if

so, to what extent. Our initial findings showed several of the receptors showing responses to being in a hypomethylated environment. TLR3 showed the greatest response to the altered methylation state, with over 100-fold decrease in expression seen in the DKO cell line when compared to the WT cells. There is a paucity of information regarding the regulation of TLR3 in an epigenetic context. There was previous studies which examined a role for epigenetic regulation of TLR3 in perinatal development, however this study just examined changes in TLR3 expression as monocytes differentiate into dendritic cells (Porrás et al., 2008), showing that, as they differentiated, TLR3 expression increased dramatically. The lack of information on TLR3 led us to pursue it as the receptor of interest from the TLR family, and as such we proceeded to examine the role of DNA methylation in the regulation of TLR3 expression and function.

TLR3 plays an essential role in the innate immune system, with its activation by viral envelope motifs and the subsequent cascade which produces type I interferon an essential part of the host defence against viral pathogens (Matsumoto and Seya, 2008, p. 3; Uematsu and Akira, 2007; Zhu et al., 2015). Thus, the ability of epigenetic modifiers to control its expression could provide a new and interesting avenue for research in antiviral immunity. In our studies in HCT 116 cells, we discovered that the removal of methylation resulted in a large decrease in TLR3 expression. Initially this was somewhat counterintuitive, as DNA methylation is generally, though not always, seen as a means of decreasing gene expression or epigenetic ‘silencing’ (Bird, 1986). This initially suggested that the epigenetic mechanism regulating TLR3 expression was not due to direct action on the TLR3 promoter, given that the TLR3 promoter region is not particularly rich in CpG islands, and also the effect seen with inhibition of methylation

was downregulation of gene expression, as opposed to an increase in expression. This did not completely exclude a direct action on the promoter as a potential mechanism, but made it far less likely. We then proceeded to assess whether pharmacological inhibition of the DNA methylation machinery would similarly affect expression, and we observed similar outcomes to those seen in the DKO cells. Furthermore, examination of the role of histone acetylation in the regulation of TLR3 produced remarkably similar effects to those seen with DNA methylation, suggesting that both mechanisms could be acting in a similar fashion, especially given how one key function of DNA methylation is the recruitment of chromatin remodelling elements such as HDACs (Buck-Koehntop and Defossez, 2013). The initial studies in the HCT 116 cells demonstrated that inhibition of HDACs and DNMTs was preventing any response to poly I:C, and as such, resulted in completely non-functional TLR3. Yet in spite of this, there was no discernible difference in total protein expression of TLR3.

The lack of cohesion between the changes in mRNA expression seen and the apparent absence of an effect at the protein level was somewhat confounding. However, changes in mRNA level that are not reflected at the protein level are not unheard of, and there is ample literature to suggest precedent for our findings. One study examined changes in somatostatin receptor 5 (SSR5) in Crohn's disease, and found that there was over 500 fold increase in mRNA expression in the diseased group when compared to controls, yet no difference in protein levels were observed between the two groups (Taquet et al., 2009). Another study examined the expression of Jagged1 (JAG1), a gene involved in notch signalling, in breast cancer. The group had shown that high levels of JAG1 mRNA were associated with a poor prognosis in breast cancer, however there was only a 65% agreement between mRNA levels and protein expression, with not all of

those showing high mRNA expression showing elevated protein levels (Dickson et al., 2007). One study which examined the correlation between cytokine mRNA expression and release following vaccination with human papillomavirus-16 L1 virus-like particles, showed that, in their examination of 20 cytokines, that 11 of them showed weak or no correlation between mRNA and protein expression (Shebl et al., 2010). Thus, there is evidence for mRNA expression and protein expression not showing correlations, as such, it is worth discussing the potential causes of the disparity seen.

One of the first and most obvious steps to examine would be the translation of mRNA into protein, a process that relies heavily on tRNA (transfer RNA). A transfer RNA is an adaptor protein which act as substrates for translation, and they play an essential role in determining how mRNA is interpreted as amino acids (Ibba and Soll, 2000). Epigenetics can not only regulate genomic DNA, but it is also believed to have significant effects at the RNA level (He, 2010). Methylation occurs at a number of structures, nucleotides and otherwise, in RNA, and its effects are believed to be strongly correlated to posttranslational modifications. tRNA is believed to be heavily regulated by methylation, with suggestions that an absence of methylation could drastically increase its function (Motorin and Helm, 2011). One could speculate that the removal of methylation, in cases such as those seen in our studies, could result in a hyperactive form of tRNA, which leads to efficient translation of mRNA into functional protein, in spite of the decrease in mRNA available as a result of the epigenetic modifications. This is purely speculation of course, but suggests a potential reason for the differences we observed in mRNA and protein expression.

Another potential cause is epigenetic alteration of micro RNA (miRNA) expression. Micro RNAs are small non coding RNAs that are involved in the control of

gene expression, usually acting to prevent translation by targeting mRNA for degradation or inhibiting translation directly (Bartel, 2004; Perron and Provost, 2008). Recent discoveries have shown that miRNAs are subject to regulation by epigenetic modifications such as DNA methylation and histone modifications (Saito et al., 2006; Scott et al., 2006). These studies show that miRNAs can be altered by epigenetic modifications, suggesting that it's possible that changes in miRNAs regulating TLR3 mRNA translation could be involved in the changes observed in our studies. MicroRNA-26a was identified to negatively regulate TLR3 expression in rats, with the study showing that miR-26a has a direct effect on TLR3 expression (Jiang et al., 2014). If the epigenetic modifications we have tested in the course of our studies have altered the expression of miR-26a, its possible that the decrease seen in TLR3 mRNA is being counteracted by the absence of a regulatory miRNA, with the result being increased translation of mRNA, with the two effects negating each other. Again, it's worth noting that this is purely speculative, but it still provides a potential explanation for the contrast seen between mRNA and protein expression.

We then examined TLR3 expression and signalling in THP-1 cells, and their progeny, macrophages. TLR3 mRNA expression in THP-1cells is at the threshold of detection, and as such, we showed that there is no measurable response to TLR3 stimulation at any level in these cells, a finding that is reflective of previous studies examining TLR expression in monocytes and monocyte derived cells (Visintin et al., 2001). One interesting result observed in the THP-1 cells was the effect of our epigenetic modifying drugs on basal TLR3 and IFNβ mRNA expression. We noted that treatment of the THP-1 cells with either 5-aza-2-dc or SAHA resulted in an upregulation of TLR3, as well as increased levels of IFNβ mRNA. These findings echoed the results

of two recent studies which showcased that epigenetic modifying drugs can activate previously silence viral elements in the genome. These elements, once activated, can then activate TLR3 and induce IFNβ expression(Chiappinelli et al., 2015; Roulois et al., 2015). We were aware of studies that showed that differentiation of monocytes into macrophages and dendritic cells led to changes in gene expression, with TLR3 being a gene that was affected by differentiation (Muzio et al., 2000; Visintin et al., 2001). We observed that differentiation of the THP-1 cells into macrophages resulted in a cell line that was responsive to poly I:C, with increases in mRNA and protein expression of TLR3 seen post stimulation. Furthermore, these cells were functionally responsive, with TLR3 stimulation resulting in signalling protein activation, and the release of inflammatory cytokines. This led us to question what factors could be changing in the differentiation process, which are leading to the development of a functional TLR3 response.

Searching for a single factor as an explanation for changes in TLR3 expression following differentiation and epigenetic modifications induced by drug treatments may have been overly optimistic. However, given that the responses we were observing in the DKO cells and following treatment with epigenetic modifying drugs, we believed that the changes in methylation and acetylation state of the chromatin could be leading to the activation or upregulation of a factor which was negatively regulating TLR3. As previously described, a study showed that IRF8 acts as a negative regulator of TLR3 expression, with its actions the result of competitive binding of IRF8 to the promoter region of TLR3, preventing the binding of pro TLR3 transcription factors (Fragale et al., 2011).

Throughout the course of our studies, we observed markedly different responses in our different cell lines, with HCT 116 cells showing high TLR3 expression and producing significant responses to poly I:C stimulation. Conversely, the THP-1 cells had very low TLR3 expression, and were not responsive to TLR3. An earlier study had shown that THP-1 cells have a very high expression of IRF8 relative to other cell types (Kubosaki et al., 2010). Based on our knowledge of the expression profile of TLR3 and IRF8 in THP-1 cells, we compared the mRNA expression of both of these genes across our different cell types.

We observed that there were significant differences in IRF8 expression across the THP-1 cells, macrophages and HCT 116 cells. The THP-1 cells had a much higher expression than both of the other cell types, with HCT 116 cells having the lowest expression of IRF8. Furthermore, the act of differentiating THP-1 cells into macrophages produced significant upregulation of TLR3, and downregulation of IRF8. This led to the macrophage cell line becoming responsive to TLR3 stimulation, with signalling protein phosphorylation and cytokine release being observed in the differentiated cells. This suggested to us that there might be some correlation between the different IRF8 expression levels we were observing, and the changes in TLR3 expression. We then proceeded to examine the expression profile of IRF8 in the HCT 116 cells and found that, in any instance where TLR3 mRNA was decreased, such as was seen in the DKO cells and the 5-aza-2-dc/SAHA treated cells, there was a large increase in IRF8 mRNA expression. These results added weight to the theory that IRF8 may be playing a role in changes we observed in TLR3 expression following the various treatment conditions examined in this thesis.

In order to gain more insight into the role of IRF8 in the regulation of TLR3, we wished to examine the effects of both the overexpression of TLR3 in our IRF8 rich cell line, the THP-1 cells, and the overexpression of IRF8 in our IRF8 deficient, TLR3 rich cell line, the HCT 116 cells. To achieve this we used a lipid-based plasmid overexpression system, which allowed us to overexpress our genes of interest in the THP-1 and HCT 116 cells. Overexpression of *hTLR3* in the WT HCT 116 cells increased further the expression of TLR3 at both the mRNA level and protein level, indicating the transfection was successful. Furthermore, the overexpression in the DKO cells led to an increase in TLR3 mRNA, as well as a huge increase at the protein level, which resulted in the DKO cells becoming responsive to stimulation with the TLR3 ligand poly I:C. A similar effect of overexpression was observed in the THP-1 cells, with a large increase in TLR3 mRNA expression seen post-transfection, and the mRNA and protein data suggesting that the cells have become responsive to poly I:C stimulation.

Following the *hTLR3* transfection and the confirmation of the rescue effect, we examined the results of the *hIRF8* overexpression. Examination of the changes in IRF8 mRNA and protein expression in the transfected cells confirmed that the overexpression was successful. Once we had confirmed the overexpression, we examined the effect of the heightened levels of IRF8 on TLR3 expression and function in the WT HCT 116 cells. We observed that overexpression of IRF8 was preventing the poly I:C induced increase in TLR3 mRNA, as well as inhibiting poly I:C induced upregulation of IFNβ mRNA. This would suggest that IRF8 was preventing the typical TLR3 response we had observed throughout the thesis. However, despite the changes observed at the mRNA level, the IRF8 overexpression did not prevent the poly I:C induced increase in TLR3

protein expression. This result is in opposition to what was observed at the mRNA level, and ultimately casts doubt as to whether IRF8 is behind the changes seen in TLR3 expression.

In light of the absence of a definitive result with the IRF8 overexpression studies, we needed to consider the other possible reasons for the changes seen in TLR3 expression and function. To this end, we first sought to exclude whether the effect seen as a result of the epigenetic modifying drugs was as a direct result of changes in the methylation status of the TLR3 promoter region. We performed bioinformatic analysis of the region to determine how rich it was in CpG content, which would be an indicator of the likelihood that it is regulated by methylation and acetylation. Generally, gene regulation by epigenetic mechanisms is correlated to the density of CpG islands in the promoter region of the gene (Deaton and Bird, 2011), and a high CpG content in the promoter region correlated with epigenetic control of gene expression.

Upon analysing the promoter region of TLR3, we found that there is only one CpG island in the 900 base pair region, and this only extends for two CG repeats (CGCG), thus the promoter region of this gene is not very CpG rich, suggesting that it may not be directly affected by epigenetic regulation. To confirm this however, we did attempt to perform methylation-specific sequencing, but due to the difficulties associated with bisulfite sequencing and the subsequent amplification of bisulfite treated DNA, we were unsuccessful. Due to the limitations involved in primer design for bisulfite sequencing, and the significant changes that DNA undergoes following the conversion process, we were unable to amplify our target region, meaning that we were unable to sequence it. Thus, despite the likelihood that this gene is not directly affected by epigenetic control,

due to both the lack of CpG repeats, and the fact that its expression decreases in a hypomethylated state, as opposed to increases, we were unable to confirm this.

In light of the lack of a definitive answer regarding TLR3 regulation, there are other possible mechanisms, which may explain the changes seen in TLR3 expression following the various epigenetic treatments. As we have seen in the different cell lines we have examined, we have observed dramatic changes in TLR3 expression that do not translate to changes in protein expression. This is most notable in the HCT 116 cells, where inhibition of DNA methylation or acetylation results in a large decrease in TLR3 mRNA expression, as well as inhibition of the function of the TLR3 receptor, with no changes observed in the TLR3 protein level. One possible explanation for this is altered trafficking of the TLR3 receptor. Intracellular TLRs undergo trafficking via the endoplasmic reticulum to the Golgi apparatus, and there are several regulatory steps involved in the export of functional TLRs. Unc93b1 is a trafficking chaperone that is involved in the ER export and partitioning of TLRs into endolysosomes (Kim et al., 2008; Lee et al., 2013). Previous studies have shown that non-functioning variants of this Unc93b1 protein have resulted in the failure of ER export of TLRs as well as the loss of TLR signalling (Brinkmann et al., 2007; Casrouge et al., 2006). Other recent studies have uncovered another trafficking protein involved in TLR trafficking, LRRC59. It was shown to have a role in loading TLRs into COPII vesicles, which are involved in transporting proteins form the ER to the Golgi apparatus (Tatematsu et al., 2015). If either of these chaperone proteins was affected by epigenetic regulation, it could result in a non-functional TLR3 response, despite the presence of adequate protein expression.

The change in expression of a positive regulator of TLR3 signalling could also be an explanation for the absence of a shift in TLR3 protein expression following epigenetic modifications. The tumor suppressor protein p53 is one of the most studied molecules in the field of cancer research, with evidence suggesting that it is highly regulated by epigenetics, miRNAs, transcription factors and a host of other factors (Saldaña-Meyer and Recillas-Targa, 2011). It has also been shown to act as a positive regulator of TLR3 expression, by positively regulating its transcription by binding to the TLR3 promoter (Taura et al., 2008). The study also showed that, in the absence of p53, there was no poly I:C induced activation of signalling proteins such as NF-κB, or activation of cytokines such as IFNβ. If the epigenetic treatments we performed in the course of our studies were inhibiting p53s actions at the TLR3 promoter, we could potentially see a change in TLR3 function, similar to what we have observed here.

Thus, it is evident that epigenetic regulation is a key contributor to the regulation of TLR3 expression and function, and that the use of epigenetic drugs has the ability to completely inhibit any observable response to TLR3 stimulation with its ligand, poly I:C. The studies undertaken in this thesis highlight the potential for these epigenetic drugs as modifiers of the immune response, and given the dramatic effects seen with the TLR3 receptor, they could have a future role in the control and tempering of the human antiviral response. Recent studies have suggested a role for targeted HDAC inhibition in the disruption and eradication of HIV (Barton et al., 2014), and DNMT inhibitors have been shown to suppress hepatitis C virus infection, replication and protein expression in cell culture (Chen et al., 2013).

However, the studies are not without their limits as well, and we would be remiss to overlook the fact that the drugs used in these treatments are pan-HDAC inhibitors, whose effects are not targeted in any way. As such, although the change in expression profile of TLR3, and the subsequent inhibition of the inflammatory response may be appealing, it is worth remembering that the non-specific actions of these drugs could be resulting in the altered expression and regulation of many different essential genes and cellular processes. This inhibition of such a broad class of enzymes is not targeted in any way, and as such the findings need to be viewed in the context of these limitations. Furthermore, the lack of clarification as to what was causing the disparity between the TLR3 mRNA and protein expression leaves us with the question as to what exactly is happening to TLR3 in the wake of the epigenetic treatments, and what factor or factors are causing these changes. The protein data for TLR3 does give cause for concern, yet throughout the course of our studies we tried to address it. Utilising two different flow cytometry antibodies, as well as western blots and immunocytochemistry, the results were consistent across all tests. However, this does not mean the protein data is unquestionable. There are very few studies which examine TLR3 protein expression in the literature, with western blotting being one of the few mechanism in which TLR3 protein data has been published. However across these studies, and across antibody manufacturers, there is no real consensus on the molecular weight of TLR3, with one study showing TLR3 antibody binding at 97 kDa (Friboulet et al., 2010), another claiming its binding at 104 kDa (G. Cui et al., 2013), and the antibody manufacturer Cell Signalling claiming the protein is 140 kDa in size. One could speculate that the lack of information on TLR3 regulation could be partly due to the difficulty associated with obtaining protein data for it. Other members of the TLR family, such as TLR4, have

been heavily investigated, yet TLR3, the main anti-viral TLR, has not. While there was some evidence to suggest that IRF8 is playing a role, and this may still be the case, there was a lack of closure on this particular issue.

Going forward, there are many paths, which the research into the regulation of TLR3 could take. The first, and most obvious route would be further investigation as to whether a potential negative regulator or TLR3 is the cause of the effects seen in this thesis. An examination of the role of Unc93b1 on the regulation of TLR3, and whether this protein was affected by the changes in methylation and acetylation, could form the basis for several future studies. Identification of the particular class of HDACs which was causing the results observed would also be a worthwhile investigation. And if a specific class of HDACs was identified the focus could shit to identifying whether one particular HDAC was the main factor involved. This would allow much more targeted control of the regulation of TLR3, which would provide a more specific means of controlling the TLR3 responses, and alleviate some of the issues that may be associated with the nonspecific pan HDAC inhibitors. Another, slightly different route, would be to move the epigenetic drug treatments into *in vivo* models, to examine the potential anti-inflammatory and anti-viral capabilities of our drugs of interest in whole organisms, as opposed to the cellular level. As we have outline previously, there is a scarcity of research in the field of TLR3 regulation, and this is even more evident at the level of *in vivo* studies. I believe there is great scope for the examination of epigenetic regulation of inflammation, be it viral or otherwise, in animal models.

7.1 CONCLUDING REMARKS

Despite the ever-present threat of viruses, even in modern society, TLR3 remains a poorly understood member of the TLR family. The goal of this thesis was to examine how epigenetic regulation may be important in TLR3s function as a PRR, and whether altering its epigenetic status would produce an altered response to stimulation. We have identified a key role for epigenetic regulation in the control of TLR3 function, showcasing that it has the ability to abolish any poly I:C induced TLR3 response, from changes in the signalling pathway, to the production and release of inflammatory mediators. Although we were unable to fully categorise the mechanism involved in the regulation of TLR3, we have shown the effects of these epigenetic modifications across a number of cell lines and different conditions. These studies have laid the foundations for further research into the regulation of TLR3, showing that it the pursuit of a targeted controller of TLR3 expression could be an incredibly powerful anti-inflammatory tool. This controller could be in the form of a more specific epigenetic regulatory effect, such as identifying a particular HDAC involved in TLR regulation, or the uncovering of a specific set of proteins involved in its regulation, be they negative regulators such as IRF8, or trafficking proteins such as Unc93b1.

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Immunology and Cell Biology (2016), 1–9

www.nature.com/icb

REVIEW

Epigenetics and innate immunity: the 'unTolld' story

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Toll-like receptors (TLRs) are a family of 13 receptors known as pattern-recognition receptors (PRRs) and have a key role in the innate immune response. The TLRs are activated by pathogen-associated molecular patterns (PAMPs) that are structurally conserved molecules present on the surfaces of bacteria and viruses. The activation of these TLRs by pathogens results in the downstream activation of genes involved in the production of proinflammatory factors. There is a lack of understanding on the mechanisms by which TLR gene expression is regulated. Epigenetics could be one such mechanism, which is concerned with changes in gene expression/products that arise without a change in the nucleotide sequence. These changes are brought about by two main mechanisms, DNA methylation and histone modifications. This review seeks to examine the current knowledge regarding the epigenetic regulation of this family of receptors and their signalling pathways.
Immunology and Cell Biology advance online publication, 22 March 2016; doi:10.1038/icb.2016.24

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are a family of 13 pattern-recognition receptors (PRRs) receptors, of which 10 are present in humans,[1] which recognise structurally conserved molecules present in bacteria and viruses.[2] Activation of the TLRs results in cytoplasmic activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), or interferon regulatory factors (IRF) 3/5/7 transcription factors, which, upon activation, translocate to the nucleus of the cell and alter transcription of proinflammatory cytokines such as interferon-α/β, interleukin-6 and tumour necrosis factor α.[2] The receptor family is recognised as having a large role in the regulation and control of the immune response to microbial and viral challenge as well as in the development of autoimmunity.[3] The TLRs have been shown to have different basal expression levels in different tissues with certain TLRs having higher levels in certain organs.[4] As is evident in Figure 1, the transduction cascade that results following TLR activation is complex and involves recruitment and activation of many factors via kinases, the nature and action of which are beyond the scope of this review (see Akira and Takeda, Toll-like receptor signalling, for a comprehensive overview of TLRs, TLR ligands and TLR signalling). The family can be divided into those that reside on the extracellular surface and those that are found intracellularly.

The extracellular members of the TLR family consist of TLRs 1, 2, 4, 5, 6 and 11; with TLR4 being the first identified TLR. It was discovered as the receptor that responds to lipopolysaccharide (LPS), the component of Gram-negative bacteria known for inducing septic shock.[5] TLR4 has been shown to be essential for defence against Gram-negative bacteria, with mutations in *tlr4* resulting in the development of Gram-negative induced sepsis while other immune function remain intact.[5] TLR2 has been shown to interact with Gram-positive bacteria.[6,7] TLR2 forms heterodimers with other TLRs, namely TLR1 and TLR6, recognising different structural motifs,[8] with TLR1/2 heterodimers responding to triacylated lipopeptides from Gram-positive bacteria and mycoplasma and TLR1/6 heterodimers recognising diacylated lipopeptides from Gram-negative bacteria and mycoplasma.[9,10] TLR5 was initially identified by the presence of the Toll/interleukin (IL-1) receptor homology domain (TIR),[11–13] which is a conserved signalling domain considered to be a defining trait of the TLR family.[14] TLR5 recognises the flagellin protein that is the structurally conserved component of bacteria flagella.[2] Studies have identified its action to be proinflammatory, acting via MyD88 to activate TNF-α which in turn stimulates cytokine production.[15]

The intracellular TLRs consist of TLRs 2, 3, 7, 8, 9 and 10, with TLR3 being the first identified. TLR3 was originally identified as recognising the structure of a synthetic analogue of double-stranded RNA known as polyinosinic–polycytidylic acid (poly(I:C)),[16] which mimics viral infection by inducing the production of type I interferon as well as other inflammatory cytokines. TLR3 is important in the immune response owing to its role in recognising amongst others, viruses such as respiratory syncytial virus, encephalomyocarditis virus, West Nile virus and certain small interfering RNAs.[2,17] The importance of TLR3 function has been shown in animal studies wherein *tlr3*-deficient mice are susceptible to lethal infection by murine cytomegalovirus. *Tlr3* knockout mice infected with murine cytomegalovirus possessed a much higher viral titre when compared with controls.[18]

TLR7 recognises imidazoquinolone derivatives such as imiquimod and resiquimod as well as guanine analogues such as loxoribine. It also recognises single-stranded RNA from viruses such as vesicular stomatitis virus, influenza type A and HIV.[2,17] TLR8 is phylogenetically similar to TLR7, and it also recognises single-stranded RNA. However, it was determined that single-stranded RNA sensing in mice

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Received 17 November 2015; revised 18 February 2016; accepted 19 February 2016; accepted article preview online 29 February 2016

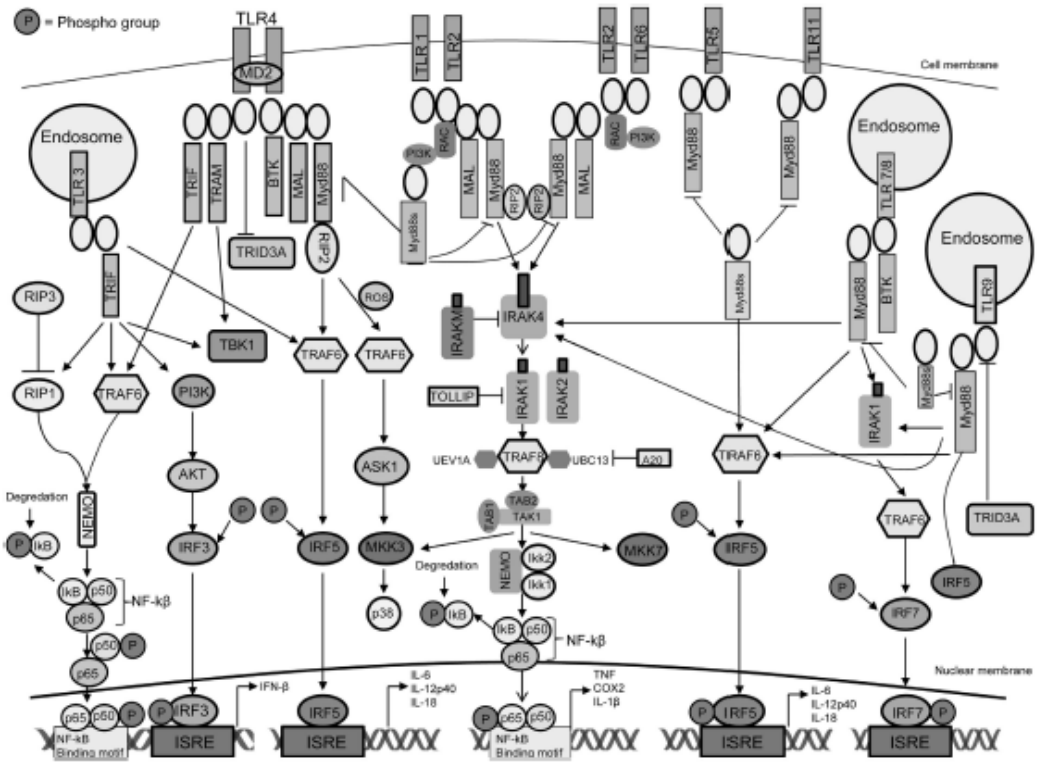

Figure 1 This figure illustrates the location of TLRs in the human cell and the signal transduction pathways that result in the downstream activation of NF-kβ/IRF3/5/7, which results in an immune response by increasing transcription of pro-inflammatory cytokines.

is not dependent on TLR8 and that single-stranded RNA sensing was normal even in the absence of TLR8.[17,2] TLR9 recognises the unmethylated CpG motifs that are a hallmark of bacteria and viruses but are rarely seen in mammalian cells. Their activation by these motifs results in activation of dendritic cells, B cells and also initiates a T_H1 response.[19] Studies have also shown that in certain cases TLR9 can recognise self CpG–DNA, which can lead to the development of autoantibodies and has been implicated in the development of certain autoimmune conditions.[20] Until recently the structure and function of TLR10 was unknown, however, one study showed that blocking TLR10 led to increased production of proinflammatory cytokines, including IL-1β, following TLR2 stimulation. The study also noted that in humans with polymorphisms in *tlr10*, there was documented increases in IL-1β, TNF-α and IL-6 upon ligation of TLR2.[21]

TOLL-LIKE RECEPTORS AND DISEASE

TLRs are purported to be involved in the pathogenesis of many disease states. Sepsis is a disease that hospitalises over 1.2 million people a year in the United States and one of the main causes is Gram-negative bacterial infections[22] and has been linked to TLR2 and TLR4. Altered regulation of *tlr2* has also been implicated in impaired wound healing in patients with type 2 diabetes mellitus.[23] Furthermore, DNA methylation differences in the promoter region of *tlr2* have been linked to pulmonary tuberculosis. Recent studies have also shown increased methylation of CpG sites in the promoter region of the gene and a resulting decrease in *tlr2* expression, which has been implicated in the development of the condition.[24] Hypermethylation of *tlr2* has also been implicated in dysbiosis of the innate immune system as a whole, with studies linking aberrant methylation of the gene to decreased global innate immune response and susceptibility to disease.[25]

Dysregulation of TLR4 signalling and polymorphisms in the *tlr4* gene have been linked to many human diseases. The D299G *tlr4* mutation, which is a germline mutation,[26] has been shown to increase the risk of infection in human patients at risk of sepsis, with studies linking this polymorphism to the increased susceptibility to Gram-negative infections.[27,28] Recent research investigating the role of the D299G mutation as well as the T399I mutation indicates that these SNPs affect constitutive receptor activity, which alters the host response to LPS challenge resulting in sub optimal response to infection.[29] The D299G *tlr4* polymorphism is also linked with ulcerative colitis and Crohns disease, with a recent study showing a significant correlation between the D299G polymorphism and both ulcerative colitis and Crohns disease.[30] TLR4 dysregulation has also been linked to the development of atherosclerosis and other acute coronary events.[31–33] In addition, polymorphisms in *tlr4* have also

been associated with psoriatic arthritis[34] and the development of rheumatoid arthritis.[35] Although recent mouse studies have shown that TLR4 antagonists can reduce atherogenesis in mice with type-2 diabetes.[36]

Non-polymorphism-related upregulation in TLR4 has also been shown to be correlated with ulcerative colitis and Crohns disease, with patient samples showing elevated intestinal levels of TLR4.[37] This suggests that epigenetic changes affecting TLR4 expression may possibly contribute to the development of inflammatory bowel disease. Endogenous suppression of TLR4 expression has also been shown to assist the maintenance of proper intestinal homeostasis, with AN160, a transcription factor abundant specifically in intestinal epithelial cells, being shown to downregulate TLR4.[38] The role of this suppressive effect is believed to be the prevention of TLR4 activation by the commensal bacteria in the gastrointestinal tract.

Dysregulation of TLR3 as well as polymorphisms and knockouts of the *tlr3* gene have also been associated with the development of immune related conditions. In mouse models, TLR3 has been linked to increased morbidity and mortality in response to vaccinia infection. *tlr3-/-* mice were shown to have much lower viral replication and significantly lower levels of cytokines, specifically IL-6, MCP-1 and TNF-α, present in the knockout mice when compared with their wild-type counterparts. All of which suggest a role for TLR3 in the pathogenesis of vaccinia infection.[39] Polymorphisms in *tlr3* have also been linked to development of type 1 diabetes also, with certain risk alleles being associated with early-age diagnosis and worse glycemic control.[40] Like TLR4, *tlr3* dysregulation has also been implicated in the development of intestinal inflammatory conditions. However, in contrast to TLR4, TLR3 was significantly downregulated in intestinal epithelial cells in Crohns disease but not in ulcerative colitis.[37,41] As with TLR4, this suggests a possible role for epigenetic-induced changes in regulation of TLR3 expression.

TLR5 has been shown to have a role in the development of inflammatory conditions such as rheumatoid arthritis and osteoarthritis. Studies have shown that in the aforementioned conditions that TLR5 expression was elevated in synovial tissue, macrophages and endothelial cells. TLR5 was shown to be instrumental in the development of the rheumatoid arthritis pathogenesis, as there was a direct correlation between TLR5 expression, levels of TNF-α in the synovial fluid, and the disease activity score. The importance of TLR5 in disease development was determined when TLR5 was blocked and there was a resultant 80% decrease in synovial TNF-α as well as a decrease in disease activity.[42]

Irregular signalling via the TLR9 receptor, as well as abnormal expression of the TLR9 receptor has been linked to certain disease states. Patients with systemic lupus erythematosus have antibodies against endogenous antigens, including nucleic acids.[43] The cause of these autoantibodies has been suggested to be mediated by signalling via the TLR9 receptor. Although under normal circumstances mammalian DNA is usually inert or inhibitory to signalling via TLR9,[44] it has been shown that signalling via TLR9 can occur when the DNA is present in DNA-immune complexes.[45,46] Thus, it is posited that in these patients where the DNA is recognised by the TLR9 receptor, that this can lead to the development of antibodies to one's own DNA, leading to the development of the systemic lupus erythematosus pathophysiology. Similarly to TLR3/4, TLR9 has also been shown to be dysregulated in inflammatory bowel disease, with peripheral B cells taken from patients with inflammatory bowel disease patients shown to have significantly higher expression of TLR9. The study also showed a positive correlation between TLR9 expression and inflammatory bowel disease severity.[47] In addition to the marked effect traditional genetic changes can have on the expression profile of TLRs, it has been suggested that epigenetic changes could also produce changes in gene regulation that could indeed lead to the development of disease.

EPIGENETIC MODIFICATIONS

Human gene expression is based on a sequence of nucleotides read in a specific order. Changing the position and sequence of these nucleotides can have drastic, even fatal, consequences. Epigenetic changes are described as changes that result in altered gene expression without changing the nucleotide sequence of these genes. These epigenetic changes are important in normal development and when disrupted can result in unwanted changes in gene expression. As such, the importance of epigenetic changes in the development of many diseases such as cancer and inflammatory bowel disease is recognised.[48–52] Epigenetics is concerned with changes in gene expression due to modifications to the structure of chromatin, which affect gene regulation by repressing or enhancing transcription factor binding through different mechanisms.[53–56] Although there are many epigenetic modifications, two distinct types will be the main focus of this review, DNA methylation and histone modifications.

DNA METHYLATION

The methylation of DNA is the most commonly found modification in mammals, usually involving the addition of a methyl group to cytosine residue. In animals it is associated with development and genomic imprinting.[57] It is regulated by the enzyme class known as DNA methyltransferases (DNMTs),[58] which transfer methyl groups to cytosine residues on a DNA strand at the 5′ carbon position of the pyrimidine ring.[59] Generally, a cytosine (C) can only be methylated if it precedes a guanine (G) residue,[60,61] forming what is referred to as CpG dinucleotides. These CpG dinucleotides occur infrequently in the genome but under normal conditions 80–90% of them are described as being methylated in both a spatially and temporally restrictive manner.[62–64] However, when these CpG dinucleotides exist as part of CpG islands, which are found largely in the promoter regions of genes or in other tissue specific genes, they typically have a high number of unmethylated CpG sites.[65] DNA methylation affects chromatin in different ways, generally resulting in epigenetic silencing. It can interfere directly with transcription by blocking transcription factor binding, for example in an unmethylated state the transcription factor erythroblastosis 1 (ETS1) can bind to DNA but when the DNA is methylated, binding does not occur.[66,67] DNA methylation also alters chromatin structure through the recruitment of chromatin remodelling elements such as histone deacetylase complexes (HDACs). Although the detailed mechanism by which the DNA methylation mediates epigenetic regulation is beyond the scope of this review (see Moore *et al*, 2013 for a comprehensive review), one of the major actions of the DNA methylation machinery is the recruitment of proteins that aid in the remodelling of chromatin.

DNA METHYLATION, INHIBITORS AND IMMUNITY

The role of DNA methylation in the regulation of gene expression is well founded, and given its role in the development of cancers, there has long been interest in inhibitors of methylation as potential antitumour therapeutics. Aberrant methylation of tumour suppressor genes has been implicated in the development of certain cancers due to the excessive methylation preventing the transcription of proapoptotic/antitumour factors.[68,69] There are a large number of characterised DNMT inhibitors and they are divided into two distinct families: the nucleoside analogues and the non-nucleoside inhibitors.

Table 1 The table summarises the known effects of epigenetic modifying drugs on immune responses showing treatments and effects

Epigenetic modification	*Treatment*	*Dose*	*Time point*	*Subject*	*Effect*	*Reference*
HDAC inhibition	Butyrate	Not available	Not available	HeLa cells	Inhibits proliferation	Riggs *et al.*[105]
HDAC inhibition (Pan Inhibitor)	LAQ824	120/40/80/nw +/− 1 μg ml^{-1} LPS	1, 3, 6, 12 or 24 h	Thp1/dendritic/macrophages/neutrophils	Inhibits Th1 function and macrophage chemotaxis	Broxton *et al.*[114]
HDAC inhibition (Pan Inhibitor)	SAHA	0.1–50 mg kg^{-1}	1-h pre-LPS injection	Mice (model of endotoxemia)	Decrease in liver damage and cytokine release	Leoni *et al.*[115]
HDAC inhibition (Pan Inhibitor)	TSA	6.25, 12.5, 25, 50, 100, 200 nM	1-h pre-LPS injection (24-h LPS stimulation)	LPS-stimulated macrophages	Decreased levels of cytokine mRNA/protein levels	Han *et al.*[117]
HDAC inhibition (Pan Inhibitor)	LBH589	2.5, 10, 20 nM	24 h	Dendritic cells	Decrease in cytokines in response to stim of TLR3/4	Song *et al.*[109]
HDAC inhibition (Pan HDAC)	TSA	100 nM	1h before stimulation for 1, 2, 4 and 20 h with TLR1/TLR2/TLR4 ligands	BMDMs (stim with TLR ligands)	Decrease in cytokines produced and altered expression of TLR 1-9	Roger *et al.*[110]
DNMT inhibition	5-Azacytidine	500 nM of AZA	72 h	63 cell lines (26 breast cancer, 14 colorectal cancer, 23 ovarian cancer)	Increased immune response	Li *et al.*[81]

Abbreviations: BMDM, bone marrow-derived macrophages; DNMT, DNA methyltransferase; HDAC, histone deacetylase; LPS, lipopolysaccharide; mRNA, messenger RNA; SAHA, suberoylanilide hydroxamic acid.

However, for the purpose of this review we will focus on just the nucleoside inhibitors (see Table 1), specifically the cytidine analogues. 5-aza-cytidine (5-aza) and 5-aza-2-deoxycytidine (5-aza-2dc) are two cytidine analogues that were first synthesised in 1964.[70] These molecules were initially used as antimetabolites and as chemotherapeutic agents in leukaemia owing to their cytotoxicity.[71] The hypomethylating action of the drugs was later defined through a series of studies that showed treatment of a non-myoblastic cell line with 5-aza-cytidine or 5-aza-2-deoxycytidine led to the differentiation of these cells into functional myotubules, a process, which would have not occurred naturally.[72,73] It was speculated that the action seen was owing to the drugs interacting with DNA and hypomethylating it and thus inactivating certain genes involved in repressing the differentiation of the nonfunctional muscle cells into functional myotubes.

5-aza-2-deoxycytidine (5-aza-2dc) is the more potent inhibitor of methylation when compared with 5-aza-cytidine (5-aza).[74] It is believed that the reason for this is owing to 5-aza being a ribose analogue, meaning it incorporates into both RNA and DNA, as opposed to just DNA which the deoxyribose analogue 5-aza-2dc does. Because of this 5-aza has less incorporation into DNA when compared with 5-aza-2dc as it also affects RNA. Its incorporation into RNA is also speculated to be related to its more significant side effects when compared with 5-aza-2dc. Because of its action 5-aza-2dc can only exert its effects on dividing cells, meaning it is somewhat more selective for rapidly dividing cancer cells, however, the ability of 5-aza to incorporate into both dividing and quiescent cells allows its effects to be more widespread, which is undesirable for a cytotoxic therapeutic.[75] The drugs are well established as anticancer drugs in the treatment of leukaemia[74] and myelodisplastic syndromes[76] owing to their efficient anticancer activity. Recently, they have been discussed as potential immunomodulatory drugs, with studies seeking to examine whether these drugs could be effective at noncytotoxic concentrations.[77]

Given the importance of DNA methylation in regulating gene expression one might expect that inhibition of methylation would exert some effect on the immune system. DNA methylation has an established role in the development, function and survival of T cells. DNA methylation and the maintenance of this methylation by DNMT1 was shown using Cre/loxP-mediated inactivation of DNMT1.[78] The study showed that without DNMT1 or DNA methylation that differentiation of naive T cells into certain lineages was impaired. This impaired function was reported to be owing to altered expression of fate-determining genes as a result of the aberrant methylation. Methylation has also been shown to be important in T-cell immunity in an indirect manner. One study showed that methylation of heparin-binding haemagglutinin protein was essential for effective immune defence against *Mycobacterium tuberculosis*. The study in question showed that methylated heparin-binding haemagglutinin provided excellent anti-tuberculosis immunity; however, the unmethylated heparin-binding haemagglutinin did not.[79] The authors showed that the methylation of heparin-binding haemagglutinin was essential for the induction of T-cell antigenicity and protective immunity against the pathogen.

There is a precedent for methylation having a role in TLR regulation, with one study showing the effect of decreased methylation on TLR2 expression in cystic fibrosis patients. The study showed that decreased methylation of the TLR2 promoter was resulting in an upregulation of TLR2, in agreement with the enhanced proinflammatory response to bacterial challenges seen in the disease.[80] Other papers have shown that methylation may have an important role in the control of the immune system as a whole, with one study showcasing

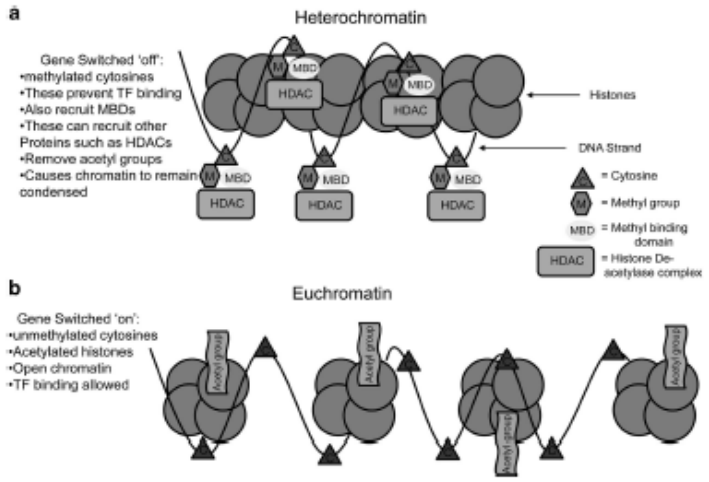

Figure 2 This figure shows the different states of chromatin and how it affects gene transcription. In (a) the cytosines are methylated, which results in recruitment of methyl binding domains (MBD), which recruit further molecules such as histone deacetylase complexes (HDACs). The removal of acetyl groups causes chromatin to remain in this condensed state known as heterochromatin. In this state TF binding cannot occur and so transcription is impaired. In (b) the cytosines are unmethylated and the acetyl groups are present, maintaining the chromatin in its open confirmation, known as euchromatin. In this state TF binding can occur as the DNA is accessible.

that DNA demethylating drugs had a broad immunostimulatory role in the immune system.[81] Within the field of immunology the epigenetic control of the immune system provides a novel and important target for future research. Drugs targeting the enzymes involved in maintaining the epigenome could provide future anti-inflammatory treatment options for a wide range of disorders. However, the level of current knowledge regarding how epigenetic modifications are affecting the immune system is still underdeveloped.

HISTONE ACETYLATION/DEACETLYATION

Transcription in eukaryotes is a highly controlled process with many different factors involved in its regulation. Acetylation of histones at specific lysine (K)-rich sites has a marked effect on transcriptional regulation by altering the structure of the histone chromatin complex.[82] The structure of chromatin has been shown to have an effect on the level of transcription of the genetic material contained within the chromatin complex. The nucleosome is the standard organisational unit of DNA in eukaryotic cells, in which 147 base pairs of DNA are coiled around a histone complex.[83] When chromatin is in this nucleosomal state it is generally repressive to transcription[84] and the nucleosomal structure coupled with the DNA–histone interactions means the genetic material is inaccessible for transcription and translation and results in epigenetic silencing. Thus, altering the state of the chromatin structure may enable transcription of genetic material.

There are several well-known protein and enzyme complexes that alter chromatin state in a myriad of ways. For example, the SWI-SNF (SWItch/Sucrose Non-Fermentable) complex alters chromatin packaging in an ATP-dependent manner.[85,86] Other methods of modifying chromatin–histone interactions involve processes such as methylation, ubiquitination, ADP-ribosylation and phosphorylation.[87] The addition of an acetyl group to the nucleosomal complex results in chromatin remodelling due to the interference of the acetyl group with the interaction between the positively charged lysine tail of the histone and the negatively charged DNA.[88] The neutralising of the positive charge on the lysine results in a weakened association between the DNA and the histone protein complex, the result of which is the remodelling of the transcriptionally inactive heterochromatin into the transcriptionally active euchromatin (see Figure 2 below). Alternatively, deacetylation of histones results in stronger ionic interactions between the positively charged histones and the negatively charged DNA resulting in the more compact chromatin structure referred to as heterochromatin. This compact structure results in transcriptionally inactive chromatin owing to its condensed state.[89] The acetylation and deacetylation of the lysine tail of the histone proteins are controlled by two distinct families of enzymes, the histone acetyltransferases and the HDACs.[88] There are three major families of histone acetyltransferases that have been previously reviewed extensively.[90–92]

In opposition to the histone acetyltransferases are the enzymes known as HDACs. There are 18 members in this family of enzymes and they are grouped into four distinct classes based on their homology to proteins identified in yeast, namely Rpd3, Hos1 and Hos2 (Class I), HDA1 and Hos3 (Class II), the sirtuins (Class III)[93–96] and the class IV HDACs which don't share homology with the others.[97] The class I, II and IV HDACs are referred to as the classical HDACs and their enzymatic activity depends on Zn^{2+} as a cofactor. Although class I and II HDACs are evolutionarily similar, class IV HDACs, despite also relying on the Zn^{2+} interaction, are not related to the others. Class III HDACs are known as sirtuins and do not require Zn^{2+} but instead rely on a NAD^{+} cofactor, which is utilised by the enzymes to accept acetyl groups.[98] Many of the HDACs are believed to exist as protein complexes comprising a number of subunits, which then target specific genomic regions via interactions with nuclear receptors, transcription factors or other proteins such as methyl binding domains.[99] HDACs are recruited to DNA is via the interaction between a methyl binding domains such as MeCP2 and HDACs. When DNA is methylated the methyl group will recruit additional proteins such as the aforementioned MeCP2 which in turn will

recruit a HDAC complex, resulting in the repression of gene transcription.[100,101]

Acetylation and deacetylation of histones has a role in the regulation of gene expression, and affects cellular proliferation and the cell cycle.[102] Indeed, HDACs have been implicated in tumorigenisis and are attractive targets due to their ability to arrest the cell cycle, induce apoptosis and inhibit angiogenesis.[103,104] Butyrate was one of the first compounds discovered to have HDAC inhibitory effects, with the compound showing reversible inhibition of cellular proliferation and increases in histone acetylation.[105] Butyrate, however, has a myriad of other effects and is not considered a specific HDAC inhibitor. Trichostatin A (TSA) was the first compound discovered to be a specific inhibitor of HDACs. It was originally classified as a fungistatic antibiotic when it was isolated from strains of *Streptomyces hygroscopicus*.[106] It was shown to have potent inhibitory effects on mammalian HDACs both *in vitro* and *in vivo* and was also shown to promote cell proliferation and differentiation.[107] Another HDAC inhibitor, suberoylanilide hydroxamic acid (SAHA), has been approved as an adjuvant therapy for the treatment of T-cell lymphomas in patients where previous therapies were ineffective.[108] Given the ability of the HDAC inhibitors to induce remodelling of chromatin and alter gene expression it was posited that they may also have an effect on the regulation of genes involved in the immune response.

HDACS, INHIBITORS AND IMMUNITY

There is little evidence at present to suggest whether HDACs or their inhibitors have a direct action on the expression of TLRs themselves. Studies have tended to examine TLR signalling or activity instead. One study showed the effect of HDAC inhibition by a novel pan-HDAC inhibiting compound LBH589 on TLR3- and TLR4-induced dendritic cell activation. They showed that upon administration of the compound the levels of cytokines released induced by the dendritic cells in response to activation by TLR3/4 was greatly decreased. There were decreases in levels of IL-6, IL-10, IL-12p70 and IL-23 released when compared with the untreated controls.[109] The decreases in the levels of these important inflammatory cytokines would suggest that the inhibition of the HDAC complex is having a huge effect on the Toll-like receptor induced immune response. However, the complete mechanism was not determined and despite the implication that TLRs 3/4 are involved, there is no evidence currently to suggest the effect is a direct one.

A study by Roger *et al.*[110] examined the effect of the HDAC inhibitor Trichostatin A (TSA) on the innate immune response to stimulation by TLR ligands and infection. They determined that treatment with the HDAC inhibitor TSA impaired the innate immune response to challenge with the various TLR ligands. The study showed that there were alterations in the levels of cytokines produced in response to the ligands as well as a change in the expression levels of the TLR1-9. These changes were not unidirectional, however, with some TLRs expression increasing and others decreasing, suggesting the relationship between histone acetylation, TLR expression and the resulting immune response is more complex than originally thought. This is one of few studies that examine the effects of HDAC inhibitors directly on the expression of the TLRs. A 2015 study has shown that histone acetylation may play a role in the development of tolerance to pathogens in the lungs. The study examined the effects of TLR stimulation on chemokine production in alveolar epithelial cells (AECs), it was found that upon repeated stimulation of AECs with TLR ligands it was noted a 'tolerance' response developed, but that addition of HDAC inhibitors circumvented this response.[111]

Similarly, another study showed that activation of TLR4 with LPS resulted in the repression of certain HDACs as well as the increase in expression of others. The study noted that LPS seemed to regulate the expression of several HDACs at the mRNA level in mouse-derived macrophages. LPS was shown to repress and subsequently induce HDACs 4, 5 and 7. Treatment of these macrophages with TSA enhanced LPS induced expression of inflammatory genes such as COX-2, Ifit2 and Cxcl2.[112] The authors also showed that over-expression of HDAC8 in mouse macrophages blocked the COX-2 inducing abilities of LPS. This paper demonstrates the negative regulatory action of the HDACs in regards to inflammatory gene expression and suggests that they may act to prevent excessive immune responses.

Given the suggested potential for HDACs as modulators of TLR expression, it has been suggested that HDAC inhibitors could be used to alter immune responses with minimal cytotoxicity when used at lower concentrations (see Table 1 for summary). Studies have shown that the HDACs are important in the regulation of certain immune cell functions.[113] In one such study, a small molecule inhibitor of HDACs, LAQ824, was found to alter the activation and function of macrophages and dendritic cells in response to TLR4 activation by LPS. Specifically, the HDACi prevented activation of T-helper 1 (Th1) cells by dendritic cells but had no effect on T-helper 2 (Th2) cells. Furthermore, the study also showed that HDACi prevented macrophage and monocyte chemotaxis, but had no effect on neutrophils.[114] Thus, in this case inhibition of the HDACs was having a highly specific effect on the immune response by altering the Th1 and Th2 balance in response to the LPS challenge.

The *in vivo* applications of SAHA have been examined in a mouse model of endotoxemia. SAHA treatment resulted in >50% decrease in circulating cytokines, including TNF-α, IL-1-β, IL-6 and IFN-γ, when compared with non-SAHA-treated mice. Furthermore, SAHA treatment suppressed cytokine-induced nitric oxide production in mouse macrophages and also prevented hepatocellular damage.[115] Recent data has also shown the effectiveness of SAHA *in vivo*, in a study that examined the immune effects of Vorinostat (SAHA) on patients receiving treatment for graft versus host disease. In patients receiving SAHA, there were decreased levels of proinflammatory cytokines when compared with controls, suggesting a clinical anti-inflammatory application for the drug.[116] TSA has also shown promise as an anti-inflammatory compound, and when compared with five other HDAC inhibitors its anti-inflammatory effects in LPS-stimulated bone marrow-derived macrophages were deemed the most potent. It showed significantly decreased mRNA and protein levels of proinflammatory cytokines TNF-α, IL-1-β, IL-6 and increased of IL-10, which is immunosuppressive.[117] Finally, a recent study has highlighted a role for HDAC7 in inflammation, with the study exhibiting HDAC7 dependent promotion of the proinflammatory effects of TLR4 activation.[118] Thus, there is increasing evidence to suggest that there may be a role for HDACs and their inhibitors as regulators of inflammation.

In a study carried out in mouse-derived macrophages, it was shown that several different broad spectrum HDAC inhibitors (pan-HDAC inhibitors) successfully inhibited the production of proinflammatory cytokines such as Edn-1, Ccl-7/MCP-3 and IL-12p40 in response to TLR4 stimulation with LPS. However, the HDAC inhibitors also increased the expression of several pro-atherogenic factors Cox-2 and Pai-1/serpine1.[119] Further studies examining TLR4 activation have also revealed a potential role for HDACs in regulating the immune response. The act of acetylating histones or inhibiting the deacetylases would usually result in an increase in transcriptional activity given the

more open confirmation of the chromatin, however, there have been cases identified where HDAC inhibitors have decreased gene expression. In one study, it was shown that the inhibitors TSA and SAHA decreased TLR-mediated increases in inflammatory gene expression. The authors identified this change was due to the direct impairment of transcription factor recruitment.[120] Thus, while the actions of the HDAC inhibitors on gene expression are not necessarily clear cut or unidirectional, there is certainly sufficient evidence to suggest that they could serve as potential methods of modulating immune responses.

CONCLUSIONS AND PERSPECTIVES

TLRs are of paramount importance in sensing many forms of pathogens and other noxious insults. Their role as the moderators of many immune related conditions such as sepsis, autoimmune disease and rheumatoid arthritis, atherosclerosis and neurodegeneration indicate the importance of researching their potential as drug targets. However, despite their importance we currently know very little about how their expression is regulated. To help better understand diseases mechanisms, researchers to date have sought to examine mechanisms where diseases have resulted from changes in the nucleotide sequence of TLR genes. With the emergence of evidence that non-nucleotide changes result in alterations in gene expression, it was reasonable for researchers to assume that epigenetic changes may affect TLRs. Thus, DNA methylation and histone modification and their related enzymes were investigated. In the process, scientists unearthed a new generation of cytotoxic anticancer drugs in the form of DNMT inhibitors and HDAC inhibitors. Rising to prominence due to their effective antitumor activity, research into the mechanism of action of these drugs would reveal their potential for use as modulators of the immune system. However, these studies are few and the full extent of how these drugs are exerting their effects is not fully understood. At present, the potential for TLRs to be manipulated by epigenetic modifying drugs remains relatively understudied. This review highlights the importance of these receptors in a medical context, and how using them as drug targets could result in new and effective anti-inflammatory medications. To achieve this, however, more needs to be understood about how epigenetic drugs affect TLRs. Determining whether the action of such of such drugs are direct or indirect should form part of future research in this area. In our opinion, the potential rewards for therapy could be great.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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